Application of Environmental DNA (eDNA) in the Detection and Quantification of Anopheles Mosquito Larvae in Artificial Aquatic Habitats

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Table of Contents

List of Figures

List of Tables

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Abstract

Background

Environmental DNA (eDNA) analysis is an emerging tool in ecological monitoring of aquatic habitats. When applied correctly, it can overcome the limitations of traditional aquatic sampling methods. This current study develops and validates a protocol for using eDNA analysis to detect and quantify mosquito larvae in laboratory and controlled field setting.

Methods

Species-specific PCR primers were designed from *An. gambiae* s.l and *An. funestus,* and qPCR primers for *An. gambiae* s.l using mitochondrial *cyt b* gene and validated on 1:10 and 1:100 dilutions of gDNA from both species.

In the laboratory eDNA testing, *An. gambiae s.s* mosquito larvae (1 larva, 3 larvae and 6 larvae densities) were held in 45 mL of distilled water. One tube from each of the three densities was then sampled simultaneously at intervals of 1 h, 3 h, 6 h and 24 h.

We also set up six 1 L habitats by adding 2, 5, 10 and 20 larvae into each with 1 negative control habitat and held for 24 h. eDNA from the lab habitats were concentration using two methods - filtration and centrifugation - and also extracted using either ChargeSwitch® or Nexttec and analyzed the eDNA using IGS assay and *cyt b* TaqMan assay.

Field testing was done in 20 artificial ponds with varying total larval numbers and relative proportions of *An. gambiae s.s.* and *An. arabiensis* larvae of 6, 24, 48 and 96 larvae. Water samples (150 mL) were taken from the ponds daily over an 8day period. eDNA was extracted from them using the ChargeSwitch® and analyzed in a *cyt b* and *IGS* TaqMan assay.

Results

First test in mosquitoes was successful under laboratory conditions but inconclusive in the field trials. Both IGS and *cyt b* assays detected and quantified DNA at very low concentrations (0.156 pg). Contamination was a major issue in the field samples and affected any conclusion from the field samples.

Conclusion

We successfully showed that eDNA analysis can be used to detect and quantify mosquito species. More work in the protocol optimization need to be done to ensure smooth transition into semi field and field settings.

List of Abbreviations

µl	Microliter
PCR	Polymerase chain reaction
qPCR	Quantitative Polymerase Chain Reaction
LSTM	Liverpool School of Tropical Medicine
ICIPE	International Centre for Insect Physiology and Ecology
pg	Picogram
COI	Cytochrome oxidase I gene
cyt b	Cytochrome b gene
IGS	Intergenic spacer region
eDNA	Environmental DNA
DNA	Deoxyribose nucleic acid
LLINs	Long lasting insecticidal nets
ACT	Artemisinin-based combination therapy
^{0}C	Degrees Celsius
UV	Ultraviolet light
CDC	Centre for Disease Control and Prevention
WHO	World Health Organization
LSM	Larval Source Management
mtDNA	Mitochondrial Deoxyribose nucleic acid

1. Introduction and literature review

1.1 Malaria control in the 21st century

More than half of the world's population are at risk of malaria. The disease is currently actively transmitted in 95 countries mostly in sub-Saharan Africa (W.H.O, 2015). A concerted effort towards malaria control through the past decade has seen global reductions in malaria (Bhatt et al., 2015, W.H.O, 2015). Mortality was reduced from over 1 million deaths in the year 2000 to about 438,000 in 2015 and new malaria cases downscaled from over 320 million in 2000 to approximately 214 million in 2015 (W.H.O, 2015). These milestones have been mainly achieved by a higher coverage of vector control strategies including better usage of long-lasting insecticidal nets (LLINs) by local populations (Lengeler, 2004) and indoor residual spraying (Pluess et al., 2010); and by a prompt case detection by microscopy and rapid diagnostic tests (Wongsrichanalai et al., 2007) and treatment of clinical cases using artemisinin-based combination therapy (ACT) for uncomplicated malaria (Bhatt et al., 2015).

Despite being so effective in terms of the number of deaths averted, these malaria control strategies have their own share of challenges. For instance, both LLINs and indoor residual spraying rely on just four classes of insecticides – pyrethroids, carbamates, organophosphates and organochlorines - that have been losing efficiency due the rise of insecticide resistance in the main African malaria vectors, *Anopheles gambiae* and *Anopheles arabiensis* (Toé et al., 2014, Cisse et al., 2015, Ranson and Lissenden, 2016). Pyrethroids are the only available class of insecticide recommend for use on LLINs, which are the main malaria vector control tool. Direct evidence on the impact of insecticide resistance on malaria control has been shown by (Kleinschmidt et al., 2006, Kigozi et al., 2012) with more indirect evidence by (N'Guessan et al., 2007). Moreover, the rise of ACTs resistance by the *Plasmodium falciparum* in Southeast Asia (Dondorp et al., 2009, Ashley et al., 2014, Packard, 2014) is posing a threat to our most efficient treatment for

malaria with fears that resistance mutations in the K13 – propeller gene in *Plasmodium falciparum* could soon spread to parts of Africa (Talisuna et al., 2012, Kamau et al., 2014).

This worsening situation calls for an urgent need for the development of novel non-chemical based tools and insecticides for vector control to support the current tools (Ranson and Lissenden, 2016). In addition, the discovery of new robust techniques for entomological monitoring of vector species and monitoring the impact of these new interventions is crucial (Hemingway, 2014).

1.2 Mosquito entomological monitoring

Entomological monitoring provides essential information to establish and maintain effective control measures such as identification of the key mosquito species responsible for malaria transmission (*i.e.* vectors) and a continuous evaluation of the control impacts in the vector populations (W.H.O, 2013a, Silver and Service, 2008). Entomological surveys apply various methods depending the vector under investigation and the control methods that are being applied. Traditionally, the survey is done by either sampling the immature stages (*i.e.* egg, larvae, pupae), or by sampling the adult mosquitoes (Silver and Service, 2008).

Sampling mosquito eggs can also provide vital information about oviposition sites and their selection criteria by females (Silver and Service, 2008). However, egg sampling is affected by difficulties in reliably detecting and quantifying the and challenges in species identification of eggs based on electron micrographs (*i.e*. when working with sterile eggs that cannot be hatched) (Linley et al., 1993). This is particularly important in the sterile insect techniques (mosquito control strategy based on releasing sterile males to mate with wild females) for *Aedes aegypti* control where sampling the sterile egg population is vital for assessing the progress of the control operation (Dejean et al., 2011b).

The sampling of adult malaria vectors is vital for evaluating IRS and LLIN efficiency/impact in a control program. For indoor biting and resting anopheline mosquitoes, sampling can be carried by collecting tubes (Russell and Baisas, 1935), oral/battery-powered aspirators,

spray sheet collection, or window/exit trap collection (W.H.O, 2013b). The sampling of outdoor biting and resting mosquitos can be carried out using human landing catches, CDC light traps (W.H.O, 2013b) and more recently tool, the Suna Trap (Hiscox et al., 2014), in addition to cattle-baited traps for zoophilic species (Figure 1). However, trapping adults may be affected by behaviour fluctuations/shifts on insect biting/resting patterns and may have strong ethical concerns associated with human landing catches (Jamrozik et al., 2015).

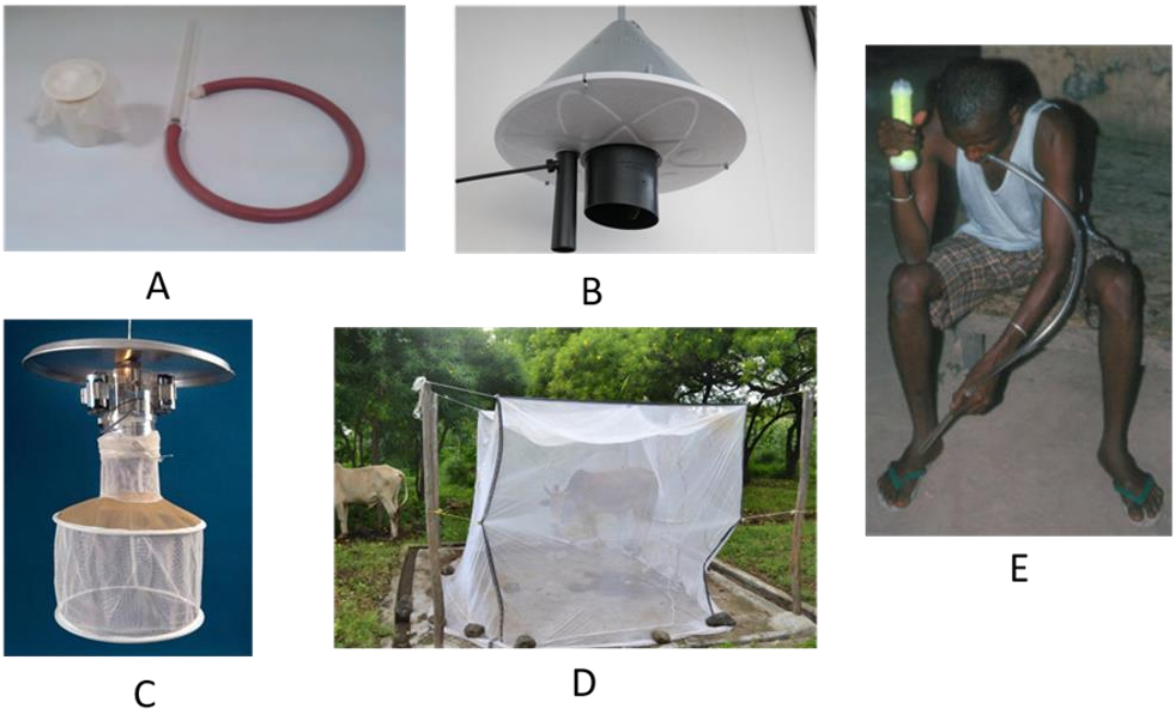


Figure 1. Common adult mosquito collection methods. A) Mouth aspirator, B) Suna Trap, C) CDC light trap, D) Cattle baited traps, E) Human landing catches (W.H.O, 2013b, Hiscox et al., 2014)

1.3 Larval control of mosquito disease vectors

Larval source management (LSM) is a mosquito control strategy that targets the immature stages of the mosquito life cycle – egg, larvae, and pupae. Its application is mainly carried by habitat manipulation, habitat modification, application of larvicides and biological control (W.H.O, 2013a). The strategy was for a very long time the primary vector control tool before the use of chemical adulticides and the main tool responsible for malaria elimination in places like Panama Canal (Dehné, 1955), Brazil (Killeen et al., 2002), Tennessee Valley (Gartrell and Ludvik, 1954) and Egypt (Shousha, 1948). More recently, integrated approaches using larviciding have been able to reduce malaria cases in Eritrea (Shililu et al., 2007), and Kenya (Fillinger et al., 2009).

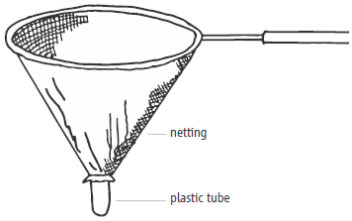
With the increasing challenges that current malaria control tools face, as outlined above, larval source management provides a viable complementary strategy (Fillinger and Lindsay, 2011). Further, the increasing number of exophilic and exophagic vectors (e.g. *An. arabiensis* and *An. rivulorum*) have been shown to sustain outdoor malaria transmission but are not adequately controlled by the indoor-based interventions – IRS and LLINs (Reddy et al., 2011, Russell et al., 2011). LSM has a natural ‘’advantage’’ in that all mosquitoes eventually must lay eggs in aquatic habitats irrespective of their biting or resting preference. There is also a wide range of WHOPES-approved safe chemical and biological larvicides that can be used for this purpose (WHOPES, 2016). However, the application of LSM presents management challenges due to resource requirements the wide array of *Anopheles* breeding sites and the heterogeneity of different habitat types (Gu et al., 2008, Fillinger and Lindsay, 2011). Such factors could considerably increase application costs and WHO officially recommend the restriction of LSM to areas where habitats are few, fixed and findable, for example in urban areas (W.H.O, 2013a). An economic analysis for LSM in two Districts of Kenya (Vihiga and Mbita) and in Tanzania (Dar es Salaam) indicates a cost benefit when compared with IRS and LLINs control measures (Worrall and Fillinger, 2011).

