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PRACTICAL TOOLS

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A novel fluorescence and DNA combination for versatile, long-term marking of mosquitoes

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Abstract

1. Current mark–release–recapture methodologies are limited in their ability to address complex problems in vector biology, such as studying multiple groups overlapping in space and time. Additionally, limited mark retention, reduced post-marking survival and the large effort in marking, collection and recapture all complicate effective insect tracking.
2. We have developed and evaluated a marking method using a fluorescent dye (SmartWater®) combined with synthetic DNA tags to informatively and efficiently mark adult mosquitoes using an airbrush pump and nebulizer. Using a handheld UV flashlight, the fluorescent marking enabled quick and simple initial detection of recaptures in a field-ready and non-destructive approach that when combined with an extraction-free PCR on individual mosquito legs provides potentially unlimited marking information.
3. This marking, first tested in the laboratory with *Anopheles gambiae* s.l. mosquitoes, did not affect survival (median ages 24–28 days, p-adj > 0.25), oviposition (median eggs/female of 28.8, 32.5, 33.3 for water, green, red dyes, respectively, p-adj > 0.44) or *Plasmodium* competence (mean oocysts 5.56–10.6, p-adj > 0.95). DNA and fluorescence had 100% retention up to 3 weeks (longest time point tested) with high intensity, indicating marks would persist longer.
4. We describe a novel, simple, no/low-impact and long-lasting marking method that allows separation of multiple insect subpopulations by combining unlimited length and sequence variation in the synthetic DNA tags. This method can be readily deployed in the field for marking multiple groups of mosquitoes or other insects.

KEYWORDS

Anopheles gambiae, dispersal, DNA, fluorescent, Mark–release–recapture

Roy Faiman and Benjamin J. Krajacich contributed equally.

1 | INTRODUCTION

Mosquito-transmitted diseases kill 600,000–800,000 people annually, as estimated by the WHO (www.who.int/news-room/fact-sheets/). While much work has been done over the past century to answer basic questions about mosquitoes such as their population sizes (Athrey et al., 2012; Lehmann et al., 1998; Taylor et al., 1993; Touré et al., 1998), longevity (Cook et al., 2006; Krajacich et al., 2017; Polovodova, 1941, 1949), dispersal patterns and distances (Gillies, 1961; Gillies & Wilkes, 1965; Huestis et al., 2019), and vectorial capacity (Afrane et al., 2008; Kramer & Ciota, 2015), methodological limitations in tracking wild mosquitoes have left many of these aspects poorly understood. One of the gold standard approaches for answering such questions is mark–release–recapture (MRR), which was first applied to mosquitoes over a century ago and has been modified and refined since (Costantini & Della, 1996; Gillies, 1961; Guerra et al., 2014; Lehmann et al., 2010; Reisen & Aslamkhan, 1979; Service, 1993; Touré et al., 1998; Zetek, 1913). All MRR techniques share the same idea: mark multiple individuals, release them and determine their new location and frequency upon their recapture.

Due to their small size (generally <5 mm long and weighing <2 mg), marking mosquitoes is limited to lightweight agents. Different types of marking materials have been used in the past including paints/stains (Gillies, 1961; Tsuda & Kamezaki, 2013; Zetek, 1913), protein markers (Hagler et al., 1992), powders and dusts (Epopa et al., 2017; Verhulst et al., 2013), internal dyes (Bailey et al., 1962), food colouring (Williams, 1962), radioisotopes (Patterson et al., 1969; Zhou et al., 2004), and more recently stable isotopes (Faiman et al., 2019; Hamer et al., 2012, 2014). While useful, these methods may have potential disadvantages such as altering the behaviour of the insect (Dickens & Brant, 2014; Naranjo, 1990), shortening its life span (Verhulst et al., 2013), limited mark persistence (Hagler et al., 2001; Walker & Wineriter, 1981) and/or limited diversity of unique tags which minimizes mark complexity (Service, 1993). From these deficits and the primary implementation of methodologies on local scales, some aspects of mosquito biology such as long-range movement (Huestis et al., 2019) and survival (Dao et al., 2014; Lehmann et al., 2010) have likely been missed, which implies our overall knowledge of basic mosquito behaviour is still limited. Additionally, due to the large population sizes of most insects and the labour-intensive nature of MRR experiments, individuals that are marked tend to be heavily diluted within the unmarked population, limiting recapture. In this paper we describe a novel mosquito marking method which addresses the issues of mark versatility, field detectability, marked mosquito survival and mark retention, and has potential for long-term marking of a diversity of insects and other species.

