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Assessing the impacts of multi-scale environmental change on vector ecology and malaria transmission in eastern Rwanda

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Doctor of Philosophy

Environmental Health Sciences

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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Doctor of Philosophy
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Abstract

Assessing the impacts of multi-scale environmental change on vector ecology and malaria transmission in eastern Rwanda

Background:
Cleaner cooking fuels are increasingly promoted to reduce morbidity and mortality from household air pollution in low and middle income countries such as Rwanda. However, smoke is a traditional insect repellent and it is unknown whether the replacement of smoky biomass fuels with cleaner fuels could alter vector behavior and exposure to malaria or other vector-borne diseases (VBDs). Additionally, Rwanda experienced a 20-fold increase in reported malaria cases in the last decade. The Ministry of Health hypothesized that environmental changes such as increased temperatures as well as insecticide resistance could have driven these trends, but no scientific studies have assessed their effects on vector ecology and the root causes of the malaria resurgence. An improved understanding of the impacts of these multi-scale environmental changes is necessary to mitigate potential risks and tailor appropriate malaria control interventions.

Methods:
In Aim 1 we employed a semi-field, Latin square design to experimentally investigate the effects of traditional and cleaner fuels on *Anopheles* mosquito behavior in rural Rwanda. Aim 2 consisted of a randomized controlled trial to assess the effects of liquified petroleum gas (LPG) adoption on vector density among houses that traditionally cook with biomass fuels. Finally, Aim 3 used a retrospective observational design to examine the effects of insecticide resistance, vector control, and regional warming on *Anopheles* bionomics and malaria incidence in eastern Rwanda.

Findings:
In Aim 1, household entry and host-seeking by lab-reared *Anopheles* mosquitoes were higher in experimental huts that cooked with LPG compared to traditional biomass fuels, whereas mosquito mortality was lower. Lower $PM_{2.5}$ and temperatures in LPG huts appeared to meditate this effect. However, in field conditions in Aim 2 we did not find a statistically significant difference in *Anopheles* or culicine density among intervention houses that received LPG stoves and fuel compared to control houses which cooked with biomass. In contrast, synanthropic fly density was reduced by 61% in intervention houses. Finally, in Aim 3 we found that insecticide resistance and regional warming were associated with the reemergence of *An. gambiae* after it was previously controlled in the early 2010s. The reemergence of this vector combined with a >2°C increase in regional temperatures drove a dramatic malaria resurgence in eastern Rwanda from 2010 to 2016, but *An. gambiae* and malaria transmission were controlled following implementation of non-pyrethroid indoor residual spraying (IRS) campaigns in the latter half of the decade.

Conclusions:
Environmental changes at multiple scales can have important implications for vector ecology and transmission of malaria and other VBDs in Rwanda. Although we found experimental evidence that the adoption of cleaner fuels can affect *Anopheles* behavior, other environmental determinants were more important drivers of mosquito density in field conditions. Reductions in flies could constitute a health co-benefit of LPG adoption in this setting. At a larger scale, insecticide resistance and regional warming are major threats to vector control and malaria prevention in Rwanda. However, the success of non-pyrethroid IRS suggests that existing control measures can mitigate vector reemergence and climate-related malaria increases, providing insecticide resistance is managed.

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Thank you.

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Abbreviations

ACT	Artemisinin Combination Therapy
Ae.	*Aedes*
An.	*Anopheles*
Anopheles gambiae s.l.	*Sensu lato*; from the Latin, “in the broad sense”, meaning any species within the An. gambiae complex, which includes eight morphologically identical species.
Anopheles gambiae s.s.	*Sensu stricto*; from the Latin, “in the strict sense”, meaning the unique species An. gambiae
CDC	The Centers for Disease Control and Prevention
Cx.	*Culex*
DHS	Demographic and Health Survey
HAP	Household Air Pollution
HLC	Human Landing Catch
HMIS	Health Management Information System
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
LLIN	Long-lasting Insecticide Treated Net
MIS	Malaria Indicator Survey
MOPDD	Malaria and Other Parasitic Diseases Division
NMCP	National Malaria Control Program
PMI	The President’s Malaria Initiative
RBC	Rwanda Biomedical Center
RDT	Rapid Diagnostic Test
VDB	Vector-borne disease
WHO	The World Health Organization

1 Chapter 1

1.1 Introduction

In the last decade, Rwanda has experienced rapid environmental change at multiple spatial and temporal scales (Akinyemi, 2017; Bizimana et al., 2015; Henninger, 2013; Ngarukiyimana et al., 2018; Tusting et al., 2017). Some of these changes, such as increasing adoption of cleaner cooking fuels or intensified agricultural production, reflect policy initiatives that seek to convey important health benefits. Others, such as changes in ambient temperature and precipitation, are possible bellwethers of climate change (Ngarukiyimana et al., 2021; Uwimbabazi et al., 2022). Many of these changes could simultaneously affect vector ecology and have important implications for exposure to malaria or other vector-borne diseases (VBDs). An improved understanding of the impacts of these changes is necessary to mitigate potential risks and tailor appropriate malaria control interventions.

Cleaner stoves and cooking fuels have been widely promoted in Rwanda to improve respiratory and cardiovascular health outcomes associated with household air pollution (HAP) (Das et al., 2018; Kirby et al., 2019; Rosa et al., 2014). There is some concern though that reductions in smoke or other volatiles from cooking fuels could affect mosquito behavior and human exposure to malaria parasites or other vector-borne pathogens. Smoke has been used as an insect repellent for centuries (Torr et al., 2011), and some studies have noted negative associations between smoke from domestic fuel burning and indoor *Anopheles* density and malaria incidence (Biran et al., 2007; Hiscox et al., 2013; Lindsay et al., 1995; Paru et al., 1995). Strikingly, an improved cookstove intervention in Malawi found an increase in malaria in children in households that received the intervention (Mortimer et al., 2017). Another recent case-control study in Guatemala found an inverse relationship between cooking with firewood indoors and arboviral infection (Madewell et al., 2020). In both cases, however, these were secondary outcomes of health impact evaluations. Few field studies have investigated the potential effects of smoke on flies. However, experimental evidence has shown that biomass smoke repels the housefly *Musca domestica* (Denloye et al., 2006), which is a mechanical vector of numerous enteric pathogens (Cohen et al., 1991; Davies et al., 2016; Greenberg, 1973; Rosef & Kapperud, 1983) .

Despite these findings, very few studies have directly investigated the impacts of the adoption of cleaner cooking fuels on vector behavior or VBD exposure (Biran et al., 2007). This information is needed for institutions involved in the promotion of cleaner cooking to understand potential adverse effects of cleaner fuel adoption and, if necessary, promote vector control interventions in tandem with clean cooking interventions. Aims 1 and 2 of this proposal address this research gap by exploring the fine-scale impacts of a household air pollution intervention trial in Rwanda on *Anopheles* density and biting behavior. For Aim 1 we employed a semi-field, Latin square design to experimentally investigate the effects of traditional and cleaner cooking fuels on *Anopheles* behavior. For Aim 2 we conducted household-level, longitudinal entomological sampling to assess the effects of cleaner fuel adoption on vector density in households participating in the trial. These were the first studies explicitly designed to investigate the impacts of household air pollution interventions on vector behavior and exposure risk.

Coarser-scale changes in land use, vector control, and climate can profoundly impact *Anopheles* population dynamics, vectorial capacity, and malaria incidence (Afrane et al., 2006; Bhatt et al., 2015; Janko et al., 2018; Kleinschmidt et al., 2018; Lindblade et al., 2000; Mordecai et al., 2013; Ryan et al., 2015). In the last decade, Rwanda has invested heavily in dam construction and rice cultivation (MINIAGRI, 2013), which can provide ideal habitat for several *Anopheles* mosquito species and has been linked to increased malaria incidence elsewhere in the East African highlands (Carnevale et al., 1999; Coosemans, 1985; Hunter et al., 1993; Ijumba & Lindsay, 2001; Rulisa, Kateera, Bizimana, Agaba, Dukuzumuremyi, Baas, de Dieu Harelimana, Mens, R Boer, et al., 2013; Tuyishimire, 2016). Extensive use of pyrethroid insecticides for vector control and agriculture have also led to high levels of insecticide resistance in Rwanda, which could reverse previous gains in malaria control (Hakizimana et al., 2018; Hakizimana et al., 2016; Hancock et al., 2018; Hemingway et al., 2016).