The success of an LSM strategy requires effective, reliable and, most importantly, replicable methods for monitoring the aquatic stages of disease vectors (Fillinger and Lindsay, 2011). This methodology is essential for selecting habitats on which to focus

interventions and for monitoring the implementation of the control measures. The traditional protocol for monitoring larval habitats focuses on a combination of larvae sampling (Table 1) with morphological identification by microscope or/and molecular identification within species complex. This in itself is time-consuming and requires a lot of labour for large areas. The heterogeneity of larval habitats and larval numbers in different habitats does not allow correlations between low and high productive habitats. Moreover, these sampling methods may also promote strong sampling bias in terms of under/overestimation of larval densities in different habitats (Thomsen and Willerslev, 2015).

Sampling mosquito larvae can be carried out by either using dippers or sweep nets (Silver and Service, 2008). In *Anopheles* mosquitoes, these techniques can be extremely time-consuming or impractical due the large size of some *Anopheles* natural breeding sites and the unpredictability in nature of use of potential breeding habitats (Minakawa et al., 2004). The conspecific sharing of larval habitats by malaria vectors (Chen et al., 2008) also requires morphological identification by microscopy or, especially in the case of morphologically identical species, molecular identification by DNA-based analyses, to identify and quantify the different species in a given habitat. These raise many challenges for larvae sampling methods, particularly for surveys on a large scale (Table 1).

Table 1: Most common mosquito larvae sampling strategies and their challenges. Diagrams adapted from (W.H.O, 2013b)

Sampling strategy	Challenges
Dippers 	• No standardization on how method is used (Silver and Service, 2008) • Species and mosquito life stage biassed (Trapido, 1951) • Affected by vegetation cover (Hess and Hall, 1943) • Massively affected by non-uniform dispersal of larvae (Service et al., 1985) • Dippers come in different sizes and shapes affecting comparability of results (Minakawa et al., 2004) • Very tedious for extensive surveys (Silver and Service, 2008) • Invasive nature of the method interfering with the habitats (Silver and Service, 2008) • OK for small habitats (though maybe not if very shallow) (Silver and Service, 2008)
Aquatic/sweep nets 	• Causes considerable interference to habitats affecting larval catches (Silver and Service, 2008) • Not appropriate for shallow and small habitats (Silver and Service, 2008) • Fewer larvae capture rates mostly skewed towards fourth instars (Silver and Service, 2008) • Good for detecting presence/absence of larvae but not quantification (Lardeux et al., 2002) • Large amount of debris and aquatic plants collected in process (Silver and Service, 2008) • Difficulty for analysing and comparing data from different pond sizes (Bruce and David, 2003)

In malaria hotspots (focused malaria transmission with a defined geographical area where the local anopheles vector population are able to sustain the basic reproductive rate, *Ro*, above 1), LSM management can be used to target outdoor biting vector in effort to break transmission (Bousema et al., 2012). This requires effective tools for predicting the high productive habitats in which to focus the control efforts (Hardy et al., 2015). Moreover, some mosquito species, such as the malaria vector *Anopheles funestus,* present limited evidence about their breeding sites (Minakawa et al., 2008) since current sampling methodologies are normally unable to collect them. In other disease vectors such as *Phlebotomus sergenti* and *Phlebotomus papatasi*, the Old World sand fly vector for cutaneous leishmaniasis and visceral leishmaniasis respectively, there is a huge knowledge gap on in the ecology of its immature stages (Moncaz et al., 2012, Cameron et al., 2016) It is therefore absolutely important that we develop quick, robust and field applicable techniques for monitoring aquatic larval species as a first step with a next move to the terrestrial vectors.

1.4 Environmental DNA (eDNA) and its application for monitoring aquatic life cycle stages

Environmental DNA (eDNA) analysis method is a relatively new strategy in the ecology of aquatic habitats that may indirectly detect the presence of an organism/species, or estimate their population density and colonization time without direct physical sampling of the organisms (Thomsen and Willerslev, 2015). eDNA is the residual DNA shed by all aquatic organisms in the form of faecal waste, urine, shedding of dead skin, gametes or post mortem degradation processes. This residual DNA can be detected by molecular biology techniques allowing inference of the presence of the organism/species without sampling them (Ficetola et al., 2008). The application of this novel technology has been further boosted by the dynamic developments in the fields of high-throughput DNA sequencing, quantitative PCR (qPCR) analysis, and most recently eDNA metabarcoding techniques (Valentini et al., 2015).

In natural habitats, eDNA concentration is affected by a number of factors such as: the density of the target species, temperature, microbial activity in the habitat, DNA depurination (nucleic acid degradation process) and exposure to ultraviolet light among other factors (Goldberg et al., 2011, Pilliod et al., 2014, Barnes et al., 2014). This degradation continues even after sampling, therefore, samples should be immediately preserved by adding ethanol and sodium acetate in a precipitation protocol (Ficetola et al., 2008) or alternatively membrane filtering the samples immediately and keeping at -20 freezers for longer storage (Thomsen and Willerslev, 2015). Water samples can alternatively be frozen at -80 °C following collection, but the storage space required for this might be a limiting factor.

Using eDNA for species detection and quantification has some advantages compared to conventional sampling methods: eDNA surveys are replicable and correlate positively when compared with traditional entomological survey methods (Dejean et al., 2011b, Minamoto et al., 2012); and the rapid diffusion of eDNA within a habitat would mean that presence of the organism can be detected by sampling anywhere within the habitat and not necessarily at the point where the organism is reducing the sampling effort hence saving time and money (Jerde et al., 2011). The non-invasive nature and relative ease of eDNA surveys have rapidly boosted its applicability as a survey tool in conservation biology/management, especially for protected species (Thomsen and Willerslev, 2015). Further, the use of high-throughput sequencing on eDNA samples can also allow the detection of multiple species in a given habitat (Goldberg et al., 2016).

The probe based quantitative PCR is the most commonly used methodology for eDNA detection and uses species specific primer-probes that targets a small fragment (*i.e.* 50 – 150bp) (Thomsen and Willerslev, 2015). qPCR is highly sensitive, specific and allows for the quantification of target DNA. The probe-primers used in this assay are developed from mitochondrial DNA (mtDNA) because of its high copy number per cell and high biological abundance in natural habitats and its high coverage in gene databases (Goldberg et al., 2016). These characteristics makes it a better target when dealing with degraded eDNA.

The use of eDNA for ecological monitoring also presents some limitations being unable to provide information on the life stage of the organism. Samples can be easily contaminated by laboratory DNA. For this reason, the molecular analysis requires dedicated well-established laboratories and sterile system that might not be available in some places (Goldberg et al., 2016). There is also a problem of confounding sources of eDNA, which is reliant on the DNA degradation rate in the habitat hence this should be established first before making any inference (Barnes and Turner, 2016). The degradation rate is reliant on multiple environmental factors making it very difficult to predict (Pilliod et al., 2014, Barnes et al., 2014). The eDNA in each habitat could have also originated from other habitats through processes like flooding and erosion.

eDNA in aquatic environments is typically degraded within a few days in warm humid habitats to a month in more cooler habitats (Pilliod et al., 2014, Strickler et al., 2015), hence any DNA detection should represent a relatively recent colonisation of the habitat (Dejean et al., 2011b, Piaggio et al., 2014). However, eDNA can settle at the bottom of water bodies, which may promote the adsorption of eDNA by soil particles. This phenomenon may protect eDNA from ultraviolet degradation, thus allowing a longer persistence of eDNA in the habitat (months or even years) than when suspended in the water (Turner et al., 2015). Therefore, it is important to avoid any activity that would disrupt the aquatic habitats during environmental sampling. Any disruption would re-suspend older DNA in the water that could promote false positives (for recent colonization) in molecular identification by detecting the presence of organisms months after they have left the habitat or died (Dejean et al., 2011a). Thus for accurate temporal inference, it is usually advisable to avoid sediments when sampling (Turner et al., 2015). unless the objective is to record historical presence when the persistence of eDNA trapped by soil particles can provide records on past colonisation and species compositions (Thomsen and Willerslev, 2015).

eDNA studies have been used to detect and monitor a variety of aquatic organisms for example Australian bullfrog (Ficetola et al., 2008), Bluegill sunfish (Minamoto et al., 2012), Asian carps (Jerde et al., 2011), New Zealand mud snail (Goldberg et al., 2013), Burmese python (Piaggio et al., 2014), Hellbenders (Spear et al., 2015). In macroinvertebrate

insects, the technique has been used to detect ice age insect diversity (Reiss, 2006, Willerslev et al., 2007), molluscs in freshwater ecosystems (Mächler et al., 2014) also on museum beetle samples (Thomsen et al., 2009). In all of the studies above, results from eDNA have been shown to be consistent with conventional sampling methods and thus can help to save resources, in terms of time, cost and sensitivity.

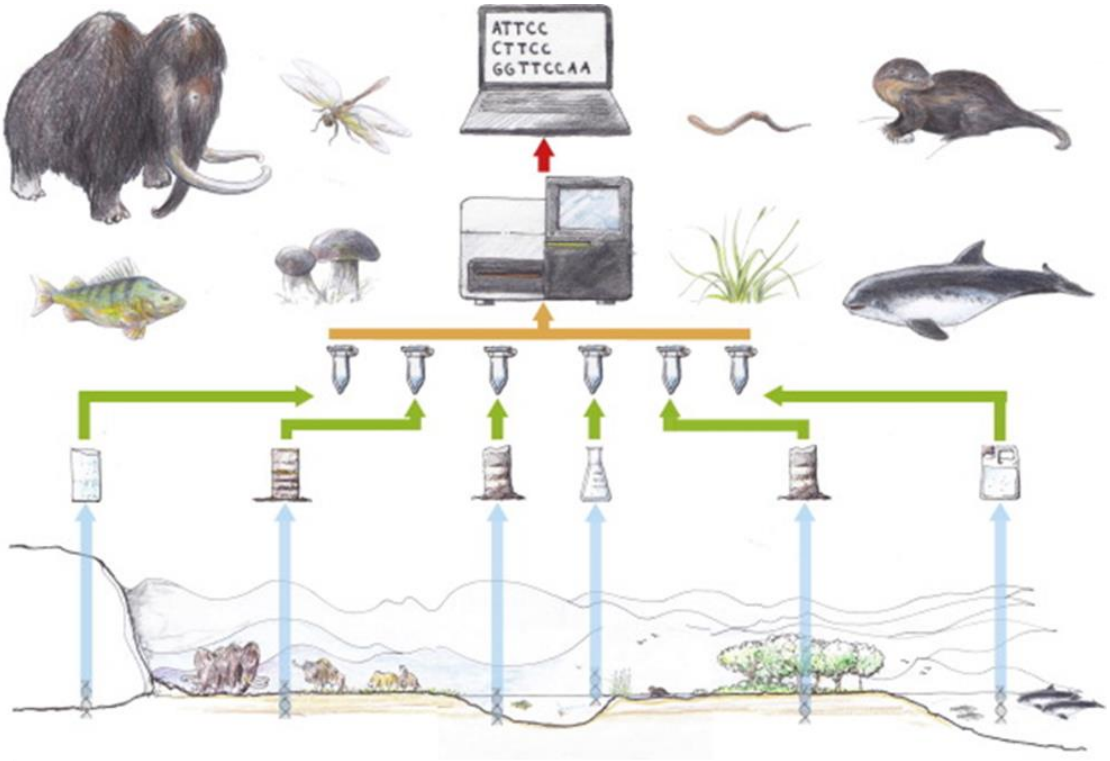


Figure 2. The overall workflow for environmental DNA (eDNA) studies with examples of organisms that have been identified from environmental samples. (Thomsen and Willerslev, 2015, Hiscox et al., 2014)

However, eDNA analysis has not been used to date to detect and quantify mosquito larvae. In this current study, we, therefore, aimed to test the applicability of this technique in the

detection and quantification of *Anopheles* mosquito larvae in a laboratory setting and in artificial habitats in a controlled field environment.

2. Aims and Objectives

2.1 Aim

To develop, test and validate eDNA as a technique to detect and quantifying *Anopheles gambiae s.l.* mosquito larvae in experimental aquatic habitats.