2 | MATERIALS AND METHODS

2.1 | SmartWater® fluorescent spray

For fluorescence marking of mosquitoes, we utilized SmartWater® (SmartWater CSI LLC). SmartWater is a proprietary, traceable water-based solution developed as a forensic marker for concealed labelling of valuable items (see: www.smartwaterfoundation.org). Within the SmartWater solution were two components: (a) a non-toxic, water-based fluorescent solution which determines the colour of the mark based on different mixtures of the base colours (APEX Invisible Blue, Red, Green or Cartax DP (Yellow-Green)), rendering the marked object visible under UV light (365 nm), and (b) Mowilith LDM 7709, a non-toxic, water-based polymer emulsion used to bind the fluorescence to the marked substrate. We used the terms 'fluorescence' and 'polymer' throughout the paper hence forth.

2.2 | DNA tag design, sizes and verification

A PCR validated set of 14 DNA tags and primer sequences were created for this project. The tags vary in size from 80 to 340 base pairs (Supporting Information; File 1) and share two 20-base universal primer regions allowing for size-based tag ID with only one pair of primers. Each tag has a unique internal sequence in addition to its unique length, enabling additional confirmation via Sanger sequencing (Sanger et al., 1977). The sequence of each DNA tag was generated with R (R version 3.6.0) to have roughly a 50% GC content (for stability and improved amplification), no start codons and no significant similarities to known sequences in the nr database of BLAST (Altschul et al., 1990). Tags were amplified using 1 μl of 0.1 μM single-stranded DNA ultramer (Integrated DNA Technologies, Coralville, Iowa, USA) or 1 μl of a 1:100 or 1:200 dilution of a 250 ng or 500 ng synthesis scale double-stranded DNA gBlock in a 50 μl PCR (GoTaq Green Master Mix; Promega) with 400 nM of primers (IDT). Reactions were amplified at 94°C for 5 min, 32 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 30 s and a final elongation of 72°C for 5 min. All amplifications were performed in heat-sealed random access plates (4titude Ltd) to reduce contamination risk, and individual-use aliquots were used to add 10 μl of PCR product per 2 ml of SmartWater spray solution (see below). PCR products were visualized on a 3% agarose/ethidium bromide gel to verify the presence of one band per reaction, and no off-target amplification.

2.3 | Application method

Mosquitoes destined for marking were placed in pint-size paper cups with a muslin net cover, onto which the spraying apparatus [adapted from (Hagler, 1997); (Figure 1)] was attached to dispense the spray solution. The spray solution was mixed shortly before spraying onto the mosquitoes as follows: 1%–8% fluorescence (colour dependent) was mixed well with 0.1%–2.5% polymer, 0.5% synthetic DNA per tag and topped with deionized water to a final volume of 2 ml. Different colours were found to behave slightly differently when sprayed (i.e. variation in fluorescence adherence to mosquito body parts), requiring adjustment of ratios (Supporting Information; File 2). The solution was vortexed at 2,000 rpm for 30 s to ensure even mixing of the components. Vortexing was repeated immediately

FIGURE 1 Spray Apparatus: Nebulizer and funnel cone over spray cup (a) with mosquitoes (b). Black paper detection cup (c) which limits background fluorescence and allows for detection of live (d) and dead (d inset) fluorescently labelled mosquitoes using a handheld UV flashlight (365 nm). Nebulization technique adapted from Hagler (1997)

before transfer of the marking solution into the nebulizer capsule. The nebulizer set (model NEB KIT 500; Drive DeVilbiss Healthcare) consisted of a capsule (spray solution reservoir, 3 ml in volume), 'T'-connector (plugged with cotton wool on one end; see Figure 1), flexible air tube and air supply tube, fed by a 1/5 horse-power airbrush compressor (model TC-20-H6-B, Master Airbrush) pre-set to 1.5 Bar pressure. A 10-cm diameter powder funnel (model 4252; Thermo Fisher Scientific) was attached with its narrow end to the flexible air tube, to allow uniform dispensing of the sprayed solution into the paper pint by covering its top with the conical end, preventing spray from exiting the pint (Figure 1).