East Africa is considered highly vulnerable to increased malaria transmission due to climate change (Ryan et al., 2020; Ryan et al., 2015). Short and long-term increases in temperature and rainfall may alter vector and pathogen population dynamics and lead to malaria outbreaks in high altitude areas of Rwanda and other areas of the East African highlands (Loevinsohn, 1994; Nkurunziza et al., 2010; Noriko & Elfatih, 2020; K. P. Paaijmans et al., 2010; Pascual et al., 2006; Siraj, Santos-Vega, Bouma, Yadeta, Carrascal, et al., 2014; Ssempiira et al., 2018). However, other anthropogenic changes such as land-use change and urbanization have non-linear interactions with climate trends and complicate model predictions (Chaves & Koenraadt, 2010; Hay et al., 2002).

The factors discussed above have been posited as potential explanations for a dramatic malaria resurgence that Rwanda experienced in the last decade (PMI, 2019; RBC, 2017; Rudasingwa & Cho, 2020; Tuyishimire, 2016; WHO, 2017a). However, no rigorous, longitudinal studies have been conducted to evaluate the actual impacts of these environmental changes on resurgent malaria transmission in Rwanda (PMI, 2019). In Aim 3 we conducted a retrospective analysis of entomological and epidemiological data in four sites in Eastern Province to investigate the impacts of multi-scale environmental change on vector ecology and malaria incidence from 2010 – 2020. This information is critical for informing effective control measures and preventing future malaria resurgences in Rwanda and similar transmission settings in East Africa.

1.2 Dissertation aims

1.2.1 Research Aim 1

The first aim, *"Assessing the effects of cooking fuels on Anopheles mosquito behavior: an experimental study in rural Rwanda",* used a semi-field experimental design to investigate the potential effects of cleaner fuel adoption on the household entry and host-seeking behavior of the primary malaria vector in Rwanda, *Anopheles gambiae.* Although anecdotal and observational evidence has suggested that smoke from biomass fuel combustion deters mosquitoes, this was the first study to directly investigate this effect in relation to the replacement of biomass cooking fuels such as wood and charcoal with cleaner-burning liquid petroleum gas. Such mechanistic information is needed in order to understand potential impacts of cleaner fuel adoption in settings where traditional biomass fuels are common.

1.2.2 Research Aim 2:

The second aim is entitled "*Effects of cooking with LPG fuel versus solid biomass on mosquito and fly density in the home: a randomized controlled trial in Eastern Province, Rwanda*". This study used longitudinal entomological sampling in the context of a randomized household air pollution trial to estimate the effects of cleaner cooking fuel adoption on mosquito and fly density. The study also investigated reported malaria and diarrhea incidence in mothers and children as secondary outcomes. This was the first study to directly investigate the effects of cleaner fuel adoption on entomological and epidemiological parameters of VBD transmission. This information is necessary for implementers of clean cooking interventions to understand potential adverse effects and promote vector control measures in tandem with clean fuels if necessary.

1.2.3 Research Aim 3:

The last aim is entitled "*The impacts of insecticide resistance and environmental change on Anopheles gambiae reemergence and resurgent malaria transmission in eastern Rwanda*". It investigated the drivers of a 20-fold increase in malaria cases that Rwanda experienced over the last decade using ten years of entomological and malaria case data from four sites in Eastern Province, Rwanda. This information is critical for informing effective malaria control interventions and predicting and preventing future outbreaks in Rwanda and similar transmission settings in East Africa.

2 Literature review

2.1 Epidemiology and entomology of malaria in Rwanda

2.1.1 Epidemiological situation

Since the early 2000s, Rwanda has made significant strides in malaria control, implementing universal bed net coverage, rapid diagnostic tests (RDTs), first-line treatment with artemisinin combination therapies (ACTs), and intermittent preventative treatment for pregnant women. These efforts resulted in an 86% decline in malaria incidence between 2005 and 2011, and similar declines in outpatient cases and inpatient deaths (PMI, 2018; RBC, 2017). These gains contributed to a substantial decline in all-cause childhood mortality from 2000 – 2010 (Eckert et al., 2017), and led the Rwanda National Malaria Control Program (NMCP) to set an ambitious goal of achieving malaria pre-elimination status by 2012 and near-zero deaths by 2018 (PMI, 2009).

However, the country witnessed an alarming malaria resurgence in the last decade. Overall reported cases increased 20-fold from a low of 225,176 in 2011 to over 4,700,000 cases in 2016 and 2017 (Figure 2-1). Test positivity increased concurrently from under 15% to >50%, indicating that the observed increase could not be attributed to improved care-seeking or reporting (PMI, 2018), and malaria deaths increased 88% (RBC, 2017). However, cases and test positivity have declined since 2018 following the massive scale-up of vector control interventions and community case management (PMI, 2022b; RBC, 2020b).

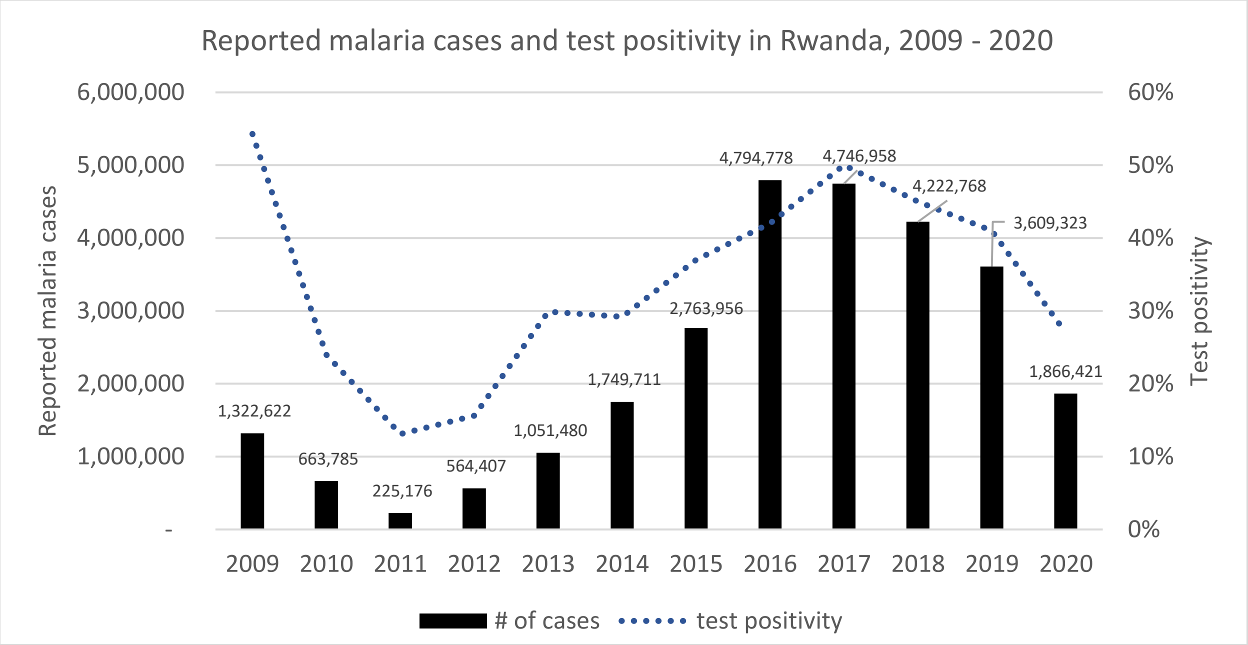

Figure 2-1 Malaria cases and test positivity reported via routine surveillance health systems. Data from the Rwanda Health Management Information System, reported in 2018 and 2022 PMI Rwanda Malaria Operational Plans.