2.2 Objectives

1. To develop and optimize eDNA primers for identification of *Anopheles gambiae* s.s., *Anopheles arabiensis* and *An. funestus* from a mitochondrial gene using conventional PCR visualised using agarose gel electrophoresis
2. To compare two extant protocols – membrane filtration and precipitation - for extracting eDNA from water samples
3. To develop, optimize and test a TaqMan qPCR assay for eDNA to differentiate between *Anopheles gambiae* s.l. and other non-vector species
4. To optimize and test a pre-existing TaqMan qPCR assay for application to eDNA to differentiate between *Anopheles gambiae* s.s. and *Anopheles arabiensis* mosquito larvae using species-specific probes
5. To determine if larval abundance correlates with the concentration of eDNA isolated in the laboratory and in experimental aquatic environments

3. Experimental Design and Methods

3.1 eDNA PCR primer design

The eDNA primers were designed from mitochondrial gene sequence (mtDNA). The gene has high copy number per cell hence making its detection probability in degraded eDNA samples higher (Thomsen and Willerslev, 2015) when compared to ribosomal gene normally used for An. gambiae species identification (Scott et al., 1993). An initial examination of conservation of mitochondrial gene sequences across *An. gambiae*, *An. arabiensis* and *An. melas* and differentiation within the same span in *Culex quinquefasicatus* and *Aedes aegypti* suggested conserved *An. gambiae* complex-specific primer design would be difficult for cytochrome oxidase I, but feasible for the cytochrome b gene.

eDNA primers were therefore designed based on the multi-copy, mitochondrial *cytochrome b* gene (*cyt b*) for *Anopheles gambiae s.l* and *Anopheles funestus*. The *cyt b* gene sequence was obtained from the full mitochondrial DNA (mtDNA) sequence of *Anopheles gambiae* s.l. (VectorBase; L20934.1). The regions 10413-11549 of full *An. gambiae* s.l mtDNA and 10405-11544 of full *An. funestus* genome were used to perform the subsequent analysis (these are the regions within the full mitochondrial sequence where the *cyt b* gene lies in each species). Both regions were screened against a non-redundant database using NCBIs Primer-BLAST tool (www.ncbi.nlm.nih.gov/tools/primer-blast/) restricting the product size for 70-120bp, to provide optimal product sizes for efficient qPCR amplification of potentially degraded DNA. Other parameters were maintained at their default settings. This search returned ten primer pairs for *Anopheles gambiae* s.l and eight primer pairs for *An. funestus* species (see appendices).

To ensure specificity we conducted a nucleotide BLAST for each of the *cyt b* sequences and selected hits from *Anopheles merus, Aedes aegypti*, *Culex quinquefascitus cyt b* sequences. *Anopheles merus cyt b* sequence was added to confirm if the *An. gambiae* s.l *cyt b* was conserved amongst all the complex members. Sequences were assembled by CodonCode Aligner software (Version 4.2.7). All primer pairs were individually matched to

the assembly to check variability of primer regions and ensure specificity of primers to either *An. gambiae* s.l or *An. funestus.*

3.2 In silico and PCR validation of the species-specific eDNA primers

The *An. gambiae* s.l and *An. funestus* primers were validated *in silico* and by using control genomic DNA. *In silico* validation was conducted by BLAST search of the primer pairs against all known sequences in the NCBI database to confirm that the primers were only binding to the target species with zero match to non-target mosquito species and also other insects such as *Musca domestica, Anisoptera, Drosophila melanogaster, Anthophila* and *Rhopaloscera* that could be associated with mosquito breeding habitats.

Specificity of the *Anopheles gambiae* and *An. funestus* primers was then tested by amplification of known 'control' genomic DNA (gDNA) from lab-reared *An. gambiae*, *An. arabiensis* and *An. funestus* species (Liverpool strain). Polymerase chain reactions (PCR) was performed with gDNA in serial dilutions of 1:10 and 1:100. From the primer pairs designed for *An. gambiae,* we selected two primer combinations (pair 5 and 8) and three primer combinations (pair 1, 4 and 8) for *An. funestus*, which each showed consistent amplification even at low concentrations with zero cross-reactivity (i.e. only amplifying DNA from the target species). Each reaction consisted of 17.8 µl PCR water, 2.5 µl 10X Dream Taq Green Buffer, 0.5 µl of 10 mM dNTP mix, 0.2 µl Dream Taq DNA Polymerase, 0.5 µl of each primer pair and 3 µl of DNA template. The thermocycler conditions were 1 cycle 95 °C for 5 minutes followed by 35 cycles of 95 °C for 1 minute, 60 °C for 1 minute, 72 °C for 1 min and a final extension at 72 °C for 5 minutes and held at 10 °C. Five microliters of each PCR products were run on a 2% agarose TAE gel pre-stained with peqGREEN (Axonlab) for 90 minutes at 100V. Band sizes were visualized under ultraviolet light in a transillumination gel documentation system (G-Box, Syngene) and compared to a HyperLadder™ 25bp (Bioline) DNA marker.

3.3 IGS and cyt b TaqMan primer probes design

In order to detect and quantify the amount of eDNA from *An. gambiae* s.s. and *An. arabiensis* in the ponds, we used a TaqMan quantitative PCR protocol designed in *An. gambiae* ribosomal DNA from the 3' 28S to 5' intergenic spacer region (*IGS*) of the genome (Walker et al., 2007); hereafter '*IGS* TaqMan'. The reaction uses a universal primer pair for *An. gambiae* s.l complex; Forward 5'-GTGAAGCTTGGTGCGTGCT-3', Reverse 5'-GCACGCCGACAAGCTCA-3' and species-specific TaqMan probes 5'VIC-CGGTATGGAGCGGGACACGTA-3' for *An. gambiae* s.s. and 5' 6FAM-TAGGATGGAGAAGGACACTTA-3' for *An. arabiensis*.

Novel TaqMan primer-probes that would distinguish *Anopheles gambiae* complex from other malaria vector species complexes was designed using *cyt b* gene. The primer-probes had product length of 150 bp and on position 1114 at the 3' end of the gene; Forward 5' TCCTAGCTATACACTATGCCGC3' and Reverse 5' ATTTGTCACGCTAACGGAGCT3', and double dye FAM labelled probes 5'-CCCACCCTTTAATTAGAATCGCTAA-3' and 5'-CGGCATAGTGTATAGCTAGGAATAAT-3' (PrimerDesign, UK)

3.4 Laboratory testing of eDNA detection

Experiments were conducted using lab-reared second instar larvae from *An. gambiae s.s* mosquitos (G3 strain). Unfortunately, we could not conduct the laboratory testing using *An. funestus* larvae due to time constraints and the inability to obtain eggs. The larvae were reared in plastic trays (20 x 18 x 7 cm) under controlled insectary conditions of temperatures 26 – 28 °C, relative humidity of 70 – 80 % and 12:12 hour light: dark cycle and fed one time a day on fine ground TetraMin® fish food.

Different larval densities, water volumes, and varying sampling times were tested to check their effect on eDNA detection. In the first experiment, we tested 12 different conditions by

adding 45 mL of distilled, autoclaved water into 12 sterile 50 mL falcon tubes. We carried four replicates with three different larvae densities (1 larva, 3 larvae and 6 larvae). One tube from each of the three densities was then sampled simultaneously at intervals of 1 hour, 3 hours, 6 hours and 24 hours. We also had 4 negative controls (with no larvae in them). The larvae were first rinsed with distilled water to reduce chances of any carry over of eDNA from the initial rearing trays into the tubes. All the experiments were set up in a PCR workstation in a room separate from the main molecular laboratory to reduce chances of contamination from aerial mosquito DNA.

In the second experiment, we added 1 L of distilled, autoclaved water to each of five 1 L glass bottles. This was followed by adding 2, 5, 10 and 20 larvae into each experimental bottle with no larvae in the control bottle and left them standing in the enclosed environment of the PCR workstation for 24 hours. We sampled three biological replicates from each of the bottles though subsequent analysis was limited to two replicates owing to reagent constraints.

3.5.1 eDNA water sampling and extractions

eDNA from the water samples was extracted by two methods described in the literature: 1) precipitation (Ficetola et al., 2008); in the precipitation method, we sampled 15 mL of water into sterile 50 mL falcon tube. We immediately added 1.5 mL of 3 M sodium acetate solution to enhance precipitation and preservation followed by 11 mL of absolute ethanol, and stored overnight at -20 °C. Chilled samples were then centrifuged at 5000 rpm, 6°C for 1 hour. The supernatant was discarded and the pellet was processed for eDNA extraction. and 2) membrane filtration (Goldberg et al., 2011); in the membrane filtration method, we directly filtered 40 mL of water samples through 0.22 µm Millipore membrane filter (HACH, UK) using a vacuum pump. eDNA from either the pellet or the membrane was extracted using the ChargeSwitch® Forensic DNA Purification Kit (Invitrogen) and Nexttec™. The ChargeSwitch® protocol had some modifications with an overnight incubation at 4°C with 1 mL of lysis buffer, and 10 µl of Proteinase K. A lysis step was carry at 56°C for 90 min. All samples were eluted with 60 µl of the proprietary elution buffer.

3.5.2 PCRs and sequencing to identify sample species

We conducted PCR amplification using two *An. gambiae* primer combinations using the same reaction conditions as described in section 3.2 above. The PCR product was cleaned by ExoSAP-IT® PCR Product Cleanup protocol (Affymetrix) adding 2 µl of ExoSAP-IT reagent in each 5 µl of amplified product. The mixture was incubated in a thermocycler for 15 minutes at 37°C, to degrade excess primers and nucleotides, followed by 15 minutes at 80°C to deactivate the ExoSAP-IT reagent. The products were sequenced commercially (SourceBioscience) and sequences aligned against reference *Anopheles gambiae* mitochondrial *cyt b* sequence using CodonCode Aligner software (Version 4.2.7) (CodonCode Corporation, UK)

3.6 Controlled field testing

3.6.1 Field site location

Field trials were conducted at the International Centre for Insect Physiology and Ecology (ICIPE) located in Mbita, western Kenya. All experiments were conducted between 14th and 23rd June 2016.

3.6.2 Artificial pond set-up

In an open field, we set up 20 artificial ponds using disinfected 50 L black plastic tubs buried into the ground leaving 2 cm of the tub aboveground. Each tub had two small 1 cm diameter overflow holes on each end covered with a mosquito netting. There was a total of four rows and 5 columns of pond with each 4 metres apart. A total of 35 L of filtered lake water was added to each pond; this water source is the standard used for rearing mosquitos in the insectaries at ICIPE.

Ponds were randomly allocated to receive 16 treatments and 4 negative controls (without mosquito larvae). In treatment ponds, we added second instar *An. gambiae* s.s and *An. arabiensis* larvae reared from the ICIPE colonies in densities of 6, 24, 48, and 96 larvae (Table 2). The *An. gambiae* s.s were Mbita strain and the *An. arabiensis* were a strain that originated from Mwea in central Kenya.

Table 2: Artificial pond set up layout showing the number of larvae for each species, *An.arabiensis* and *An. gambiae* s.s, added to each pond.

Pond ID	***An. arabiensis*** (no. of larvae)	***An. gambiae* s.s.** (no. of larvae)	**Larval Totals**
1	2	4	6
2	4	2	6
3	0	0	0
4	32	64	96
5	64	32	96
6	16	32	48
7	32	16	48
8	2	4	6
9	4	2	6
10	0	0	0
11	8	16	24
12	16	8	24
13	16	32	48
14	32	16	48
15	8	16	24
16	0	0	0
17	0	0	0
18	16	8	24
19	32	64	96
20	64	32	96

All ponds were covered throughout the experiment period (birds removed some covers but they were put back in position immediately after noticing) with a mosquito net to prevent wild female mosquito oviposition. We also measured and recorded water turbidity, conductivity, dissolved oxygen level, pH and temperature of each pond 3 times over the experimental period (9 days).

3.6.3 Sample collection

The sampling began 24 hours after the larval introduction and continued over an 8day period – 5 days with the larvae in the ponds and for 3 days after removal of all the larvae including emerged adults and exuviate. Each pond was sampled on an alternate day. During the first 5 days, we collected one replicate of each treatment (8ponds) and 2 negative control ponds on each sampling day (Table 2). Three collection tubes with 50 mL of water were gently drawn (*i.e.* avoiding disturbance) from equally spaced points around the pond in order to increase our chances of collecting eDNA from the larvae (Biggs et al., 2014, Piaggio et al., 2014). Sampling was done in sterile 50 mL falcon tubes by one person with fresh sterile gloves (*i.e.* changed between ponds). Water samples were immediately put on the ice and once all daily samples were collected, transported to the laboratory for downstream analysis.

On the fifth day, we aspirated all the emerged adults (with some escaping into the air) and removed the larvae, pupae and exuviates that were shed during emergence. Adults, larvae, and pupae from each pond were placed in labelled 1.5 mL Eppendorf tubes and preserved by adding absolute ethanol for storage at -20C.

3.6.4 Water sample filtrations

Three samples of 50 mL of water from each pond were vacuum filtered through one 0.22 µm Millipore membrane filter (HACH). After filtration, the membrane was air-dried for two minutes at room temperature and placed in a labelled Ziploc bag. All the equipment; Buchner funnels, tweezers, collecting jar, were disinfected between each sample by washing in 10% bleach and rinsing in 70% ethanol and autoclaved at the end of each day. All filtrations were performed in a biosafety cabinet that was disinfected before each use. Membranes were placed in a tightly-sealed plastic container containing silica gel (*i.e.* dry conditions) for transportation.