For spraying, 2 ml of mixed solution was pipetted into the vertically held nebulizer capsule, followed by activation of the compressor for 5–6 s, dispensing ~250 µl, with the funnel cone covering the mosquito pint. A visual verification of the spray cloud was done immediately before covering the mosquito pint to rule out the possibility of blockage or non-treatment. The mosquito pint was tapped gently to encourage mosquitoes to take flight during the spraying, ensuring uniform coverage of the mosquitoes by the nebulized spray solution. After 5 s of spraying of each pint, mosquitoes were inspected visually. Adequate marking resulted with sedentary mosquitoes, typically seen standing on the cup floor or flying poorly. Full mobility was regained ~15 min after spraying. Sprayed mosquitoes were left in the paper pint no less than 2 hr to allow full drying of the sprayed solution and full recovery of the mosquitoes before subsequent release.

2.4 | Effect of SmartWater and DNA spraying on longevity, blood feeding, reproduction and development of *Plasmodium* parasites

To verify that the application of SmartWater and/or DNA does not affect the survival of mosquitoes, we compared the life span of mosquitoes after application of combinations of SmartWater fluorescent components (colours) and polymer concentrations versus a water control. Survival analyses were performed using Kaplan–Meier survival curves and pairwise log-rank tests adjusted for multiple comparisons implemented in the 'SURVMINER' package in R (Kassambara et al., 2019). These experiments were performed using *Anopheles gambiae* G3 strain mosquitoes at the NIH, or a recently adapted strain of *Anopheles coluzzii* in Mali under standard insectary conditions (27°C, 85% RH, 12:12 Light:Dark cycle). The durability of the SmartWater marking was tested with a simulated 'rain' of a weekly water misting of the mosquito rearing cages from a laboratory spray bottle (model F11620-0050; Bel-Art- SP Scienceware®). Qualitative intensity of marking post-rain was tracked weekly by removing two or three mosquitoes from both treatments and inspecting their

brightness side-by-side under a UV light. A cursory examination of blood feeding and reproduction was also performed, in which groups of 20 mosquitoes (5 total groups per colour of varying fluorescence/polymer concentrations) were given two bloodmeals and allowed to oviposit, at which point egg counts were estimated per bloodfed female. Feeding proportions were compared via an adjusted Dunnett's test with the 'BINMTO' package in R (Schaarschmidt, 2018).

The potential effects of marking on the transmission of *Plasmodium falciparum* parasites were also investigated using a standardized membrane feeding assay (SMFA) following previous methodology (Sagara et al., 2018; Wu et al., 2008). Briefly, an in vitro 15-day old culture of *P. falciparum* (NF54 line) containing stage V gametocytes was diluted with washed O + RBCs (Interstate Blood Bank) and an AB + serum pool (not heat-inactivated) from US malaria-naive subjects (Interstate Blood Bank) to final concentration of 0.07%–0.1% stage V gametocytes and 38.5% haematocrit. For each individual assay, 260 μl of diluted culture was fed to five groups (in duplicates) of 40–50 starved 3- to 5-day old *Anopheles gambiae* (G3 strain) female mosquitoes, using a Parafilm membrane on a mosquito feeder, kept warm with 40°C circulating water. After feeding, mosquitoes were kept at 26°C and 80% humidity to allow parasites to develop, and survival was tracked daily. On Day 8 after the feed, mosquito midguts were dissected and stained with 0.05%–0.1% mercurochrome solution in water for 20–30 min. Infectivity was measured by counting oocysts in 20–25 mosquitoes per treatment. On Day 15 after the feed, salivary glands of at least five mosquitoes per group were dissected and sporozoites were observed by phase-contrast microscopy.

3 | RESULTS

3.1 | Post-marking survival, oviposition and mark persistence

We tested multiple combinations of fluorescence/polymer components to determine the optimal concentrations in terms of marking visibility under UV and minimal effects on survival (Supporting Information; File 3). Different colour combinations required varying amounts of polymer for life-long retention on sprayed mosquitoes (Figure 2). The intensity of the fluorescence varied by colour, but at least four colours provided clear long-term marking: Cartax (yellowish-green), orange (mixture of red and green), blue and magenta (Figure 2). The intensity of marking found with the red mixture was variable in its current formulation and was not used in later experiments.