Nationally representative health surveys also demonstrated an increase in malaria prevalence during the 2011-2017 period; microscopy-confirmed prevalence among children under five increased from 1.4% in 2010 to 2.2% by 2014-2015, and then to 7.2% by 2017 (ICF, 2017; National Institute of Statistics of Rwanda et al., 2015; NISR & Macro, 2012). Prevalence increased the most dramatically in Eastern Province, from 3.4% in 2010 to 18% in 2017 (Figure 2-2). The reasons for these large increases are not definitively understood, though the Rwanda National Malaria Control Program and its partners cited numerous potential explanations including land use changes, increased treatment seeking, improved reporting, low intervention coverage, insecticide resistance, and climate change (PMI, 2018; RBC, 2017; WHO, 2017a).

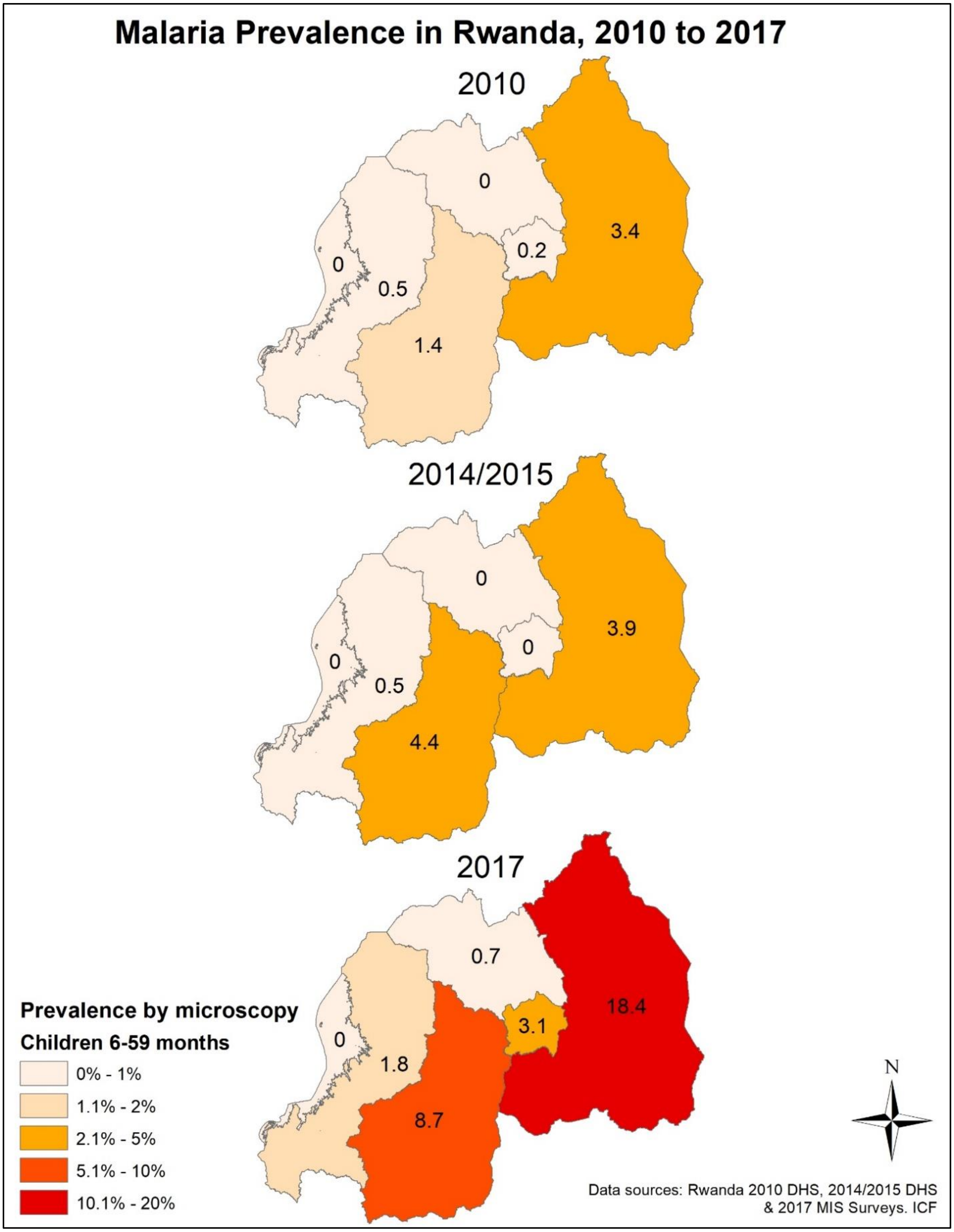

Figure 2-2. Malaria prevalence in Rwanda via microscopy among children age 6-59 months. Data are from the 2010 Demographic and Health Survey, 2014-2015 Demographic and Health Survey, and 2017 Malaria Indicator Survey, ICF

In response to the observed increase in malaria cases, the NMPC implemented a revised malaria contingency plan to reduce transmission (RBC, 2017). Interventions included expanding free access to treatment, and strong investments in malaria case management by community health workers (CHWs), who now diagnose and treat more than 50% of all malaria cases (PMI, 2019). This was accompanied by enhanced vector control efforts, including universal net distributions in 2016-2017 and expanding indoor residual spraying (IRS) coverage from 5 to 12 districts. These interventions resulted in a reduction in overall cases by 2020 and a continued decline in malaria mortality rates (RBC, 2020b).

2.1.2 Entomological situation

The NMCP maintains 12 sentinel sites for routine entomological surveillance. An analysis of data from seven of these sites was conducted for the time period of 2010 to 2013 (Hakizimana et al., 2018). Out of more than 300,000 mosquitoes collected via human landing catch, 26.2% were identified as anophelines. Of these, 94.3% of these were *Anopheles gambiae* sensu lato (s.l.), 0.4% *An. funestus* and 5.3% other *Anopheles* species. Two species within the *An. gambiae* species complex are found in Rwanda: *An. gambiae* sensu stricto (s.s.) and *An. arabiensis*. *An. arabiensis* accounted for 84.4% of the species complex in this study. *Plasmodium falciparum* infection rates averaged 2.8% for *An. gambiae* s.l. However, the relative importance of *An. gambiae* s.s. and *An. arabiensis* for malaria transmission in Rwanda has not been evaluated in detail.

Universal bed net coverage and agricultural use of insecticides have resulted in increased rates of insecticide resistance in Rwanda. Resistance to pyrethroid insecticides increased in most entomology sentinel sites from 2011 to 2013, prompting a shift to carbamate insecticides for IRS (Hakizimana et al., 2016). Pyrethroid resistance has continued to increase; in 2018 more than 75% of 30 monitoring sites detected resistance to permethrin and Alpha-cyhalothrin, and 47% of sites reported resistance to deltamethrin. The NMCP switched to organophosphates for IRS in 2016 as part of an insecticide resistance management plan, and then switched to a noenicitinoid-pyrethroid combination starting in 2019 (PMI, 2020; RBC, 2020a).

2.2 Importance of culicine mosquitoes and associated arboviruses in Rwanda

Culicine mosquitoes are responsible for transmission of a wide variety of arboviruses (e.g. Dengue Virus (DENV), Zika Virus (ZIKV), Chikungunya Virus (CHIKV), and West-Nile Virus (WNV)). *Aedes aegypti* is the principal vector for DENV, CHIKV, and ZIKV and Yellow Fever Virus, and is found in Rwanda in addition to other *Aedes* species (Demanou, 2014). *Culex quinquefasciatus* is the principal vector for WNV and a secondary vector for *W. bancrofti*, as well as for Rift Valley Fever virus (RVFV) (BØGh et al., 1998; Farajollahi et al., 2011; Sang et al., 2017). It is found throughout Rwanda, and is considered an important vector species (Hakizimana et al., 2018; WHO, 2009). *Mansonia* species are also found throughout Rwanda (Hakizimana et al., 2018), and are vectors of O'nyong'nyong virus, RVF, and other arboviruses (Braack et al., 2018).