3.6.5 DNA extractions

DNA was extracted from the membranes using the ChargeSwitch® Forensic DNA Purification Kit (Invitrogen) (as described in section 3.5.1). DNA extraction from the larvae and adult mosquitoes was conducted by individually grinding them in STE buffer (100 mM

NaCl, 10 mM Tris-HCl, pH8.0, 1 mM EDTA, pH 8.0), heating to 95°C for 5 mins, centrifuging for 3 mins at 13,000 rpm and the supernatant used directly as PCR template.

3.6.6 TaqMan standard curves determination and cyt b and IGS assays on the eDNA samples

We prepared our qPCR standards by conducting a 1:5 dilution series of both *An. gambiae* and *An. arabiensis* gDNA and a third gDNA mixture from both species. The Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher) was used to determine the DNA concentration of the starting template for each series. These dilutions were also used as controls in all subsequent TaqMan assays. The total qPCR reaction volume was 20 µl and consisted of 1 µl primer probe pair (PrimerDesign, UK), 10 µl TaqMan® Gene Expression Master Mix (Applied Biosystems, USA), 4.5 µl nuclease free water and 4.5 µl eDNA template. The samples were then run on an Agilent Mx3005P qPCR System with a thermal profile; 1 hold at 95 °C for 10 minutes followed by 50 cycles of 92 °C for 15 seconds and 57 °C for 60 seconds.

In the Walker TaqMan assay, fluorescence was recorded through the FAM and VIC channels. A significant increase in FAM dye indicated the presence of *An. arabiensis,* while an increase in VIC dye indicated the presence of *An. gambiae* s.s. In the novel eDNA TaqMan assay, fluorescence was recorded through the FAM channel. Significant increase in FAM dye indicated the presence of *An. gambiae* s.l. eDNA in the sample.

4. Results

4.1 Primer design and PCR validation

Initial examination of conservation of mitochondrial gene sequences across *An. gambiae*, *An. arabiensis* and *An. melas* and differentiation within the same span in *Culex quinquefasicatus* and *Aedes aegypti* suggested conserved *An. gambiae* complex-specific primer design would be difficult for cytochrome oxidase I, but feasible for the cytochrome b gene. Using Primer-BLAST ten specific primer pairs for *An. gambiae* s.l. were designed (Appendix I). Following matching each primer to the assembly of the other mosquito species (above), primer pairs 1, 4, 5 and 8 were chosen for further assessment. These pairs exhibited at least three polymorphic bases towards the 3' end of either the forward or reverse sequence differentiating them from the culicine mosquito species , which should ensure specificity during the primer annealing process in the PCR (Wright et al., 2014). These four primer pairs were also selected based on their self-complementarity values (binding of primers to itself), which was less than three, and regions on the gene where they were located to ensure they covered different parts of the gene (Appendix II). We were also able to design cyt b primer pairs for *An. funestus* complex which were additionally checked for 3' specificity as before against *Anopheles gambiae s.l.*, *Aedes* and *Culex* sequences (Appendix III). Applying the same criteria as for *An. gambiae* s.l. above, we selected final primer pairs 1, 5, and 8 for the *in situ* and PCR validations (Table 3).

All the *An. gambiae* s.l. eDNA primers showed 100% amplification on gDNA from *An. gambiae* s.s. (N=4 samples) at 1:10 concentration. At 1:100 dilutions, only primer pairs 5 and 8 showed consistent amplification (100% and 75% respectively), hence they were selected to be tested on eDNA from water samples. All the eDNA primers for *An. funestus* had 100% amplification at both 1:10 and 1:100 dilutions.

Table 3: Summary of criteria for primer selection. The red ticks represent *An. gambiae* s.l. primers and the blue ticks represent *An. funestus* primers.

Primer No.	Species specificity	Self-complementarity	Variants*	PCR template dilution	
				1:10	1:100
1	✓	✓	✓	×	×
1	✓	✓	✓	✓	✓
2	✓	✓	×	×	×
2	✓	✓	×	×	×
3	✓	×	×	×	×
3	✓	✓	×	×	×
4	✓	✓	✓	×	×
4	✓	✓	×	×	×
5	✓	✓	✓	✓	✓
5	✓	✓	✓	✓	✓
6	✓	×	×	×	×
6	✓	✓	×	×	×
7	✓	×	×	×	×
7	✓	✓	×	×	×
8	✓	✓	✓	✓	✓
8	✓	✓	✓	✓	✓
9	✓	✓	×	×	×
10	✓	✓	×	×	×

* Identification of at least 3 nucleotide differences in at least one of the primers

4.2 Nexttec™ Vs ChargeSwitch® nucleic acid purification protocols

We compared two DNA extraction protocols (Nexttec™ and ChargeSwitch®) to check on which protocol was more efficient at extracting eDNA from water samples (obtained as 15ml taken from high density *An. gambiae s.s.* insectary rearing trays, and prepared using the precipitation method – see Methods). Nexttec is a fast (4 minutes) column-based one-step DNA purification protocol with typically high DNA yield while ChargeSwitch is a magnetic bead-based protocol that isolates DNA based on changes in pH of the surrounding extraction buffers (90 minutes). Nexttec-extracted samples did not show any amplification with either *An. gambiae* s.l primer pairs 5 or 8, while ChargeSwitch-extracted DNA showed 100% amplification for both (Figure 3). Owing to this clear difference in performance, ChargeSwitch® was chosen for all subsequent eDNA extractions.

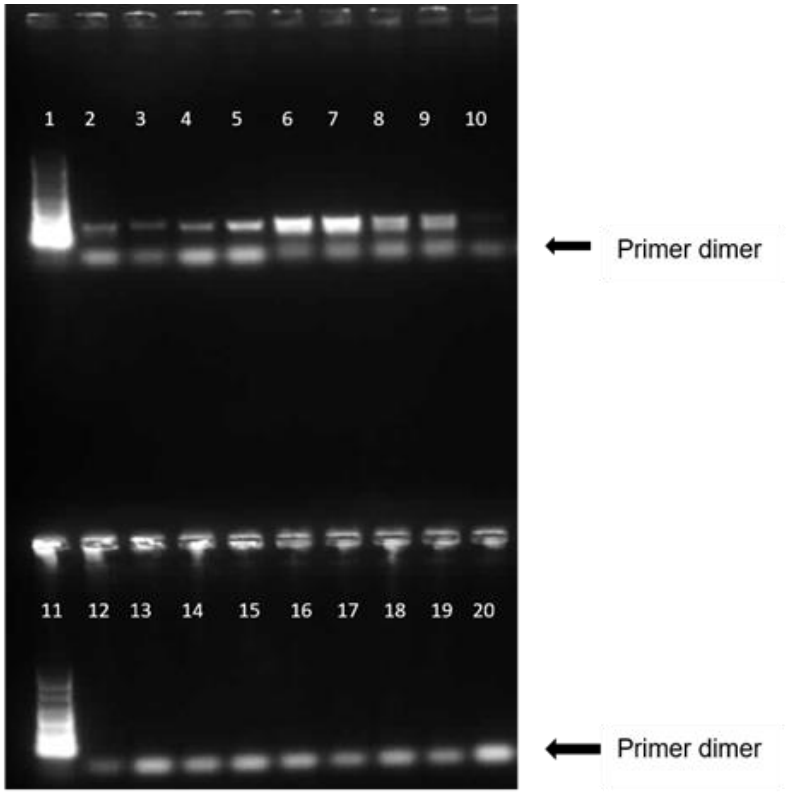


Fig. 3. Agarose gel image of PCR products comparing the two DNA isolation methods, Nexttec and ChargeSwitch on eDNA water samples. Lanes 1 and 11: 100bp DNA ladder, Lanes 2 – 5: ChargeSwitch samples with primer set 5 and lanes 6 – 9 using primer set 8, Nexttec samples: Lane 12 -15 with primer set 5, lane 16 -19 with primer set 8. Lane 10 and 20 are non-template controls.

4.3 Comparison of eDNA concentration protocols - membrane filtration vs. precipitation – with different larval conditions

The two protocols tested for eDNA concentration, filtration and precipitation, were compared against each other according to PCR success. In the precipitation method, 40/46 samples (87%) produced PCR bands. Albeit from a smaller test set, the membrane filtration method resulted in a similar outcome with 7/8 samples (87.5%) with PCR products (Table 4). Results suggested that at least 6 h in the habitat was required for an eDNA signal from a single larva (Table 4). However, increasing the larval density to three or six typically gave a consistent signal after just 1 h, though samples from habitats occupied by

larvae for 24 h gave stronger signals. Increasing the water volume of the habitat from 50ml to 1 L for 24 h with a minimum of two larvae had no direct effect on capacity to detect eDNA. This suggested that larval number and duration of occupancy might be of more importance than simply larval density.

When both eDNA concentration protocols were compared directly for larval numbers of 1,3 and 6 held for 6 h, the membrane filtration protocol had stronger eDNA bands especially at the highest larval density (Table 4). However, this could have reflected the difference in extraction volumes used for the two protocols. A sample of N=7 PCR products sequenced aligned perfectly to the *cyt b* gene sequence from the complete *An. gambiae* s.l. mitochondrial sequence (accession number L20934.1).

Table 4: PCR detection of eDNA samples processed by precipitation and filtration protocols. The band strengths were scaled accordingly: weak, mid, and strong. The band strength column with two labels indicate variation between the replicates.

No. larvae pooled	Volume of water	Hours	Extraction volume	Extraction method	No biological replicates	+ PCR results	Band strength
1	50 mL	1	15 mL	Precipitation	3	0/3	0
3	50 mL	1	15 mL	Precipitation	3	3/3	weak
6	50 mL	1	15 mL	Precipitation	3	3/3	weak
1	50 mL	6	15 mL	Precipitation	2	1/2	weak
3	50 mL	6	15 mL	Precipitation	3	3/3	weak
6	50 mL	6	15 mL	Precipitation	3	2/3	weak, mid
1	50 mL	24	15 mL	Precipitation	3	2/3	weak
3	50 mL	24	15 mL	Precipitation	3	3/3	weak
6	50 mL	24	15 mL	Precipitation	3	3/3	mid
2	1 L	24	15 mL	Precipitation	4	4/4	weak
5	1 L	24	15 mL	Precipitation	6	6/6	weak
10	1 L	24	15 mL	Precipitation	4	4/4	mid, strong
20	1 L	24	15 mL	Precipitation	6	6/6	weak, strong
1	50 mL	6	40 mL	Filtration	2	2/2	weak
3	50 mL	6	40 mL	Filtration	3	2/3	weak
6	50 mL	6	40 mL	Filtration	3	3/3	weak, mid, strong

4.4 Analysis of laboratory eDNA samples based on qPCR

4.4.1 Standard curves

The novel TaqMan assay developed based on the *cyt b* gene was designed to differentiate between *Anopheles gambiae s.l.* (including the three main malaria vectors *An. gambiae s.s.*, *An. coluzzii*, and *An. arabiensis*) and other species, including other *Anopheles*. The assay uses double-dye FAM-labelled probes that are expected to be highly specific. Any sample with a dye signal above a threshold value (Ct) is recorded as positive for *Anopheles gambiae* s.l.

A qPCR TaqMan standard curve was performed by conducting a fivefold dilution series from *An. gambiae* s.s. gDNA of known concentration. From this standard curve we aimed to determine: 1) linearity (on a log scale) of the qPCR across DNA concentrations; 2) detection limits for the assay; 3) quantitation equations for DNA present in an unknown sample (Fig. 4).