To evaluate the negative impact that polymer and colours may have on mosquitoes, survival was compared for Cartax (yellow-green), blue, magenta and orange to a water control (Supporting Information; File 3, two replicates, ~15 mosquitoes/group, adjusted *p*-values ≥ 0.21, 0.11, 0.96, 0.98 by pairwise log-rank test for blue, Cartax, magenta and orange respectively). A cursory pilot trial indicated there may be a slight, non-significant increase in mortality at

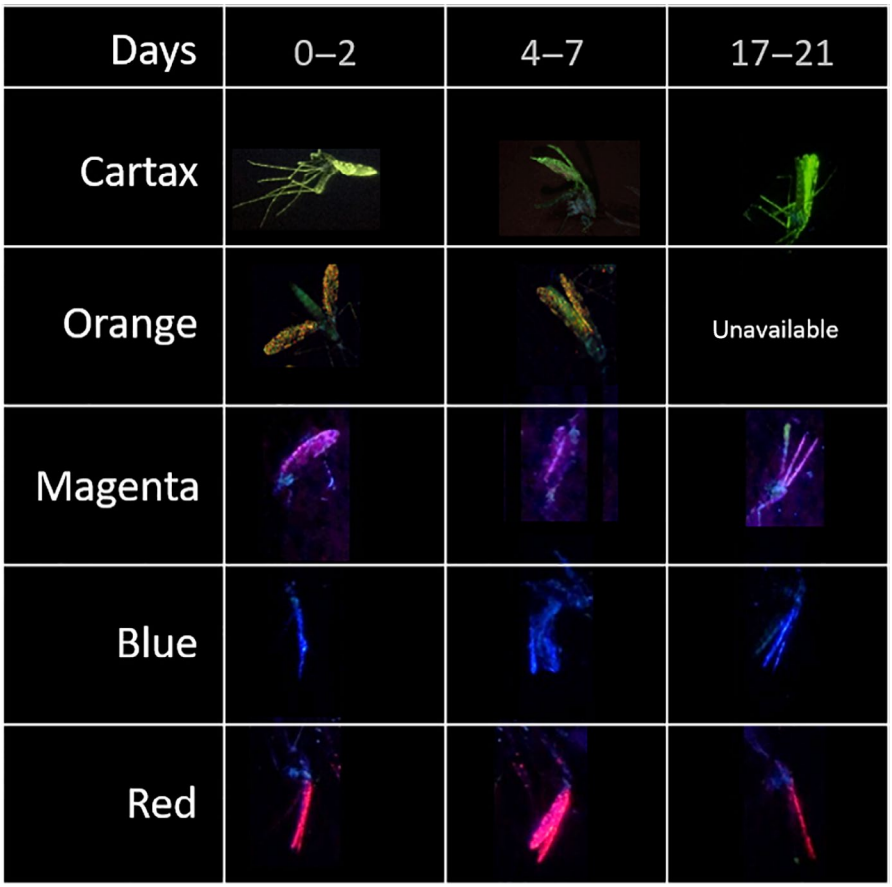


FIGURE 2 Composite of representative SmartWater marking intensity (pictures) over time (days post-application). From top: Cartax (0.5% fluorescence, 0.1% polymer), 0–21 days. Orange (5% fluorescence, 0.5% polymer), 0–7 days post-marking (17–21 day image unavailable). Magenta (5% fluorescence, 2.5% polymer), 0–17 days post-marking. Blue (5% fluorescence, 1% polymer), 0–17 days post-marking. Red (5% fluorescence, 2.5% polymer), 0–21 days post-marking. The lowest concentrations of fluorescence and polymer that provided long-term marking were chosen