Dengue antibodies were detected in seroprevalence studies in Rwanda between 1987 and 1993 (Amarasinghe et al., 2011). A more recent seroprevalence study found high CHIKV seroreactivity (63%) among Rwandan blood donors and substantial WNV seroreactivity (10.4%), although the authors suggested the high CHIKV results could be due to cross-reactivity with O'nyong'nyong virus (Seruyange et al., 2019). The majority of positive results were from Eastern Province where our studies took place. The same authors found 1.4% prevalence of ZIKV IgG in Rwandan blood donors (Seruyange et al., 2018). RVFV antibodies have been detected in cattle in Rwanda, and human cases have occurred in neighboring countries (LaBeaud et al., 2007; Shoemaker et al., 2019; Umuhoza et al., 2017).

2.3 Importance of synanthropic flies as mechanical vectors of enteric pathogens in Rwanda

Synanthropic flies, also referred to as filth flies, include numerous species in the families of Muscidae (house flies and latrine flies), Calliphoridae (blowflies and bottleflies), and Sarcophagidae (flesh flies). They are adapted to live in close association with humans and breed in animal manure, human excrement, garbage, or decaying organic material. They are capable of mechanically vectoring enteric pathogens via mechanical dislodgment from the exoskeleton, fecal deposition, and regurgitation (Greenberg, 1973). They have been implicated in the transmission of more than 100 pathogens, including

Salmonella, *Campylobacter*, *E-coli*, Helminths, *Shigella* spp., *Vibrio* spp., and Giardia (Adenusi & Adewoga, 2013; Bidawid et al., 1978; Graczyk et al., 2001; Olsen, 1998; Rosef & Kapperud, 1983; Songe et al., 2016; Tan et al., 1997). *Musca sorbens* flies are also considered important mechanical vectors of trachoma (Emerson et al., 2000; Emerson et al., 2004).

Only one study in Rwanda examined the association between flies and diarrhea, and found that the presence of flies in or around the household was associated with an increased odds of self-reported diarrhea among household members after controlling for other confounders (adjusted OR: 1.69, 95% CI: 1.29-2.81) (Uwizeye et al., 2014). Numerous studies in other settings have found similar associations between fly density and food contamination and diarrhea incidence (Boadi & Kuitunen, 2005; Collinet-Adler et al., 2015; Doza et al., 2018; Lindeberg et al., 2018). A randomized controlled trial investigated the impacts of fly control on incidence of diarrhea in children in Pakistan. Caregiver-reported diarrhea in children was reduced by 23% in villages which received insecticide spraying to reduce fly density compared to control villages (Chavasse et al., 1999). Similarly, a study in the Gambia reported 22-26% reductions in childhood diarrhea after insecticide spraying reduced fly density by 75% (Emerson et al., 1999). Another study in Israel found an 85% reduction in clinic visits for shigellosis among soldiers following a fly control intervention (Cohen et al., 1991). However, no effect was seen for norovirus seroconversion, highlighting potential pathogen-specificity in the role of flies as enteric disease vectors (Cohen et al., 2002).

Filth flies including those in the Muscidae, Calliphoridae, and Sarcophagidae families are found in Rwanda (Lutz et al., 2018; Park et al., 2019; Pont & Crosskey, 1980). The roles of individual species in the transmission of enteric pathogens in Rwanda has not been well characterized.

2.4 Effects of biomass smoke on disease vectors and vector-borne disease risk

2.4.1 Background

Although smoke has been used as an insect repellent for centuries (Torr et al., 2011), there have been very few rigorous studies on the actual impacts of smoke on insect vectors or incidence of the diseases they vector (Biran et al., 2007). Additionally, most published studies examine the impacts of burning specific plant parts (e.g. leaves, seeds, or essential oils) as putative insect repellents (e.g. (Adhikari & Chandra, 2014; Denloye et al., 2006; Kamalakannan et al., 2009; Ramkumar et al., 2015; Seyoum et al., 2002)). Relatively fewer studies have specifically examined entomological or epidemiological impacts of solid biomass fuel combustion for domestic cooking or heating (e.g. wood, crop residues, dung, or charcoal). These effects may be direct or indirect, and species and context specific. The sections below provide a brief review of the literature on the direct and indirect impacts of solid biomass combustion on anopheline mosquito behavior and malaria epidemiology.

2.4.2 Direct impacts on *Anopheles* mosquito behavior:

Repellence*:*

Smoke, heat, or other volatiles from combustion of biomass fuels may function as a mosquito repellent, causing them to orient away from the source (Dethier et al., 1960). In laboratory experiments, Denloye and colleagues investigated the repellent effects of smoke by placing *An. gambiae s.l.* mosquitoes into a two-chambered glass cage and igniting pellets made of pulverized waste-wood, palm kernel cake, and *Adansonia digitata* leaves in one chamber. They found that 37% of mosquitoes were repelled into the neighboring chamber when pellets were burned, compared to 0% when no pellets were introduced (Denloye et al., 2006). However, it is difficult to discern the impacts of the inactive wood pulp ingredients in the pellets from *Adansonia digitata* leaves, which have been shown to have some insecticidal properties (Krishnappa et al., 2012). This was the only laboratory study which directly investigated repellency impacts of solid biomass smoke. In Ethiopia, Dube found that burning dried *Corymbia citriodora* and *Ocimum suave* leaves had a stronger repellent effect than fresh leaves in laboratory studies with *An. arabiensis* and *Ae. aegypti* (Dube et al., 2011).

Table 2-1 presents a summary of field experimental and observational studies that investigated the repellent effects of biomass combustion on *Anopheles* mosquitoes. Bockerie and colleagues experimentally investigated the impacts of burning an unspecified species of wood in bedrooms before bedtime, which was a common mosquito control practice in the Bo region of Sierra Leone. They found significantly more *An.*

gambiae s.s. females in exit traps outside the house in which wood fires were lit each night for 15 days, suggesting that the presence of smoke induced an exophilic response in mosquitoes which had previously entered the house (Bockarie et al., 1994). They did not directly report repellence in terms of differences in overall house entry by mosquitoes, although the smoky huts appeared to have fewer overall mosquitoes when the results of window traps and indoor spray catches were summed.

Table 2-1. Field experimental and observational studies on the impact of smoke on *Anopheles* abundance

Study	*Study location*	*Design*	*Outcome/ method*	*Fuel burned*	*Anopheles species*	*Protective efficacy*	*Significant effect*
Bockarie et al. (1994)	Sierra Leone	Field experimental	Indoor resting, window traps	Unspecified wood	*An. gambiae*	35%	?
Kweka et al. (2008)	Tanzania	Experimental huts	Deterrence	Dried leaves and plant parts	*An. arabiensis*	79 - 89%	Y
Kweka et al. (2008)	Tanzania	Field experimental	Deterrence	Dried leaves and plant parts	*An. arabiensis*	73-82%	Y
McCann et al. (2017)	Kenya	Observational	Presence (indoor resting)	Unspecified wood and charcoal	*An. arabiensis, gambiae, & funestus*	53-74%	Y
Charlwood et al. (2018)	Kenya	Observational	Presence (indoor resting)	Unspecified - likely wood	*An. gambiae s.l.*	49%	N
Hiscox et al. (2013)	Lao PDR	Observational	Hut entry via light traps	Unspecified wood	*Anopheles spp.*	78^%	Y
Maia et al. (2016)	Tanzania	Field experimental	Resting abundance	Blank mosquito coil	*An. arabiensis*	16%	N
Maia et al. (2016)	Tanzania	Field experimental	Resting abundance	Blank mosquito coil	*An. funestus*	-10%	N
Pålsson et al. (1999a)	Guinea Bissau	Field experimental	Resting abundance	*Daniella oliveri*	*An. gambiae s.l. & pharoensis*	74.7-77.9%	Y

In semi-field experimental hut trials in Tanzania, Kweka and colleagues investigated the repellent effects of smoke from burning dried plant material from five local plant species; *Ocimum suave, Ocimum kilimandscharicum*, *Azadirachta indica*, *Eucalyptus globules*, and *Lantana camara* (Kweka et al., 2008). They released 100 unfed *An. arabiensis* and *Culex quinquefasciatus* mosquitoes inside two experimental huts with a person sleeping under an untreated bed net in each hut, and burned the plant materials between 7 and 10pm in the treatment hut. They estimated repellency as the proportion of mosquitoes which were found in veranda and window exit traps the next morning, and found that all burning materials had strong repellent effects compared to untreated huts, ranging from 79 to 89%. They repeated the experiments in eight houses within the community and found similar repellent effects. The plant materials used in this study mostly consisted of dried leaves, which are less relevant to the impacts of biomass (mostly wood) combustion for domestic fuel use. However, *Eucalyptus* and *Lantana* are commonly used cooking fuel species in Rwanda, and dried leaves are often used to light fires (Ndayambaje & Mohren, 2011).