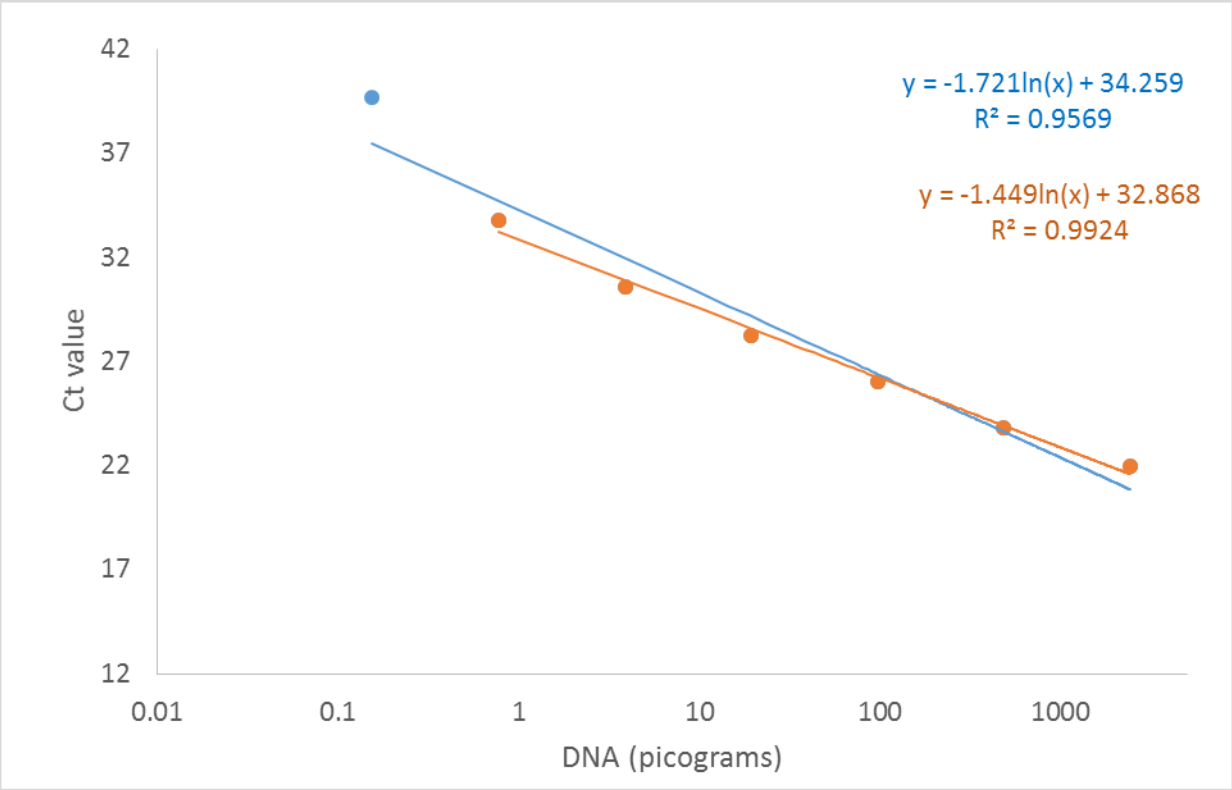


Fig 4. TaqMan standard curve for *cyt b* across a fivefold dilutions series of gDNA from *Anopheles gambiae* s.s. The blue line shows a regression line fitted through all points, with associated equation in blue font (upper equation). The orange line shows a line plotted through points excluding the lowest concentration sample, with associated equation in orange font (lower equation).

The *cyt b* standard curve exhibited evidence of non-linearity driven by a visually outlying point at the lowest dilution resulting in a poor fit of the regression line (Fig. 4). In subsequent repetitions using the same standard series on eDNA sample plates, the same pattern was not observed (data not shown). Therefore, the lowest dilution was excluded, leading to a good curve fit, for calculation of the predictive equation which was used for future calculations of concentration from samples. The low limit for detection was lower than that included in the dilution series (minimum of 0.156 pg) though because quantitation below 0.778 pg was extrapolated from the curve, accuracy at low DNA concentrations might be biased.

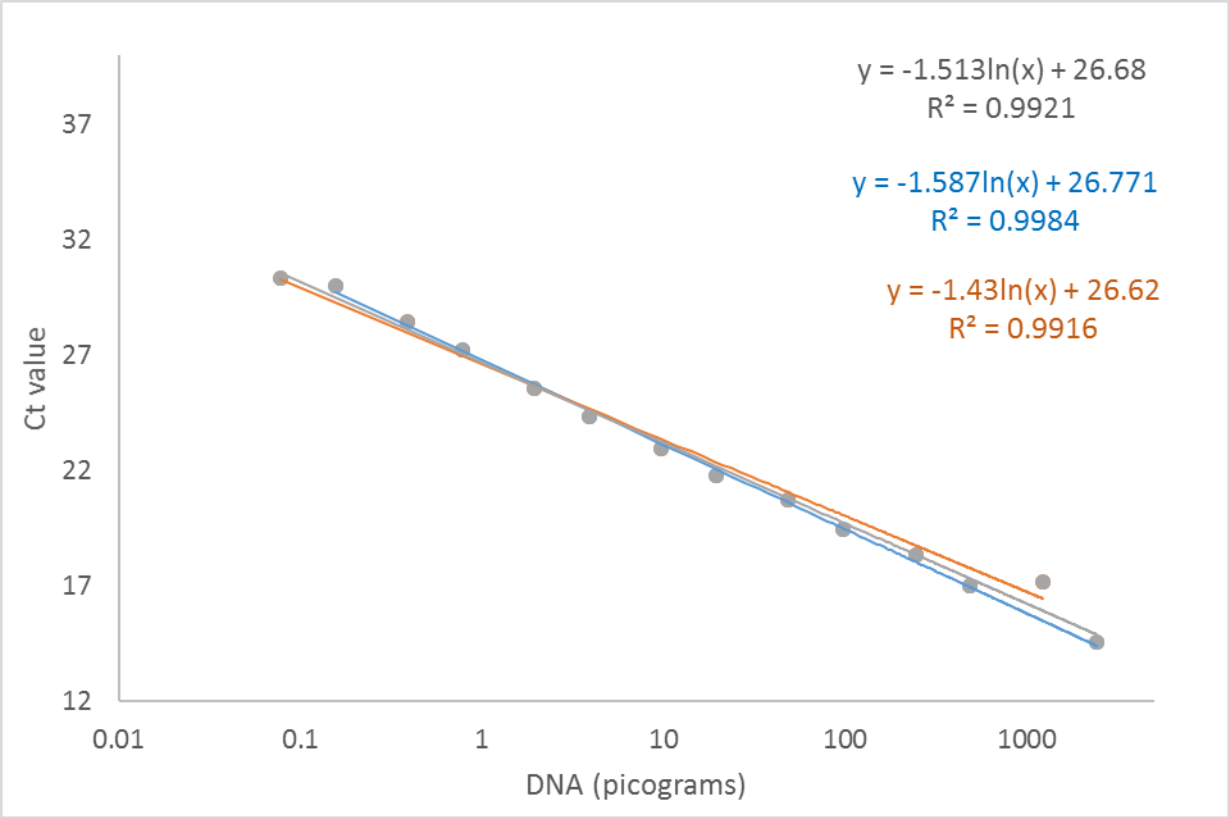


Fig 5. TaqMan standard curve for *IGS* (Walker et al.2007) assay across a fivefold dilutions series of gDNA from *Anopheles gambiae* s.s (blue line and equation), An. arabiensis (orange line and equation) or both (grey line and equation).

In contrast to the *cyt b* assay the IGS assay was linear across the range of concentrations tested, with good model fit for both *An. gambiae* and *An. arabiensis* DNA detection (Fig. 5). In fact, the difference between the two models was very slight and the model fit for the combined data was almost identical, permitting the use of this single predictive equation for DNA detection from subsequent samples. As with the *cyt b* assay the IGS assay readily detected DNA at the lowest dilution of 0.156 pg and quantitation also appears accurate throughout the range of dilutions.

4.4.2 Analysis of laboratory samples

Both the IGS and the *cyt b* assay could detect *Anopheles gambiae* s.s. eDNA in the undiluted samples of 2, 5, 10 and 20 larvae from the 1L laboratory habitats (Table 5). To test the limit of detection of the two assays, we conducted a fivefold dilution (1/5, 1/25 and 1/125) and ran a TaqMan with four replicates for both assays. The assays could only detect eDNA in the lowest larval number (N=2) samples without dilution (2/L). With 5 larvae in the starting water sample both the assays could detect eDNA at 1/5 dilution (1/L), with inconsistent, possibly erroneous, detection by IGS also at the lowest dilution of 1/125. For the 10 larvae water sample, detection at the lowest dilution (0.08/L) was possible but in a minority of replicates, whereas from the 20 larvae starting sample consistent detection was observed at the lowest dilution (0.2/L) with both assays.

There was a significant positive correlation between larval densities and eDNA detection (38 positive samples for IGS and 32 for *cyt b* -Table 5) in both assays: IGS, $r = 0.503$, $p = 0.047$; and *cyt b,* $r = 0.558$, $p = 0.025$. However, there was also a stochasticity in eDNA detection across the samples which reflected larval number in addition to larval concentration (Table 5). For example, 0.2/ L was readily detected from an initial sample containing 20 larvae but not in one originating from 5 larvae, and similarly, 0.08/L was undetectable when water originated from a sample with 2 larvae but was detected at least occasionally from 10 larvae habitats. Such stochasticity suggests that accurate eDNA quantitation at low densities could be difficult. This experiment suggested that depending on starting larva numbers, concentrations of 0.04 larvae/L could be detectable, or 0.2/L detectable with complete reliability in either assay.

The eDNA concentration calculated using the IGS assay was consistently lower than that of *cyt b* across all the habitats and dilutions. The *cyt b* assay is based on a mitochondrial gene that has greater copy number per cell hence is likely to giving lower Ct values in the qPCR compared to a ribosomal gene with a lower abundance (Goldberg et al., 2016). However, the detection limits for the IGS and *cyt b* assays appear comparable.

Table 5: Comparison of results from the *IGS* and *cyt b* TaqMan assays showing the number of positive samples in each of four replicates and the average concentrations for p samples. The larval density was a fivefold dilution series (d1,2,3) from the initial number of larvae in the water sample, C. Minimum sample dilutions at which eDNA was detected are highlighted in red.

2 Larvae		Density	Positives	Av Conc	std dev	5 Larvae		Density	Positives	Av Conc	std dev
IGS	C	2	2/4	0.0002	0.0002	**IGS**	C	5	4/4	0.0006	0.0002
	d1	0.4	0/4	NA	NA		d1	1	3/4	0.0003	0.0002
	d2	0.08	0/4	NA	NA		d2	0.2	0/4	NA	NA
	d3	0.016	0/4	NA	NA		d3	0.04	2/4	0.0030	0.0008
	NTC	0	0/2	NA	NA		NTC	0	0/2	NA	NA
Cyt b	C	2	2/4	0.0306	0.0422	***Cyt b***	C	5	4/4	0.0885	0.0244
	d1	0.4	0/4	NA	NA		d1	1	1/4	0.0501	NA
	d2	0.08	0/4	NA	NA		d2	0.2	0/4	NA	NA
	d3	0.016	0/4	NA	NA		d3	0.04	0/4	NA	NA
	NTC	0	0/2	NA	NA		NTC	0	0/2	NA	NA
10 Larvae		**Density**	**Positives**	**Av Conc**	**std dev**	**20 Larvae**		**Density**	**Positives**	**Av Conc**	**std dev**
IGS	C	10	4/4	0.0078	0.0035	**IGS**	C	20	4/4	9.0527	1.0964
	d1	2	4/4	0.0017	0.0010		d1	4	4/4	1.7046	0.0821
	d2	0.4	2/4	0.0004	0.0002		d2	0.8	4/4	0.3758	0.0884
	d3	0.08	1/4	0.0002	0.0001		d3	0.2	4/4	0.0818	0.0182
	NTC	0	0/2	NA	NA		NTC	0	0/2	NA	NA
Cyt b	C	10	4/4	0.7848	0.4672	***Cyt b***	C	20	4/4	27.2678	5.5316
	d1	2	3/4	0.2078	0.1813		d1	4	4/4	6.8140	1.8393
	d2	0.4	1/4	0.1270	NA		d2	0.8	4/4	1.7338	0.2231
	d3	0.08	1/4	0.0346	NA		d3	0.2	4/4	0.3709	0.1233
	NTC	0	0/2	NA	NA		NTC	0	0/2	NA	NA

4.3 Analysis of field eDNA samples

The IGS (Walker et al., 2007) and the *cyt b* assay were used to detect the relative proportions and quantify the eDNA concentration of each species. The artificial ponds had varying total larval numbers and relative proportions of *An. gambiae s.s.* and *An. arabiensis* larvae (Table 6 and 7, full data set in Appendix IV). The lowest larval density used in the ponds was 6 larvae; this value was determined from the laboratory trial which revealed reliably detectable density of 0.2 larvae/L (translating to 7 larvae in a 35L pond, but required an equal number for division between (Appendix IV). Based on artificial pond size this density was also concordant with the estimate of typical larval densities in natural breeding habitats (3 to 10 larvae per M^2) (Ndenga et al., 2011). On days 1 – 5 (larvae were introduced on day 0) water samples were taken with the larvae present in the ponds and on days 6 – 8 samples were taken after removal of all the larvae, adults and exuviae (on day 5 after water sampling). Ponds were analysed on alternate days (owing to water processing time constraints) yielding a total of 80 samples (10 from each sampling day), which were processed by filtration at ICIPE, then DNA extracted from dried filters and analysed in each TaqMan assay with two technical replicates for the *cyt b* assay and one for the IGS assay (owing to reagent limitation).