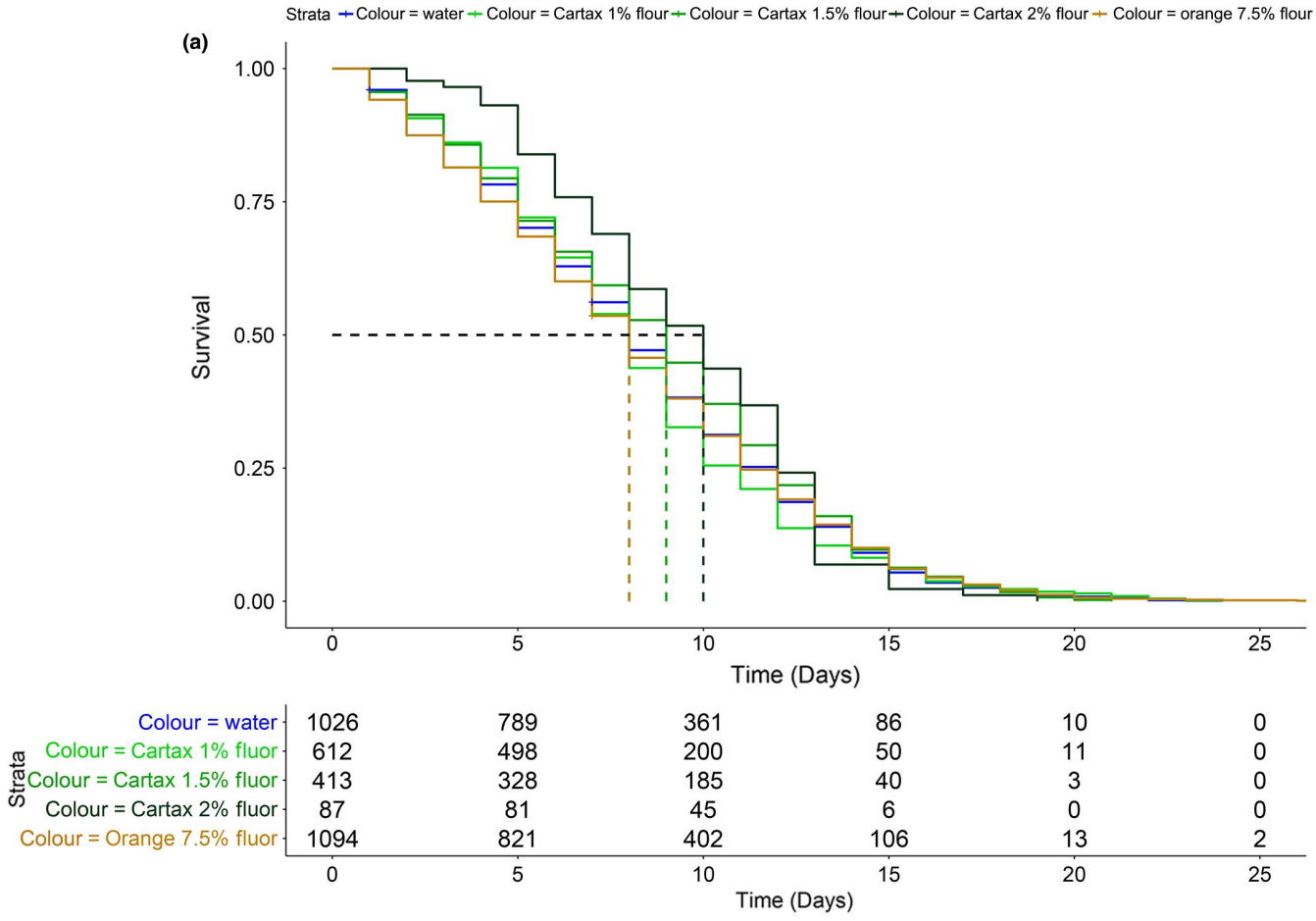
polymer concentrations >2.5%, but these higher polymer concentrations are not necessary for strong and long-term marking.

In a subsequent survival study based on the results of the preliminary study above, no increased mortality was observed with SmartWater and DNA spray when applied to recently colonized, laboratory-reared *An. coluzzii* mosquitoes in a village in Mali (26–28°C, 70%–85% RH, 2–26 cups per group, 87–1,094 mosquitoes, Figure 3). In this study, groups of mosquitoes varying in sizes (based on availability) were used as controls to assess the impact of marking on survival in wild mosquitoes in a MRR study conducted in parallel (published separately).