In a field observational study in Kenya, McCann et al. measured *Anopheles sp.* abundance in 525 houses using pyrethroid spray catch (PSC), and found that cooking in houses the night before was associated with reduced catches of all anopheline mosquitoes (McCann et al., 2017). In Tanzania, on the other hand, Charlwood et al. found no difference in the indoor density of *An. gambiae s.l.* mosquitoes collected via Prokopacks and CDC light traps in smoky versus non smoky houses (Charlwood et al., 2018). In rural Lao PDR, Hiscox and colleagues found that wood smoke from cooking fires underneath the house or indoors was protective against household entry by *Anopheles* and *Culex* mosquitoes (Hiscox et al., 2013).

Attractant effects:

Mosquitoes are activated and attracted by a combination of olfactory, thermal and visual cues (Davis & Bowen, 1994; Lehane, 2005). Carbon dioxide (CO_2) is emitted by all vertebrate hosts via respiration, and is widely considered the most important attractant for host-seeking mosquitoes (Gillies, 1980; Takken & Knols, 1999). Increases in ambient CO_2 levels as little as 0.01% above baseline can stimulate female mosquitoes to activate and search a blood meal (Healy & Copland, 1995; Webster et al., 2015). Varying levels, or pulses, of CO_2 appear to be more effective in attracting host-seeking mosquitoes, most likely because this mimics CO_2 pulses from the breath of humans or other hosts (Kline, 2002; McPhatter & Gerry, 2017). Heat and human odor are also important attractants for host-seeking mosquitoes and interact with CO_2 (McMeniman et al., 2014).

CO_2, heat, and real or synthetic host odors are widely used in mosquito traps. CO_2 for mosquito traps can be produced by catalytic conversion of propane, CO_2 tanks, dry ice, or sugar-yeast formulations (Kline, 2002; Lima et al., 2014; Oli et al., 2005). Heat is also generated by propane traps and may serve as a further attractant (Kline, 2002). At the same time, CO_2 and heat are major byproducts of fuel combustion for cooking fuels (Edwards, 2014). While cleaner burning fuels such as LPG generally result in reduced exposure to pollutants such as black carbon and particulate matter, they emit more CO_2 per kilogram of fuel used due to more complete combustion. Indeed, LPG emits an average of 2532 grams of CO_2 / kilogram of fuel used (range 1390 - 3120), compared to 1610 g/kg (1320 – 2700) for unvented traditional wood stoves. CO_2 emissions from burning unvented charcoal are similar to LPG at 2559 g/kg (2091 – 3026) (Edwards, 2014).

Despite the fact that fuel combustion produces two key attractants for mosquitoes, CO_2 and heat, very few studies have investigated whether fuel combustion for domestic cooking or heating could serve as an attractant for host-seeking mosquitoes. The few studies that do exist have investigated the effects of propane or LPG use for mosquito traps such as the Mosquito Magnet, and generally find an attractive effect for *Anopheles*, *Culex*, and other mosquito genera (Chaiphongpachara et al., 2018; Rubio-Palis et al., 2012). It is possible that smoke or other volatiles from fuel combustion could counteract any attractant effects of emitted CO_2, although this could be less important for cleaner fuels such as LPG. Additionally, it is possible that extremely high levels of CO_2 from fuel combustion do not actually attract mosquitoes, or that the attractant effect is minimal because fuel combustion produces relatively constant plumes of CO_2 rather than pulses. Nevertheless, this gap in the literature merits further investigation.

Deterrence:

In addition to repellence, solid biomass fuel may act as a deterrent, meaning mosquitoes are impeded from host-seeking or blood feeding (Dethier et al., 1960). We did not find any laboratory studies which examined differences in blood feeding when mosquitoes were exposed to burning solid biomass fuel compared to no fuel. Table 2-2 presents a summary of all field and observational studies that investigated the impacts of biomass combustion on host-seeking by *Anopheles* mosquitoes.

Table 2-2. Review of field experimental and observational studies on the impact of smoke on *Anopheles* host-seeking

Host-seeking deterrence

Study	*Study location*	*Design*	*Outcome/ method*	*Fuel burned*	*Anopheles species*	*Protective efficacy*	*Significant effect*
Bockarie et al. (1994)	Sierra Leone	Field experimental	Blood fed status	Unspecified wood	*An. gambiae s.s.*	not reported	N
Kweka et al. (2008)	Tanzania	Experimental hut	Blood fed status	Dried leaves and plant parts of 5 local plant species	*An. arabiensis*	60 - 98%	Y
Kweka et al. (2008)	Tanzania	Field experimental	Blood fed status	Dried leaves and plant parts of 5 local plant species	*An. arabiensis*	61 - 100%	Y
Vernède et al. (1994)	PNG	Field experimental	Human landing catch	Coconut husks	*An. karwari*	66% (17-86)	Y
Vernède et al. (1994)	PNG	Field experimental	Human landing catch	Coconut husks	*An. koliensis*	38% (-15-67)	N
Vernède et al. (1994)	PNG	Field experimental	Human landing catch	Mango wood	*An. karwari*	35% (-58-73)	N
Vernède et al. (1994)	PNG	Field experimental	Human landing catch	Mango wood	*An. koliensis*	-21% (-123-35)	N
Paru et al. (1995)	PNG	Field experimental	Human landing catch	Five wood species	*Anopheles spp.*	76% (50-88)	Y
Moore et al. (2007)	Bolivia	Field experimental	Human landing catch	*Attalea princeps* husks burned on charcoal	*An. darlingi*	35% (-13 - 82)	N
Maia et al. (2016)	Tanzania	Field experimental	Blood fed status	Blank mosquito coil	*An. arabiensis*	47%	Y
Maia et al. (2016)	Tanzania	Field experimental	Blood fed status	Blank mosquito coil	*An. funestus*	44%	Y
Lindsay et al. (1995)	Gambia	Observational	Host-seeking	Unspecified wood	*An. gambiae s.l.*	14% (1 - 26)	Y

In field-based experimental studies, Paru and colleagues examined the effect of burning wood and/or leaves from local plant species on indoor biting, as measured by human landing catch. They found an average 79% reduction in biting associated with wood smoke from *Artocarpus altilis*, *Coffea robusta*, *Gnetum gnemon,* and *Leucaena leucocephala* (Paru et al., 1995). Moore et al. conducted field experimental trials in the Brazilian amazon in which they burned various local plant species and measured deterrence via outdoor human landing catch in a Latin square design. They found that burning 250 g of charcoal deterred about 20% of *Mansonia sp.* and *An. darlingi* bites when compared to burning a locally sourced, insecticide treated mosquito coil. Adding seeds from *Attalea princeps* increased deterrence (Moore et al., 2007). In Papua New Guinea, Vernède et al. reported that wood fires from coconut husks (*Cocos nutifera*) reduced outdoor human landing catches of *An. kamtari* and *An. koliensi,* whereas wild mango wood (*Mangifera* spp.) did not have a significant effect. Fires from both types of biomass fuel reduced culicine biting by more than 55% (Vernède et al., 1994).