4.3.1 IGS assay

Overall in the IGS assay (Figure 6), 11 samples were positive for *An. gambiae* s.s., 9 for *An. arabiensis*, and 3 for both species, from 13 different ponds in total (Table 6). In the first 5 days of sampling (with larvae in the ponds), the *An. gambiae* s.s. probe gave positive signals in 9 ponds compared to the *An. arabiensis* probe that was positive in 3 ponds. In the period between day 6 – 8 (after removing all larvae, adults and exuviate), 5 ponds were positive for *An. gambiae* s.s and ponds were positive for *An. arabiensis*. Larval detection within the ponds over the 8 day sampling period was very inconsistent, with one species being positive in a sampling day and negative (or positive for the other species)

when the same pond was sampled again (Table 6). For example, pond 8 was positive for *An. gambiae* s.s. when sampled at day 2 but was positive for *An. arabiensis* when sampled at day 6.

There was no correlation between the number of *An. arabiensis* and the eDNA concentration in the ponds (N = 12 ponds; r = - 0.247, p = 0.438), nor between the number of An. *gambiae s.s.* and the eDNA concentration in the ponds (N = 14 ponds; r = - 0.198, p = 0.497). In the ponds that both species were detected (pond 12, 13 and 16), there was no relationship between density of each species and its eDNA concentration. For example, *An. arabiensis* had higher eDNA concentration (0.25 pg) in pond 13 than in pond 12 (0.16 pg) despite a 1:2 ratio with *An. gambiae* s.s. in the former and 2:1 in the latter. Three of the four experimental negative control ponds (10, 16, and 17) were positive for eDNA from *An. gambiae s.s* (pond 10 on day 1 and pond 17 on day 6) and *An. arabiensis* (pond 10, day 6) with pond 16 being positive for both species at day 6. Notably in each negative control pond a positive sample for each species was detected on only one sampling day (Table 6). The calculated concentrations from the control ponds (range 0.00089-0.3759) spanned the full range of those in the ponds containing larvae, barring one outlier (pond 2, day 1) for which the concentration estimate is improbably high. This suggests that all estimates from the ponds with larvae cannot be considered as reliable owing to potential for contamination. For the IGS assay two qPCR negative controls were run; neither exhibited any Ct.

Table 6. Calculations of eDNA concentration in the field artificial ponds using *IGS* (Walker et al. 2007) assay**.** Data shown is for only ponds that were TaqMan positive

Sampling Day	Pond No.	No of larvae		Total No. larvae	Ct		DNA concentration (pg)	
		An. arabiensis	*An. gambiae s.s.*		FAM	HEX	*An. arabiensis*	*An. gambiae s.s*
1	1	2	4	6	38.23	No Ct	0.02392	NA
	2	4	2	6	No Ct	23.09	NA	10.72706
	10	0	0	0	No Ct	37.37	NA	0.00085
	12	16	8	24	39.05	38.18	0.01590	0.00050
2	8	2	4	6	No Ct	35.83	NA	0.00236
3	3	0	0	0	No Ct	38.74	NA	0.00035
	6	16	32	48	No Ct	38.09	NA	0.00053
4	13	16	32	48	No Ct	36	NA	0.00211
5	6	16	32	48	No Ct	36.47	NA	0.00155
	10	0	0	0	37.2	No Ct	0.03993	NA
	11	8	16	24	No Ct	38.89	NA	0.00031
	12	16	8	24	38.88	No Ct	0.01731	NA
6	8	2	4	6	34.17	No Ct	0.18045	NA
	9	4	2	6	No Ct	39.06	NA	0.00028
	13	16	32	48	33.5	35.95	0.25188	0.00218
	16	0	0	0	32.7	30.03	0.37509	0.10925
	17	0	0	0	No Ct	36.75	NA	0.00129
	18	16	8	24	38.29	No Ct	0.02321	NA
7	2	4	2	6	No Ct	37.69	NA	0.00069
	6	16	32	48	39.24	No Ct	0.01447	NA
	11	8	16	24	38.51	No Ct	0.02080	NA
8	15	8	16	24	33.23	No Ct	0.28811	NA
	17	0	0	0	38.12	No Ct	0.02526	NA

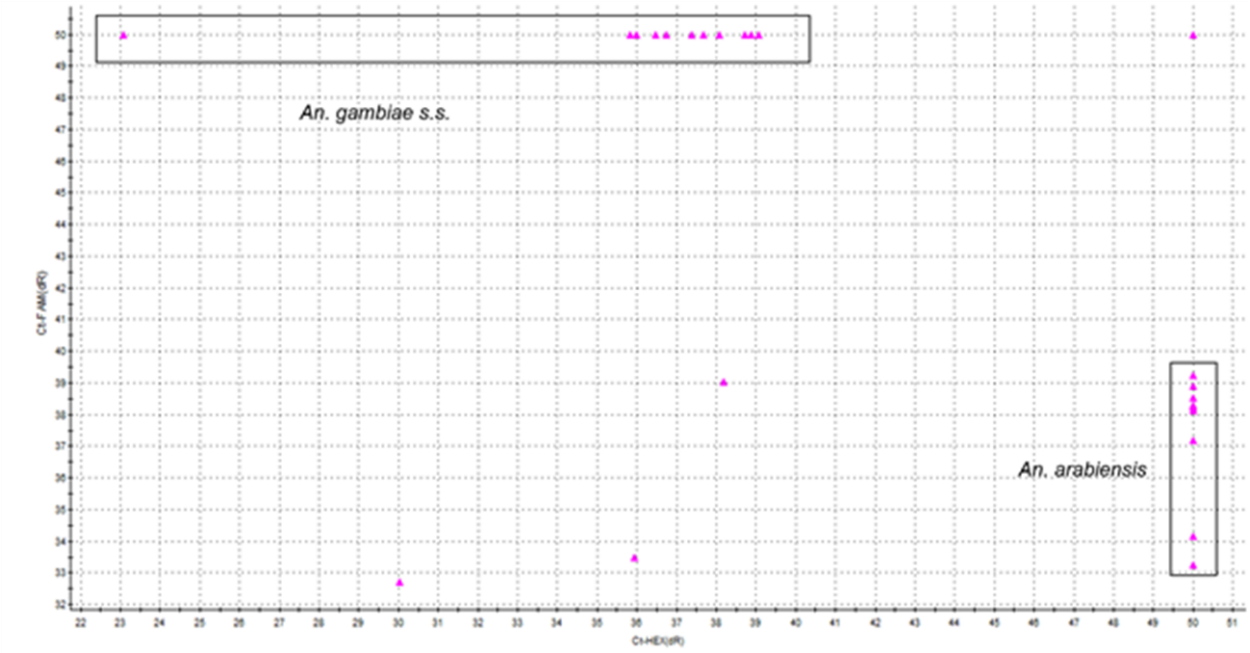


Fig. 6. A dual color scatter plot of allele discrimination in the *IGS* TaqMan assay for *An. gambiae s.s.* and *An. arabiensis* pond sample eDNA. The points not within the labelled boxes are positive for both species or for neither (point at maximum Ct on both axes). The FAM dye (y-axis) indicates *An. arabiensis* DNA (i.e. no HEX signal) while the HEX dye (x-axis) is for *An. gambiae s.s.* (i.e. no FAM signal).

4.3.1 Cyt b assay

There were 25 samples (31.25%) positive for *An. gambiae* s.l in the *cyt b* assay, these were from 12 out of the 20 ponds sampled (Table 7). Nine positive samples were taken between days 1 – 6 (with larvae in) compared to 16 positive between date 5 – 8 (after removing larvae, adults and exuviate). As before, there was no correlation between the total number of larvae in a pond and the average concentration of eDNA (r = - 0.156, p = 0.456). Whilst the IGS assay gave positive results in water samples from 14/20 ponds while *cyt b* assay gave positive results in 12/20 ponds. Overall 12 samples were positive in both assays, whilst a further 13 were positive in the *cyt b* assay alone. When comparing concentration calculations of the same sample collected on the same day, the *cyt b* assay gave higher concentrations than the IGS assay, as expected from the earlier results of the laboratory samples. Crucially, two out the four negative control ponds were positive for eDNA, both also detected as positive in the IGS assay. Pond 16 was positive on day 6

alone, whilst pond 17 was positive on consecutive sampling days at the end of the experiment. The concentrations from the control pond positives (2 – 612 pg) spanned most of the range of estimates from the ponds with larvae, again barring the outlying estimate from pond 2, day 1, also detected as extreme using the IGS assay (above). For the *cyt b* assay two qPCR negative controls were run on each plate (i.e. N=4); neither exhibited any Ct.

Table 7. Calculations of eDNA concentration in the field artificial ponds using the *cyt b* assay**.** Data shown is for only ponds that were TaqMan positive in at least one replicate

Sampling day	Pond No	No of larvae		Total No. Larvae	Ct		DNA concentration (pg)		Average DNA concentration	Std dvtn
		An. arabiensis	*An. gambiae s.s*		Replicate 1	Replicate 2	Replicate1	Replicate 2		
1	2	4	2	6	19.32	11.71	129.6071	19816.0964	9972.8517	13920.4501
2	8	2	4	6	42	23.71	0.0000	7.1206	3.5603	5.0350
	15	8	16	24	NA	20.46	NA	61.0100	61.0100	NA
	20	64	32	96	30.8	17.97	0.0657	316.3264	158.1960	223.6301
3	11	8	16	24	NA	24.71	NA	3.6768	3.6768	NA
4	13	16	32	48	15.71	21.46	1408.8030	31.5035	720.1533	973.8978
	18	16	8	24	NA	18.97	NA	163.3404	163.3404	NA
5	4	32	64	96	24.84	NA	3.3741	NA	3.3741	NA
	7	2	4	2	NA	25.71	NA	1.8986	1.8986	NA
6	8	2	4	6	NA	22.46	NA	16.2674	16.2674	NA
	9	4	2	6	34.1	NA	0.0074	NA	0.0074	NA
	13	16	32	48	27.5	24.71	0.5816	3.6768	2.1292	2.1887
	15	8	16	24	29.75	NA	0.1315	NA	0.1315	NA
	16	0	0	0	16.97	NA	612.6006	NA	612.6006	NA
	17	0	0	0	NA	21.46	NA	31.5035	31.5035	NA
	18	16	8	24	22.68	NA	14.0659	NA	14.0659	NA
	20	64	32	96	22.7	NA	13.8812	NA	13.8812	NA
7	2	4	2	6	NA	18.97	NA	163.3404	163.3404	NA
	11	8	16	24	27.02	NA	0.7987	NA	0.7987	NA
8	8	2	4	6	NA	25.71	NA	1.8986	1.8986	NA
	13	16	32	48	35.23	NA	0.0035	NA	0.0035	NA
	15	8	16	24	NA	22.46	NA	16.2674	16.2674	NA
	16	0	0	0	28.19	24.71	0.3686	3.6768	2.0227	2.3393
	18	16	8	24	28.03	NA	0.4097	NA	0.4097	NA
	20	64	32	96	28.73	21.46	0.2580	31.5035	15.8807	22.0939

Owing to the contamination problem indicated by relatively high levels of DNA detected in negative controls, no further analysis was attempted on the field data. Also, for the same reason, plans to genotype the adults and larvae recovered from the experimental ponds (to assess numbers surviving from each species) were discontinued.

5. Discussion

5.1 Summary of results

This is the first study in mosquitos to demonstrate that analysis of water samples from aquatic breeding habitats can be used to detect presence of larvae in a laboratory setting. This process involved as a first step, developing, validating and optimising convectional PCR primers for both *An. gambiae* s.l. and *An. funestus* and developing *cyt b* TaqMan primer-probes for *An. gambiae* s.l. An eDNA concentration and extraction protocols were also developed and optimized. The IGS TaqMan originally designed for distinguish *An. gambiae* s.s. from *An. arabiensis* mosquitos was also optimised for eDNA work and its efficacy compared to the novel *cyt b* TaqMan assay using laboratory eDNA water samples. Finally, we went further to demonstrate the applicability of this tool in a controlled field setting in western Kenya. Unfortunately, the field data was highly affected by contamination in the negative control samples hence no conclusive results could be drawn from it.