Additionally, we assessed the application of a weekly simulated rain on one colour (Cartax) and found it had no effect on the visibility of fluorescence on all mosquitoes tested for the duration of the experiment (0–21 days, Supporting Information; File 5). Finally, feeding and oviposition rates of mosquitoes marked with the fluorescence/DNA mixture did not differ from that of the water-sprayed control using the Dunnett's test (Supporting Information; File 6; Feeding and Oviposition, n = 20/group, 35%–90% feeding rate per fluorescence group, 60% water-only control). The application also had no significant effect on the development of *P. falciparum* oocysts (mean oocysts 5.56–10.6, adjusted p-values > 0.95 for all groups, Supporting Information; Files 7 and 8).

3.2 | Durability of synthetic DNA as a high-information marker

We tested the incorporation of synthetic DNA tracer tags into the nebulized SmartWater mixture to provide increased group



(b)

	Water	Cartax 1% fluor	Cartax 1.5% fluor	Cartax 2% fluor
Cartax 1% fluor	0.40	NA	NA	NA
Cartax 1.5% fluor	0.37	0.22	NA	NA
Cartax 2% fluor	0.37	0.22	0.79	NA
Orange 7.5% fluor	0.83	0.48	0.37	0.37

FIGURE 3 Survival analysis for Cartax (yellow-green) and orange colours with added DNA tag: Kaplan–Meier survival curves (a-top) and risk table (a-bottom) for five variations of colours tested on laboratory mosquitoes in Mali. Adjusted p-values from pairwise log-rank test of survival curves are shown for each comparison (b), with all comparisons between treatments showing no significant difference in survival. Polymer concentrations were 0.3% for Cartax and 2.5% for orange

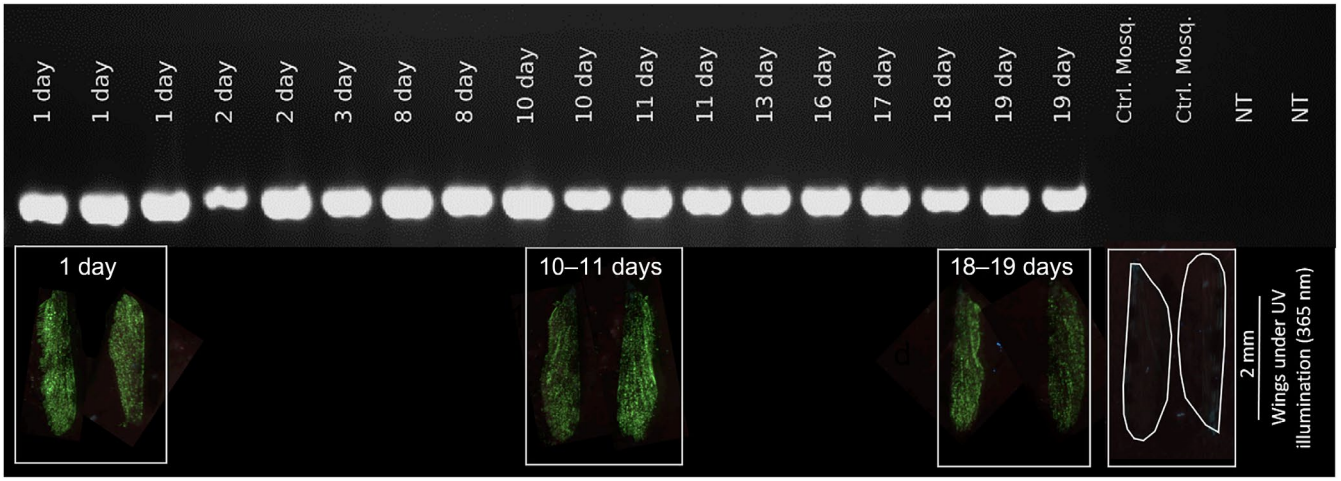


FIGURE 4 Leg PCR (DNA) and wing fluorescence retention: 220 base pair DNA tag directly amplified from the leg of laboratory-reared *Anopheles coluzzii* upon death (lanes 1–18, day of death post-marking listed). Two unsprayed control *Anopheles gambiae* (lanes 19–20) and two no template (NT) PCR controls (lanes 21–22) are included as negative controls. Wings of corresponding *A. coluzzii* mosquitoes under UV illumination (365 nm) placed below DNA bands to illustrate visual detectability by duration post-treatment in days (all wings from same spray treatment group; two wings selected in random per age group). No significant reduction in intensity of UV was found among the different ages (see Supporting Information; File 9)

resolution, for example, different tags for mosquitoes released from different release zones and at different dates into the marking mixture and provide a panel of 14 size-discriminative DNA tags amplifiable by one pair of primers (Supporting Information; File 1).