In another field-based experimental study in Guinea Bissau, Pålsson found that burning the bark of *Daniella oliveri* significantly reduced overall mosquito catches on bed nets, which is a proxy for biting behavior. However, this effect appeared to be primarily mediated by reduced *Mansonia* spp. catches, and was not significant for anopheline or culicine mosquitoes (K. Pålsson & T. G. T. Jaenson, 1999a). In a follow-up study, the authors found a 75-79% reduction in outdoor human landing catches when collectors sat near a smoldering *D. oliveri* bark fire, compared to no-fire controls (K. Pålsson & T. G. T. Jaenson, 1999b). Similarly, Lindsay and Janneh found a 77% reduction in outdoor human landing catches when collectors were seated next to a smoldering fire of 70 grams of *D. oliveri* bark and resin (Lindsay & Janneh, 1989). In Tanzania, Kweka et al. measured the proportion of blood-fed mosquitoes in outdoor traps, and reported between 60 – 100% feeding inhibition for the five different plant materials that were burned in experimental huts and village houses (Kweka et al., 2008). Maia et al. also found a dramatic reduction in the number of blood-fed *An. arabiensis, An. funestus,* and *Culex spp.* mosquitoes sampled via Prokopacks in houses in Tanzania in which an untreated ‘blank’ mosquito coil was burned the night prior (Maia et al., 2016). In Sierra Leone on the other hand, Bockarie et al. found no direct difference in blood-feeding rates among *An. gambiae s.s.* caught in smoke-filled houses versus non-smoky houses (Bockarie et al., 1994).

In a field-based observational study in rural Gambia, Lindsay and colleagues found that the absence of woodsmoke indoors was associated with increased exposure to *Anopheles* mosquitoes, as measured by the number of mosquitoes found under bed nets after a child slept under it (Lindsay, 1995). Also in the Gambia, Kirby et al. observed a reduction in *An. gambiae s.l.* CDC light trap catches in houses that burned *Daniella oliveri* bark and resin (Kirby et al., 2008). In contrast, a field observational study in Uganda in 1933 found a positive association between smoke and *Anopheles* biting indoors (Gibbins, 1933).

Resting behavior:

In addition to repellence and deterrence, biomass fuel fires may affect resting behavior of mosquitoes. For example, Charlwood and colleagues found that *An. gambiae s.l.* mosquitoes were much more likely to rest on walls rather than ceilings in smoky houses compared to non-smoky houses in Tanzania (Charlwood et al., 2018). A similar effect was seen in an earlier study in Tanzania (Smith, 1962). In Sierra Leone, Bockarie et al. found that the presence of indoor wood fires did not reduce blood feeding, but resulted in fewer mosquitoes resting indoors after taking blood-meals (Bockarie et al., 1994). This could alter the efficacy of IRS, which requires mosquitoes to land on a treated indoor wall or roof surface in order to be exposed to residual insecticides (Sherrard-Smith et al., 2018).

Mortality

Denloye and colleagues also experimentally investigated the impacts of pellet combustion on *An. gambiae* mortality and found that 22.5% to 35% of mosquitoes died when exposed to smoke from the waste-wood, palm cake, and *Adansonia digitata* pellets, compared to 2.5% mortality in unexposed mosquitoes (Denloye et al., 2006). Most other laboratory studies found only marginal differences in mortality when burning ‘blank’ mosquito coils compared to negative controls. For example, Jantan et al. measured a 24-hour mortality rate of 6.7% when *Aedes aegypti* were exposed to smoke from untreated coils made from *Cinnamomum iners* and coconut shell, compared to 0% mortality for unexposed mosquitoes (Jantan et al., 1999).

2.4.3 Indirect impacts on *Anopheles* mosquito behavior

Changes in cooking fuel may have a number of indirect effects on *Anopheles* mosquito behavior, which are not directly related to fuel combustion itself. For example, indoor cooking with biomass fuel can lead to soot buildup on long-lasting insecticide treated nets (LLINS). While soot in itself does not necessarily reduce the effectiveness of LLINs (Curtis et al., 1990; Kayedi et al., 2008), it can induce owners to more frequently wash the net (Bhatt et al., 2012; Miller et al., 1999). Frequent washing reduces the effectiveness of the nets by deteriorating the insecticides.

The adoption of cleaner cooking fuels may be also be accompanied by changes in house ventilation that could impact human exposure to *Anopheles* mosquitoes. For example, many stove interventions focus on increasing ventilation in cooking spaces by widening eave or window spaces, installing chimneys, hoods or flues, or encouraging the opening of windows during cooking (Apte & Salvi, 2016; Bruce et al., 2002; McCracken et al., 2007; Schilmann et al., 2019; Weaver et al., 2017). Some of these household modifications could facilitate household entry by host-seeking mosquitoes, and the presence of open eaves, unscreened windows, or other openings in houses has been widely shown to increase indoor *Anopheles* density and malaria risk (Killeen et al., 2019; Lindsay, 1995; Lwetoijera et al., 2013). On the other hand, the adoption of clean cooking fuels such as LPG could reduce the perceived need for ventilation. Individuals may therefore close eave spaces or chimneys that were otherwise required for venting smoke from biomass fuel burning and thereby block mosquito entry. However, it is important to note that ventilation is still important for LPG stoves to reduce exposure to nitrogen and dioxide and other pollutants (Kephart et al., 2021).

Similarly, households might alter their primary cooking location when they adopt cleaner-burning fuels. For example, households in Rwanda frequently cook either outside or in a separate kitchen structure (Kirby et al., 2019). One study in Kigali reported that individuals preferred cooking indoors after received LPG stoves, whereas another study in Western Province reported higher rates of outdoor cooking among intervention households following a rocket cookstove intervention (Kirby et al., 2019; Seguin et al., 2018).

These changes in cooking location could alter human exposure to mosquitoes. For example, most *Anopheles* mosquitoes bite indoors at night, but earlier and outdoor biting has been reported in Rwanda and elsewhere in sub-Saharan Africa (Hakizimana et al., 2018; Russell et al., 2011; Sherrard-Smith et al., 2019). Additionally, changing the location of cooking stoves may alter the repellent or attractant effect that they provide. For example, fuel combustion indoors could increase ambient CO_2 concentrations, which are highly attractive to host-seeking mosquitoes (McMeniman et al., 2014; McPhatter & Gerry, 2017), or repel mosquitoes from entering the house because of high smoke levels (Hiscox et al., 2013). These effects could vary substantially depending on the type of fuel used, cooking location and duration, season, and vector species.

Similarly, a number of studies have suggested that the adoption of artificial lighting could directly and indirectly alter exposures to *Anopheles* mosquitoes and malaria risk (Barghini & de Medeiros, 2010). In Burkina Faso, for example, a case-control study found that electrification was associated with two-fold higher odds of malaria, after adjusting for relevant confounders. Similar results have been observed in cross-sectional studies in Uganda and Malawi (Pellegrini & Tasciotti, 2016; Tasciotti, 2017). Nighttime artificial lighting may attract host-seeking mosquitoes, which are attracted by specific wavelengths of visible light (Costa-Neta et al., 2017; Owens & Lewis, 2018). The adoption of artificial lighting can also alter human behaviors such as time spent outdoors at night or timing of sleep (Beale et al., 2017), both of which influence exposure to night-biting vectors. Electricity could also reduce dependence on biomass fuels for cooking and heating, reducing repellent effects from biomass smoke (Yamamoto et al., 2010). On the other hand, electricity may co-occur or contribute to other improvements in socio-economic status and housing which are generally associated with reduced malaria incidence. This could account for the negative association between electricity and malaria that has been observed in cross-sectional studies in Rwanda, Kenya, and elsewhere (Kateera et al., 2015; Sultana et al., 2017). Cleaner fuel adoption could also co-occur or result from general improvements in SES, in which case it would be difficult to attribute any changes in vector behavior or malaria incidence directly to fuel-use practices themselves.