5.2 Strengths and weaknesses of this study

5.2.1 Laboratory trials

There was good linearity of the DNA quantification standard curves in both the *cyt b* and IGS assays. The similarity in IGS eDNA quantification for both species makes relatively accurate quantification possible in habitats where the two species are sympatric without having to calculate correction factors for dye bias in the TaqMan. It should be noted that calculated eDNA concentration from the two assays are not comparable, due to the bias of using a multi copy gene (*cyt b*) vs a gene that occur in relatively low copies (IGS), but it should be fine to use either. It is difficult to say which assay works better at present as they both lost sensitivity at very low eDNA concentration.

Contamination is a major problem for any eDNA study but at the laboratory stage we had no problem with contamination. The eDNA extraction and concentration protocols worked fine at the laboratory stage in LSTM but a more peristaltic pumps in the field will be required to allow more water to be processed.

The detection at very low larval numbers was possibly inconsistent and had high stochasticity in both assays. This makes eDNA analysis better for detecting multiple larvae in a cryptic species situation and as a quick assay to estimate relative species numbers. This also implies that detection of these small short-lived (aquatic stage) species could be difficult and might require many samples to be collected from a single breeding site.

5.2.2 Field assay testing

5.2.2.3 Possible causes of low number of positive field samples

The low number of positive samples in the filed experiments could have been caused by 1) DNA degradation within the ponds and on the filters. This was unlikely though as larval survival in the ponds was high and the constant release of eDNA could have created a balance with any degradation. Some positive ponds had strong eDNA concentrations indicating degradation was unlikely. 2) Stochastic effect of sampling relatively small water volume. This was likely as each sample was only 150 mL from a 35 L pond. 3) Suboptimal filtration process. Constant power blackouts in the field site that could have led to loss of samples as filtration vacuum was lost. 4) Suboptimal DNA extraction conditions. Extractions was done from a whole filter in a 1.5 mL eppendorf tube possible leading to all surfaced to getting good contact with the lysis buffer. There was also a poorly optimised filter amount to extraction buffer volume.

The optimised protocol at the LSTM did not therefore transfer well to the field site and laboratory in ICIPE. More laboratory a field optimization will therefore, be necessary in order to ensure smooth transition. Preferably, the protocol should be optimized and customized to a laboratory/geographical location rather than simply transferring protocols.

Table 8. Summary of possible causes of contamination in the field samples and suggested ways to mitigate these in future.

Contamination source	Chances of occurring	Possible solutions
Directly within the ponds	• Unlikely. Signal in the negative controls were (3 out of 4 controls) but no signal in most of the ponds with larvae in them.	• Conduct experiments in a screen house semi-field • Sample more water per pond *i.e.* 500 mL – 1 L
Transfer among the sampling tubes	• Highly possible during the collection as all samples were stored in the same ice box. • Less likely during processing stage as all the filtration equipment were pre-autoclaved and rinsed in 10% bleach and 70% ethanol between samples.	• Collection tubes should be kept in different ice boxes whenever possible. • Put in place enough equipment for all samples so you only autoclave at end of filtration day • Strict decontamination protocol (UV, 50% bleach)
Contamination of stored filters	• Possible as samples were stored in separate bags but within a larger bag for transport	• Conduct extractions at the offshore field site to avoid sample transfer between labs
Extraction stage	• Possible but aerosol contamination was unlikely because not encountered with lab samples	• Include extraction negative controls to detect any contamination
qPCR stage	• Unlikely. High number of samples not positive and the qPCR negative controls were all negative	• Run more replicates per sample (5 – 15)

5.3 PCR primer design

We developed and validated an eDNA PCR primer pair for *An. gambiae* s.l complex and *An. funestus* mosquitos. The primers designed from the mitochondrial *cyt b* gene due to its high copy number per cell making it to be biologically available in more copies in environmental water samples. The *cyt b* was chosen since an initial bioinformatics examination of the mitochondrial sequences from *An. gambiae* s.l., and similar sequences from *Aedes aegypti*, *Culex quinquefasciatus* and *An. funestus* revealed that conserved sequences using was not possible the cytochrome oxidase I gene (COI) but was feasible using the *cyt b* gene. The primers were first used in an unlabelled agarose gel assay for preliminary testing and protocol assessment before moving further to design the qPCR assay.

5.4 Comparison of eDNA isolation protocols

When comparing the efficacy of the two DNA isolation techniques, ChargeSwitch® performed better than the Nexttec™ technique. Since eDNA occurs in very low concentrations in nature, an extraction technique that captures the maximal amount of eDNA from the water samples is vital (Deiner et al., 2015). Previous studies using eDNA have tested a wide range of other techniques. Qiagen DNeasy Blood and tissue kit (Ficetola et al., 2008, Goldberg et al., 2011) and the MoBio Ultra-Clean Soil DNA isolation (Jerde et al., 2011) kit are the most commonly used methods although some studies have also used the CTAB/phenol chloroform technique (Oh et al., 2011). The choice of extraction technique could be guided by factors such as the type of sample, cost, and field applicability. The ChargeSwitch® protocol is a technique optimized to extract forensic DNA that mostly occur in low concentrations and could be the reason it had better performance with low copy eDNA. A further optimization of the ChargeSwitch® protocol is however required in order to determine optimal amount of extraction buffers. A comparison assay on its performance (in terms of DNA yield) against the DNeasy Blood and tissue kit and Ultra-Clean Soil DNA isolation is also key.

5.5 Comparison of two eDNA concentration protocols

We did not find any significant differences between the commonly used eDNA concentration protocols – membrane filtration and precipitation. This could be because we used the same eDNA extraction protocol (ChargeSwitch®) for the two procedures masking out any differences. Similar conclusion was reached by other researchers who tried to compare different eDNA concentration methods. However, when they varied the capture and the extraction protocol, they noticed that a combination of filtration and DNeasy Blood and tissue kit gave the highest DNA recovery (Deiner et al., 2015). Filtration generally uses large volumes of habitat water compared to precipitation and could also be a contributing factor towards high eDNA yield. For the purposes of this study, we picked the membrane filtration method due to its sensitivity, and how easily adaptable it was for field use as it only required a vacuum pump, biosafety hood and source of power. It was also relatively easier to transport back membranes from the field site to a more specialized laboratory for the extractions, unlike precipitation where all the extractions had to be done at the field site. Precipitation method also required the use of a refrigerated centrifuge, which was not available at our field site.

5.6 IGS and cyt b analysis of the laboratory water samples

The high sensitivity and specificity and ability to quantify DNA have meant that TaqMan qPCR assays are increasingly popular for eDNA studies (Amberg et al., 2015). We were able to develop a novel *cyt b* TaqMan assay for detecting and quantifying *An. gambiae* s.l larvae in an aquatic habitat. We also successfully tested a published IGS assay (Walker et al., 2007) for use with eDNA. This assay has species specific probes so can accurately distinguish between *An. gambiae* s.s. and *An. arabiensis*. Results from testing this assay on a mixture of gDNA from the two species revealed that there was no species dye bias in the quantification process evident by a linear curve from the standard curve plots for the IGS assay (Fig. 6). The curve was even linear at lower concentrations than those previously suggested in a paper by (Bass et al., 2007) making it a feasible assay for use in degraded eDNA samples. The low limit for detection for the *cyt b* assay was lower than that included in the dilution series (minimum of 0.156 pg) though because quantitation

below 0.778 pg was extrapolated from the curve, accuracy at low DNA concentrations might be biased. Future work should ensure that the standard curve lower limits are within the range of sample concentrations to avoid such bias in during concentration calculations. This can be done by conducting a longer dilution series for the standards.

The two assays had relatively similar detection rates in the laboratory samples with both having high stochasticity in detection probability at low larval densities (Table 5). For example, relatively low eDNA concentrations were detected from the high-density habitats (10 and 20 larvae habitats) but similar concentrations could not be detected in the low-density habitats, indicating the eDNA productivity in a habitat is a stochastic process that could be highly dependent on larval densities. Using the two assays for quantification in low density habitats might therefore be an issue and might require further tests to resolve. With the difficulties in detecting few larvae in a habitat, tests should be done using new technologies such as the droplet digital PCR (ddPCR) (Hindson et al., 2011) which has been shown to be more accurate in quantifying eDNA in very low concentrations (Doi et al., 2015) and can detect as low as 1.25-fold, lower than the 2 fold change detected in qPCR (Hindson et al., 2011).

5.7 Controlled field testing

The IGS (Walker et al., 2007) and the *cyt b* assay were used to detect the relative proportions and quantify the eDNA concentration of each species in the field samples. The lowest larval density used in the ponds was 6 larvae; this value was determined from the laboratory trial which revealed reliably detectable density of 0.2 larvae/L (translating to 7 larvae in a 35L pond, but required an equal number for division between species. Based on artificial pond size this density was also concordant with the estimate of typical larval densities in natural breeding habitats (3 to 10 larvae per M^2) (Ndenga et al., 2011), the highest density in the field ponds was 96 larvae.

Contamination was a major issue in the field with 3 out of the 4 negative control habitats contaminated with *An. gambiae* s.l DNA. The contamination could have resulted from 1)

directly in the ponds with wild mosquitos visiting the habitats and laying on top of the moist net covers 2) the habitat water used for the experiments which was filtered lake water 3) during sample processing (*i.e.* sampling, filtration and extraction) 4) contamination of the stored filters and 5) during the qPCR analysis. These have been outline in details in Table 8 with the likelihood of them of occurring and suggestion on how to mitigate them going forward. No qPCR negative controls had any Ct ruling out the contamination at the genotyping stage.

Owing to the high numbers of contamination in the negative control ponds, we could not make any conclusions from the field data. The filed trial however, raised a chain of important questions that will need to be answered sequentially as the technique is transferred from the laboratory to a controlled field setting and further in the field for use in natural mosquito habitats some of which are included in the suggestions for further work section below.

5.8 Suggestions for further work

More laboratory investigation should be done to establish the detection of the two assays, IGS and *cyt b*, at very low concentrations. Future qPCR DNA standards should also include a longer dilution series to establish the linearity of the standard curves at low concentrations. This would ensure more accurate concentration calculations rather from extrapolation. Laboratory protocols should also be optimised in situ *i.e.* all the DNA extractions should be done at the field laboratory and an agarose gel run to check if the protocol is working if running a qPCR is not possible. The DNA extraction method from filter need potentially more optimization to establish the optimal sample vs buffer volumes.

At the controlled field testing in artificial ponds, the water processing mechanism should be changed to adapt to field conditions. Hand-held vacuum pumps should replace powered pumps in area where power is not stable/available. Before moving to the open field, experiments can first be setup in a semi-field screen house. This could help answer many questions such as degradation and contamination before the strategy in transferred into the field.

Initial open field trials in natural breeding habitats should involve taking water samples and then collecting all the larvae in the habitat and also any emerging adults using an emergence trap. This would be helpful for comparability between the qPCR signal and the species identification genotyping.

5.9 Conclusion

Larval sampling of mosquito disease vectors in vital in monitoring and evaluating control programs. It provides information of vector species distribution and cryptic mosquito species as they establish their populations in new ecological regions. The current two commonly used larval sampling strategies faced many challenges such as non-standardization, invasiveness and biasness therefore necessitating development of better tools.

This study has, for the first time, demonstrated the application of eDNA protocol in mosquito species detection and quantification using water samples. Initials trials in the laboratory have been successful but trials in the field conditions have been inconclusive due to contamination and high rate of negative samples. Ecological sampling using eDNA protocols have been shown to be more sensitive, reliable and replicable than traditional sampling tools (Thomsen and Willerslev, 2015).

The findings from this study suggest that both the IGS and *cyt b* assays can be used to detect eDNA at quite low concentrations, though quantification from *cyt b* assay might be an issue due to non-linearity in the standard curve at low concentrations. eDNA analysis technique can be a better option for detecting multiple larvae in a cryptic situation and in habitats where traditional sampling methods are logistically difficult such as rice fields. This tool should therefore complement rather than replace already established methods.