For initial testing of DNA tags, we chose one size (120 bp) to be incorporated with SmartWater solution, though detection of multiple-sized amplicons on mosquitoes is possible (up to four tags with two primer sets on one mosquito have been tested, data not shown). To reduce cost/time needed for DNA extraction, we evaluated direct PCR detection by using a portion of the mosquito and adding it directly into the PCR mixture for amplification. We found that mosquito legs provided a reliable PCR-based detection of the sprayed tag over the course of the mosquito's life span without obvious drop in band intensity up to 21 days post-spray application (Supporting Information; File 9).

Similarly, in a replicate experiment on colony mosquitoes, no drop in band intensity was found up to 19 days post-spray (Figure 4). Wings and abdomens also provided life-long DNA-based detection (Figure 4).

4 | DISCUSSION

We have developed and tested a new marking technique that combines fluorescence and multiple DNA tags, enabling rapid separation of marked from unmarked mosquitoes and near-unlimited mark complexity. Our results demonstrate that it can be effectively applied onto large numbers of mosquitoes with a clear mark that remains detectable for at least 3 weeks with no obvious drop in the intensity of both visible and molecular tags. No impact on mosquito survival, blood feeding, reproduction and capacity to support *Plasmodium* infection was found. Thus, we find this methodology a straightforward and highly versatile technique for marking mosquitoes and most likely other insect groups and is well-suited to laboratory and field applications at scale.

While the widespread utilization of fluorescent powders and dyes for MRR studies of insects has produced decades of valuable information (Guerra et al., 2014), methodological limitations leave considerable room for improvements. Our investigation of synthetic DNA tags co-sprayed with SmartWater® for mosquito marking demonstrate that the fluorescent mark allows rapid identification of recaptures and the DNA tags facilitate complex experiments by marking with unique tags multiple groups, for example several release sites, various dates, indoors versus outdoors caught wild mosquitoes, laboratory raised versus wild mosquitoes and their combinations. Moreover, we show that DNA amplified directly from legs without extraction, produced bright, life-long detection (up to 3 weeks, highest value tested) with no loss of signal (Figure 4). Synthetic DNA has been used as a non-harmful tracer of groundwater sources for several decades in hydrological studies (Sabir et al., 1999), and recently similar DNA barcodes have been developed for safe labelling in food production (SafeTraces). These DNA tags expand experimental possibilities and shift the decision about the number of tags to logistical considerations, including the ability to amplify and apply many unique tags for multiple groups per day. Using the four easily discriminated fluorescent colours in combination with the 14 validated DNA tags used in this study allows for up to 56 unique marking groups with a relatively simple experimental design and modest material and diagnostic costs (leg-based, extraction-free PCR costs are ~$0.30/sample with results in several hours; see Supporting Information, File 10). The size-based discrimination of tags with shared primers coupled with Sanger sequencing of the unique internal sequences allows for a blend of rapid detection and more stringent confirmation of tag identity needed for certain studies.

The use of SmartWater-based fluorescent dyes were chosen assuming the mark would be less hazardous, more durable and would minimize the risk of transfer between insects during mating or cohousing. SmartWater conforms with the five prerequisites of an ideal marking material as was defined by Hagler and Jackson (Hagler & Jackson, 2001): highly durable (>30 day retainment in laboratory study), inexpensive (supplied gratis by SmartWater Foundation for scientific research), non-toxic, easily applied and clearly identifiable. Detection of marked mosquitoes in the field can be achieved through use of a handheld UV flashlight, easily allowing separation of marked from unmarked individuals. We did see a slight drop in fluorescence over the life span of the mosquitoes (Figure 4, and Supporting Information; File 9); however, this drop does not appear to be significant enough to limit detection over a likely mosquito life span and marked mosquitoes remain clearly detectable under UV light.