2.4.4 Epidemiological outcomes - malaria

Relatively few studies have investigated the impacts of solid biomass fuel combustion on malaria incidence. Most strikingly, a recent RCT on the impact of clean cookstoves on pneumonia incidence in children in Malawi found an increase in malaria incidence rate in children in the intervention group, compared to those in the control group (IRR = 1.32, 95% CI: 1.03–1.69) (Mortimer et al., 2017). In a case-control study in Burkina Faso, Yamamoto et al. found that higher PM_{10} exposures were associated with lower incidence of clinical malaria episodes in children (Yamamoto et al., 2011). The same authors also conducted a case-control study with women and children diagnosed with severe malaria. They found that controls were exposed to slightly higher levels of particulate matter (PM2.5) and carbon monoxide (CO), although the effects on severe malaria risk were not statistically significant (Yamamoto et al., 2007). In Thailand, Chaveepojnkamjorn et al. found that not using a smoky fire was a risk factor for malaria infection, measured by microscopy in a cross-sectional survey (Chaveepojnkamjorn & Pichainarong, 2005). Similarly, van der Hoek et al. found that using traditional fumigants was protective against malaria episodes in a village in Sri Lanka, although they did not specify what type of plant material was used (van der Hoek et al., 1998).

Other epidemiological studies have found null or positive associations between biomass combustion and malaria. In the Gambia, Snow and colleagues found no difference in malaria prevalence among children from houses which burned *Daniella oliveri* bark, or ‘churrai’ as an insect repellent, compared to those who did not (Snow et al., 1987). However, some authors have noted that households which use traditional insect repellents may be differentially more exposed to mosquito biting, which may confound the relationship between repellent use and malaria outcomes (Biran et al., 2007).

Additionally, Jaenson and colleagues did not find an association between reported use of firewood for mosquito control and self-reported febrile illness episodes among adults and children (Jaenson et al., 1994). In Mexico, Danis-Lozano found (1996) found no association between use of unspecified smoke and malaria infection (Danis-Lozano et al., 1999). Ghebreyesus and colleagues noted that having a kitchen inside the house was a risk factor for malaria in a hyperendemic area of Ethiopia (Ghebreyesus et al., 2000). However, this finding may be subject to residual confounding if kitchen location is related to socioeconomic status, which is an important risk factor for malaria. One cross-sectional study by Semakula and colleagues examined Malaria Indicator Survey (MIS) data from four countries and found that use of firewood as a cooking fuel was associated with an increased malaria risk compared to houses which used charcoal (Semakula et al., 2015). This finding may also be subject to confounding, given that charcoal use is related to socio-economic status, which is highly correlated with malaria risk (Degarege et al., 2019).

Besides impacting vector behavior and human exposure, changes in cooking fuel may have indirect effects on human malaria outcomes. For example, exposure to household air pollution can alter or reduce innate immune function (Gordon et al., 2014; Lee et al., 2015), which can make individuals more susceptible to malaria or other infectious diseases. The adoption of cleaner cooking fuels could improve immune function and reduce malaria infections or severe malaria outcomes. This effect has not been widely studied, although malaria infection during pregnancy has been shown to mediate adverse pregnancy outcomes associated with HAP exposure (Amegah et al., 2013). One other study found that cooking fuel influences bed net use, although this is likely an artifact of uncontrolled confounders such as geographic location and socio-economic status (Tapera, 2019).

2.4.5 Impacts of fuel combustion on culicine mosquito behavior and arbovirus transmission

The impacts of biomass fuel combustion on *Anopheles* mosquito behavior and malaria epidemiology was reviewed above. Here we focus on the impacts of fuel combustion on culicine mosquitoes (i.e., *Aedes*, *Culex*, and *Mansonia* mosquitoes). A number of laboratory studies have focused on insecticidal properties of burning leaves or specific plant parts as putative insect repellents, using untreated mosquito coils made out of wood or other plant parts as a ‘blank’ smoke control (Kamalakannan et al., 2009; Ramkumar et al., 2015). However, only a single study included no smoke as a negative control, and found slightly higher mortality among *Aedes aegypti* exposed to smoke from blank wood coils (6.7%) compared to no smoke (0.0%) (Jantan et al., 1999). Propane-powered mosquito traps have been shown to be effective in attracting *Cx.* spp. mosquitoes in laboratory and field experiments (Cooperband & Cardé, 2006; Kline, 2002). However, no studies have directly addressed the impact of LPG combustion for domestic cooking on culicine mosquito attraction.

In a field-based experimental study in Brazil, Moore and colleagues found that smoke from *Attalea princeps* burned on a charcoal fire reduced human landing catches of *Mansonia* spp. by 51%, and a charcoal-only fire reduced biting by 20% compared to a locally sourced mosquito coil (Moore et al., 2007). In Papua New Guinea, Vernède et al. found that smoke from wild mango wood (*Mangifera* sp.), and coconut husks (*Cocos nutifera)* reduced culicine human landing catches by 57% and 62%, respectively. In the Gambia, Lindsay and Janneh found that smoke from burning *Daniella oliveri* bark reduced human landing catches of a mixed group of culicines, including *Cx. thalassius* Theobald, *Cx. neavei*, *Cx. tritaeniorhynchus, and Aedes simpsoni* (Lindsay & Janneh, 1989). In Guinea Bissau, Pålsson et al. measured 85% and 75% reductions in mixed anopheline and culicine human landing catches when collectors sat next to smoldering fires with *Hyptis suaveolens* and *Daniellia oliveri* compared to no fire (K. Pålsson & T. G. T. Jaenson, 1999b). In rural Thailand, Nagao et al. (2003) noted that the use of firewood for cooking was associated with reduced larval densities of *Aedes aegypti*, although this may be an artifact of uncontrolled confounders such as distance from urban centers (Nagao et al., 2003).

A single epidemiological study has investigated the relationship between biomass fuel use and incidence of *Aedes*-borne arboviruses. Madewell and colleagues conducted a retrospective case-control study on the association between household air pollution exposure and incidence of Dengue, Chikungunya, and Zika in patients hospitalized with acute febrile illness or diarrhea in Guatemala. After adjusting for relevant confounders, they found that cooking with firewood ≥5 times per week was inversely associated with arbovirus risk (AOR: 0.54; 95% CI: 0.36–0.81). Similarly, cooking with firewood in the main house and cooking with firewood on an open hearth were protective (AOR: 0.22; 95% CI: 0.08–0.57 and AOR: 0.50; 95% CI: 0.33–0.78) (Madewell et al., 2020). The authors suggested this effect could be due to repellent effects of biomass fuel combustion on *Aedes aegypti*, although no entomological sampling was conducted. No studies have investigated the relationship between cooking fuels and other culicine-borne pathogens such as Yellow Fever Virus, West Nile Virus, or Rift Valley Fever.

Human behavioral changes associated with the adoption of LPG or other clean cooking fuels may also indirectly affect exposure to culicines and arboviruses. For example, fuel interventions may result in changes to cooking location or to cooking time, which could affect exposure to indoor or outdoor biting culicines. Bensch and Peters found an improved biomass cookstove intervention resulted in more outdoor cooking and shorter cook times (Bensch & Peters, 2015). A large cookstove intervention in western Rwanda also resulted in increased rates of outdoor cooking (Kirby et al., 2019; Rosa et al., 2014). On the other hand, the adoption of cleaner fuels such as LPG may result in increased indoor cooking, especially in countries where outdoor cooking is common (Langbein et al., 2017). *Ae. aegypti* primarily bite mostly indoors during the morning and early evening, which coincides with cooking times for many families (Captain-Esoah et al., 2020; Ndenga et al., 2017). They also commonly rest in kitchens and other indoor areas of the house (Chadee, 2013). On the other hand, *Culex* mosquitoes bite both outdoors and indoors, generally during the night. (Rozendaal, 1997; Uttah et al., 2013).