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7. Appendix

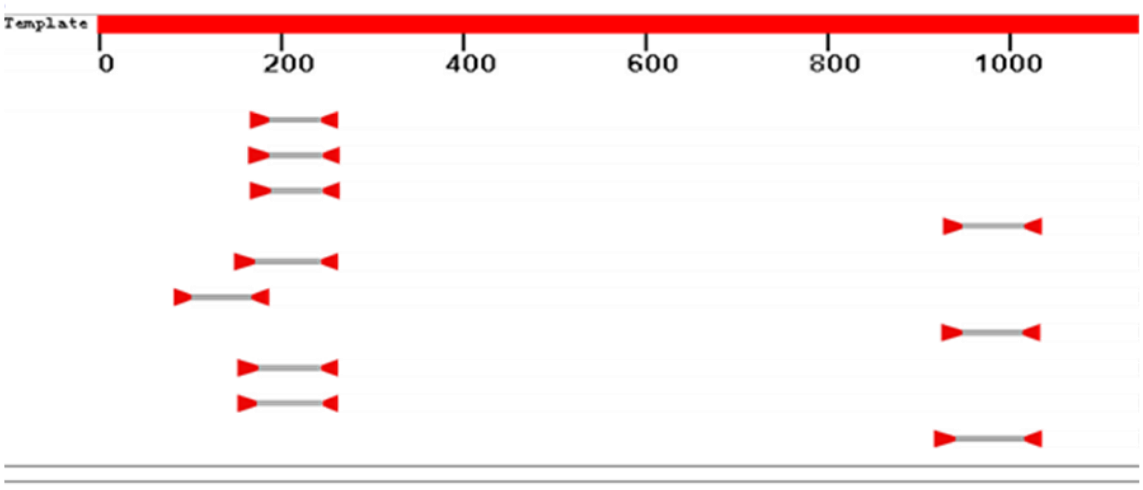
7.1 Appendix I: *An. gambiae* s.l. eDNA PCR sequences

Designed from mitochondrial *cyt b* sequences using NCBI – Primer Blast. **F** is forward sequence, **R** is the reverse sequence and **bp** is base pairs.

Primer Pair	Sequence (5' - >3')	Product Size (bp)	Region
1	**F** TGCCGCAGATATTGAAACAGC **R** AGCTCCGTTAGCGTGACAAA	90	10580 – 10600 10676 – 10657
2	**F** ATGCCGCAGATATTGAAACAGC **R** AAGCTCCGTTAGCGTGACAA	99	10579 – 10600 10677 – 10658
3	**F** GCCGCAGATATTGAAACAGCTT **R** GAAGCTCCGTTAGCGTGACA	98	10581 – 10602 10678 – 10659
4	**F** ACACTCTAGCAAGTTTCGAGGA **R** ACTGGTCGAGCTCCAATTCA	108	11336 – 11357 11443 – 11424
5	**F** TCCTAGCTATACACTATGCCGC **R** AGCTCCGTTAGCGTGACAAAT	113	10564 – 10585 10676 – 10656
6	**F** TCCGCCTGATGAAATTTTGGT **R** AGCTGTTTCAATATCTGCGGC	105	10497 – 10517 10601 – 10581
7	**F** CACACTCTAGCAAGTTTCGAGG **R** TGGTCGAGCTCCAATTCAAGT	107	11335 – 11356 11441 – 11421
8	**F** AGCTATACACTATGCCGCAGAT **R** AAGCTCCGTTAGCGTGACAAA	110	10568 – 10589 10677 – 10657
9	**F** AGCTATACACTATGCCGCAG **R** AGCTCCGTTAGCGTGACAA	109	10568 – 10587 10676 – 10658
10	**F** ACCTTTCACACACTCTAGCAAGT **R** ACTGGTCGAGCTCCAATTCAA	117	11327 – 11349 11443 – 11423

7.2 Appendix II: Regions on *An. gambiae* s.l. *cyt b* gene for primer pairs 1, 4, 5, and 8

Appendix II. Regions on the *cyt b* gene where primers 1,4, 5, and 8 are located. Selection was done to ensure they covered different parts of the gene



7.3 Appendix III: *An. funestus* complex eDNA PCR primer sequences

Designed from mitochondrial *cyt b* sequence using NCBI – Primer Blast. **F** is forward sequence, **R** is the reverse sequence and **bp** is base pairs.

Primer Pair	Sequence (5' - >3')	Product Size (bp)	Region
1	**F** TCCGAGGATTACAATTTTACCCA **R** GGGTCTTCTACTGGTCGTGC	103	11345 – 11368 11428 – 11448
2	**F** TTCCGAGGATTACAATTTTACCCA **R** ACTGGTCGTGCTCCAATTCA	95	11344 – 11368 11419 – 11439
3	**F** CCGAGGATTACAATTTTACCCACTA **R** CTTCTACTGGTCGTGCTCCA	98	11346 – 11370 11423 – 11443
4	**F** ATTCCGAGGATTACAATTTTACCCA **R** GGTCTTCTACTGGTCGTGCT	104	11342 – 11367 11426 – 11446
5	**F** ACACATATGAGAAAATTCCGAGGAT **R** GTCTTCTACTGGTCGTGCTCC	117	11328 – 11353 11424 – 11445
6	**F** TTACACATATGAGAAAATTCCGAGG **R** GGTCTTCTACTGGTCGTGCTC	120	11326 – 11351 11425 – 11446
7	**F** CGAGGATTACAATTTTACCCACTAA **R** TCTTCTACTGGTCGTGCTCC	98	11346 – 11371 11424 – 11444
8	**F** TGCAATTCTTCGATCAATTCCTAAT **R** AATTGTAATCCTCGGAATTTTCTCA	117	11243 – 11268 11335 – 11360

7.4 Appendix IV: Full data set of field pond experiments

Sampling day	Sample Code	Pond No.	No. of Larvae in pond		Sampling Date	Cyt b assay			IGS assay	
			ARAB	GAM		Rep 1	Rep 2	Rep 3	HEX	FAM
1	1	1	2	4	16062016	No Ct	No Ct	No Ct	No Ct	38.23
	2	2	4	2	16062016	19.32	11.71	40.86	23.09	No Ct
	3	3	NT	NT	16062016	No Ct	No Ct	No Ct	No Ct	No Ct
	4	4	32	64	16062016	No Ct	No Ct	No Ct	No Ct	No Ct
	5	5	64	32	16062016	No Ct	No Ct	No Ct	No Ct	No Ct
	6	6	16	32	16062016	No Ct	No Ct	No Ct	No Ct	No Ct
	7	7	32	16	16062016	No Ct	No Ct	No Ct	No Ct	No Ct
	8	10	NT	NT	16062016	No Ct	No Ct	No Ct	37.37	No Ct
	9	11	8	16	16062016	No Ct	No Ct	No Ct	No Ct	No Ct
	10	12	16	8	16062016	No Ct	No Ct	No Ct	38.18	39.05
2	11	8	2	4	17062016	42	23.71	No Ct	35.83	No Ct
	12	9	4	2	17062016	No Ct	No Ct	No Ct	No Ct	No Ct
	13	13	16	32	17062016	No Ct	No Ct	No Ct	No Ct	No Ct
	14	14	32	16	17062016	No Ct	No Ct	No Ct	No Ct	No Ct
	15	15	8	16	17062016	No Ct	20.46	No Ct	No Ct	No Ct
	16	16	NT	NT	17062016	No Ct	No Ct	No Ct	No Ct	No Ct
	17	17	NT	NT	17062016	No Ct	No Ct	No Ct	No Ct	No Ct
	18	18	16	8	17062016	No Ct	No Ct	No Ct	No Ct	No Ct
	19	19	32	64	17062016	No Ct	No Ct	No Ct	No Ct	No Ct
	20	20	64	32	17062016	30.8	17.97	No Ct	No Ct	No Ct
3	21	1	2	4	18062016	No Ct	No Ct	No Ct	No Ct	No Ct
	22	2	4	2	18062016	No Ct	No Ct	No Ct	No Ct	No Ct
	23	3	NT	NT	18062016	No Ct	No Ct	No Ct	38.74	No Ct
	24	4	32	64	18062016	No Ct	No Ct	No Ct	No Ct	No Ct
	25	5	64	32	18062016	No Ct	No Ct	No Ct	No Ct	No Ct
	26	6	16	32	18062016	No Ct	No Ct	No Ct	38.09	No Ct
	27	7	32	16	18062016	No Ct	No Ct	No Ct	No Ct	No Ct
	28	10	NT	NT	18062016	No Ct	No Ct	No Ct	No Ct	No Ct
	29	11	8	16	18062016	No Ct	24.71	No Ct	No Ct	No Ct
	30	12	16	8	18062016	No Ct	No Ct	No Ct	No Ct	No Ct
4	31	8	2	4	19062016	No Ct	No Ct	48.47	No Ct	No Ct
	32	9	4	2	19062016	No Ct	No Ct	No Ct	No Ct	No Ct
	33	13	16	32	19062016	15.71	21.46	39.32	36	No Ct
	34	14	32	16	19062016	No Ct	No Ct	No Ct	No Ct	No Ct
	35	15	8	16	19062016	No Ct	No Ct	No Ct	No Ct	No Ct
	36	16	NT	NT	19062016	No Ct	No Ct	No Ct	No Ct	No Ct
	37	17	NT	NT	19062016	No Ct	No Ct	No Ct	No Ct	No Ct
	38	18	16	8	19062016	No Ct	18.97	No Ct	No Ct	No Ct
	39	19	32	64	19062016	No Ct	No Ct	No Ct	No Ct	No Ct
	40	20	64	32	19062016	No Ct	No Ct	No Ct	No Ct	No Ct
5	41	1	2	4	20062016	No Ct	No Ct	No Ct	No Ct	No Ct
	42	2	4	2	20062016	No Ct	No Ct	No Ct	No Ct	No Ct
	43	3	NT	NT	20062016	No Ct	No Ct	No Ct	No Ct	No Ct
	44	4	32	64	20062016	24.84	No Ct	43.73	No Ct	No Ct
	45	5	64	32	20062016	No Ct	No Ct	No Ct	No Ct	No Ct
	46	6	16	32	20062016	No Ct	No Ct	45.16	36.47	No Ct
	47	7	32	16	20062016	No Ct	25.71	No Ct	No Ct	No Ct
	48	10	NT	NT	20062016	No Ct	No Ct	46.97	No Ct	37.2
	49	11	8	16	20062016	No Ct	No Ct	No Ct	38.89	No Ct
	50	12	16	8	20062016	No Ct	No Ct	48.71	No Ct	38.88
6	51	8	2	4	21062016	No Ct	22.46	45.69	No Ct	34.17
	52	9	4	2	21062016	34.1	No Ct	No Ct	39.06	No Ct
	53	13	16	32	21062016	27.5	24.71	44.65	35.95	33.5
	54	14	32	16	21062016	No Ct	No Ct	No Ct	No Ct	No Ct
	55	15	8	16	21062016	29.75	No Ct	49.2	No Ct	No Ct
	56	16	NT	NT	21062016	16.97	No Ct	38.14	30.03	32.7
	57	17	NT	NT	21062016	No Ct	21.46	No Ct	36.75	No Ct
	58	18	16	8	21062016	22.68	No Ct	45.54	No Ct	38.29
	59	19	32	64	21062016	No Ct	No Ct	No Ct	No Ct	No Ct
	60	20	64	32	21062016	22.7	No Ct	No Ct	No Ct	No Ct
7	61	1	2	4	22062016	No Ct	No Ct	No Ct	No Ct	No Ct
	62	2	4	2	22062016	No Ct	18.97	No Ct	37.69	No Ct
	63	3	NT	NT	22062016	No Ct	No Ct	47.85	No Ct	No Ct
	64	4	32	64	22062016	No Ct	No Ct	No Ct	No Ct	No Ct
	65	5	64	32	22062016	No Ct	No Ct	No Ct	No Ct	No Ct
	66	6	16	32	22062016	No Ct	No Ct	No Ct	No Ct	39.24
	67	7	32	16	22062016	No Ct	No Ct	No Ct	No Ct	No Ct
	68	10	NT	NT	22062016	No Ct	No Ct	No Ct	No Ct	No Ct
	69	11	8	16	22062016	27.02	No Ct	No Ct	No Ct	38.51
	70	12	16	8	22062016	No Ct	No Ct	No Ct	No Ct	No Ct
8	71	8	2	4	23062016	No Ct	25.71	No Ct	No Ct	No Ct
	72	9	4	2	23062016	No Ct	No Ct	No Ct	No Ct	No Ct
	73	13	16	32	23062016	35.23	No Ct	No Ct	No Ct	No Ct
	74	14	32	16	23062016	No Ct	No Ct	No Ct	No Ct	No Ct
	75	15	8	16	23062016	No Ct	22.46	No Ct	No Ct	33.23
	76	16	NT	NT	23062016	28.19	24.71	48.09	No Ct	No Ct
	77	17	NT	NT	23062016	No Ct	No Ct	No Ct	No Ct	38.12
	78	18	16	8	23062016	28.03	No Ct	No Ct	No Ct	No Ct
	79	19	32	64	23062016	No Ct	No Ct	49.49	No Ct	No Ct
	80	20	64	32	23062016	28.73	21.46	No Ct	No Ct	No Ct