DNA tags amplified from ultramers (200 bp or less) or gBlocks (>200 bp) have limited cost, with one ~$100 tag generating enough spray material for 40,000 spray applications on cups of up to ~50 mosquitoes (Supporting Information; File 10). Design, amplification and detection of DNA tags requires laboratory-standard molecular biology knowledge (i.e. primer design, checking BLAST for sequence uniqueness, etc.), and a thermocycler/gel electrophoresis equipment now standard in many laboratories, including entomological laboratories in malaria endemic regions. Spray application only requires an air compressor (~$60–100 depending on model), power source (i.e. 12 V adaptor in car) and disposable nebulizer kits (<$3 per nebulizer) which allow for marking thousands of mosquitoes/day depending on number of marks used and team size.

Potential downsides to this application method were found through initial laboratory testing followed by mock implementation, that is, marking without release of laboratory mosquitoes followed by marking assessment (e.g. coverage and retention) and survival in village conditions. It was found that care had to be taken to use the best laboratory practices against cross-contamination as minute amounts of DNA could produce a faint band. As the approach is essentially a nested PCR (a technique known for high sensitivity and possibility of false positives), this result is not unexpected but must be accounted for. This means that all spray aliquots of the DNA tags should be prepared in a designated area and amplified in heat-sealed individual-use tubes to prevent any possible cross-contamination after amplification. At a minimum, the use of pre- and post-amplification areas, the use of one-directional workflow (i.e. do not go from post-amplification to pre-amplification areas), single use reagent aliquots and use of designated pipettes and laboratory coats are recommended (Aslanzadeh, 2004). Furthermore, mosquito leg testing should always be done with proper negative control legs to indicate whether there is potential cross-contamination. In the studies in Mali, we found that bright external fluorescence and bright electrophoresis bands present on a mosquito was a reliable indication of true marked mosquitoes. However, it was common for there to be small particles of fluorescence from potentially environmental particles (Reeves et al., 1948), autofluorescence of sugar meals, eggs and/or genitalia (see Supporting Information; File 10), and low degree of contamination from reused spray cups/covers and aspirators used to process many marked mosquitoes. Care must be taken to not reuse items exposed to many marked mosquitoes, and it is important to separate between spray areas (stations) using different DNA tags and separate them from detection stations. Finally, the method presented here still demands labour-intensive manual application of the mark on the collected/reared mosquitoes by spraying groups of adult mosquitoes, thus facilitating MRR studies. Future works would benefit from self-marking techniques (e.g. automated eave tubes or window marking units) which could potentially increase the output of marked individuals several fold with little-to-no increase in labour (Knols et al., 2016) and allowing in situ marking. Increasing the numbers of marked individuals significantly will increase recaptures and subsequently robustness of the data, thus opening the door to studies on greater spatial and temporal scales as well.

5 | CONCLUSIONS

The SmartWater and DNA tagging mixture provided a highly flexible, modestly priced and long-lasting mark that had no detectable effects on mosquito survival, despite exceptionally large experiments to evaluate such effect. No negative impacts were detected on blood feeding, reproduction or malaria transmission capacity. This technique is well-suited for use in MRR studies on mosquito behaviour, population size estimation, movement analysis, transmission dynamics and impact of control measures, especially when multiple groups require higher spatial resolution than is available with currently used adult-marking methodologies. Currently, large MRR experiments using this novel marking method are being performed in the field in Mali showing promising results.

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AUTHORS' CONTRIBUTIONS

R.F., B.J.K. and T.L. conceived the ideas and designed the methodology; R.F., L.G., B.J.K., A.D., A.S.Y., O.Y., Z.L.S., M.D., D.S., O.M., A.M., D.S., M.C., S.K., S.G., M.B.C. and T.L. collected the data; B.J.K., R.F., B.P.G., O.M., A.M. and T.L. analysed the data; R.F., B.J.K. and T.L. led

the writing of the manuscript. All the authors contributed critically to the drafts and gave final approval for publication.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The files necessary to reproduce the survival, oocyst and feeding figures in this paper and the original gel images for other figures are available online (Faiman & Krajacich, 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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