The adoption of clean cooking fuels could also impact LLIN integrity and use, as described in the previous section. Because many culicine mosquitoes such as the *Cx. pipiens* species complex are nighttime biters and seek hosts both indoors and outdoors (Uttah et al., 2013), alterations in bed net use or net integrity could impact human exposure to culicine mosquitoes.

2.4.6 Impacts of cooking fuels on domestic filth fly behavior and enteric pathogen exposure risk

Smoke from burning plant parts is used widely to repel *Musca domestica* and other filth flies (Baana et al., 2018). However, most laboratory studies have investigated the insecticidal effects of specific plant extracts rather than burning whole plant parts (Cheraghi Niroumand et al., 2016; Kumar et al., 2012; Pavela, 2008; Sharma et al., 2011). In one laboratory-based experiment, Denloye and colleagues found that the smoke from burning pellets made of pulverized waste-wood, palm kernel cake, and *Adansonia digitata* leaves deterred 37.5% of *Musca domestica* flies into a separate chamber, compared to 0.0% of flies which were not exposed to smoke (Denloye et al., 2006).

Smoke is also traditionally used to deter tsetse flies (*Glossina* spp.), which are vectors of *Trypanosoma brucei*, the causative agent of human African sleeping sickness and livestock nagana. In field experimental studies, Torr et al. found that smoke from wood and dung fires reduced tsetse fly catches by 50 – 90% (Torr et al., 2011). In another study, however, Vale et al. (2013) found that the presence of woodsmoke from a fire in or just outside houses was not protective against house entry by tsetse flies (Vale et al., 2013). On the other hand, *Chrysops silacea*, the principal vector of Loiasis, appears to be highly attracted to smoke from wood fires. In early studies in the Democratic Republic of Congo, Duke showed that the presence of woodsmoke increased *Chrysops* catches more than six-fold (Duke, 1955). Fires from kerosene stoves did not produce the same attractant effect, suggesting that flies were attracted to woodsmoke odors or volatiles rather than to heat or general combustion byproducts such as CO_2.

Cooking location is likely related to fly density as well. For example, the presence of open latrines or open defecation sites is positively associated with the density of filth flies such as *Chrysomya putoria* (S. W. Lindsay et al., 2012; T. Lindsay et al., 2012; Wolfe et al., 2017). Similarly, higher temperatures in food storage areas along with higher fly densities are associated with increased log10 *E. coli* counts on stored foods (Doza et al., 2018). Cookstove interventions which result in a change in cooking location may thereby indirectly affect fly exposure by altering the kitchen's proximity to latrines or other sources of contamination, or affect the temperature of the food storage environment (Sharma & Jain, 2019). Food storage practices also mediate food contamination by flies (Parvez et al., 2017), and could be influenced by cookstove interventions.

2.4.7 Literature gaps:

The existing research on the impacts of domestic fuel combustion on vector ecology and vector-borne disease transmission is subject to a number of important limitations. First, no entomological or epidemiological studies were explicitly designed to investigate the differences between different types of cooking fuels or the effects of cleaner fuel adoption, although the CAPS trial in Malawi did measure malaria incidence as a secondary outcome and found a 33% increase in malaria incidence among children whose households received improved cookstoves compared to control households who cooked with traditional stoves (Mortimer et al., 2017). Further studies are needed to compare entomological and epidemiological outcomes across different fuel types such as LPG, wood and charcoal, as well as with different stove types. No studies investigated the epidemiological impacts of biomass fuel use on other *Anopheles*-vectored pathogens such as *Wuchereria bancrofti,* or O'Nyong-Nyong Virus, both of which are transmitted by *Anopheles* species in East Africa (Rezza et al., 2017; Ruberanziza et al., 2009), and evidence for culicine-transmitted viruses and fly-borne pathogens is extremely limited. A single study found that cooking with biomass fuels indoors was associated with reduced arbovirus risk, and suggested that this was mediated by the deterrent effects of smoke on *Aedes aegypti* (Madewell et al., 2020).

Issues with comparability also complicate interpretation of the literature. For example, differences in cooking location, fuel type, length of cooking time, ventilation of the cooking area, and vector species all could affect mosquito behavior and human exposure. Future studies should carefully characterize these covariates and assess their importance. Additionally, geographic variations in vector and human behavior may reduce the generalizability of findings. For example, one indoor air pollution intervention in western Rwanda found that participants were more likely to cook outside after the intervention, whereas another study in central Rwanda reported that participants were more likely to cook indoors.

Another key issue with the entomological literature is the difficulty in isolating the exact mechanism by which fuel combustion impacts mosquito behavior. Most studies, for example, only investigated the overall effects of burning fuels, and it remains unclear which attributes of the fuel combustion (e.g. chemical volatiles, heat, CO_2, particulate matter, or other factors) are the primary influencers of vector behavior when changes are observed. Further research should attempt to isolate these factors, which could prove important for ensuring that HAP interventions do not increase exposure to arthropod vectors. Finally, very few studies simultaneously investigated repellence, deterrence, and exitorepellence (i.e., induced exophily), which is critical for gaining a more complete understanding of how fuel combustion impacts vector behavior and human exposure risk (WHO, 2013).

2.5 Potential impacts of land-use, climate change, and vector control on vector ecology and malaria transmission in Rwanda

As mentioned previously, the NMCP conducted a situational analysis following the dramatic malaria resurgence that occurred in Rwanda from 2011 to 2017. The analysis identified numerous potential causes of resurgent transmission, which included rice cultivation/agricultural environmental modification, increased temperatures and rainfall, insecticide resistance, and low or inconsistent coverage of vector control interventions (PMI, 2019; RBC, 2017). Other studies have suggested that higher rates outdoor biting and shifts in biting timing could be reducing the effectiveness of indoor interventions such as LLINs and IRS (Hakizimana et al., 2018). Here we review the potential effects of these hypotheses, relying on literature in Rwanda or elsewhere in East Africa whenever possible.

Reports from the NMCP and President's Malaria Initiative (PMI) noted a spatial and temporal correlation between areas of expanded rice cultivation and increased transmission (PMI, 2019; RBC, 2017). Rice cultivation can produce ideal habitat for some *Anopheles* species, particularly *An. arabiensis* (Lacey & Lacey, 1990; Sinka et al., 2010), which has become the most common malaria vector in Rwanda (Hakizimana et al., 2018). Early stages of rice transplanting and growth provide ideal water and sunlight conditions for larval development (Diuk-Wasser et al., 2006; Githeko et al., 1996; Mutero et al., 2000; Muturi et al., 2007; Mwangangi et al., 2006; Mwangangi et al., 2010; Sinka et al., 2010; Yasuoka & Levins, 2007), and rice volatiles may attract gravid females (Wondwosen et al., 2016).

Conversion of natural marshlands for agriculture can increase water temperatures and local ambient air temperatures, increasing vectorial capacity in highland areas (Afrane et al., 2006; Lindblade et al., 2000; Munga et al., 2005; Munga et al., 2006; Wamae et al., 2010). Insecticide and fertilizer applications can also favor population growth due to reduced predation and alterations in dissolved oxygen, turbidity, and nutrient availability (Chandler & Highton, 1976; Mogi, 1993; Mutero et al., 2004; Muturi et al., 2007;

Mwangangi et al., 2006; Service, 1977). Many of the same environmental changes are associated with increased *Anopheles* populations in newly constructed dams and irrigation systems (Yasuoka & Levins, 2007).

In areas of stable malaria transmission, higher *Anopheles* populations from rice cultivation are not necessarily associated with increased disease burden, a phenomenon known as the “paddy paradox” (Ijumba & Lindsay, 2001). This may be due to decreased adult fitness due to high larval densities, changes in *Anopheles* species composition, or increased living standards and use of malaria interventions among populations engaged in rice cultivation (Diuk-Wasser et al., 2005; Dolo et al., 2004; Ijumba & Lindsay, 2001; Ijumba et al., 2002; Lindsay et al., 1991).

However, in areas of unstable transmission such as the East African Highlands, rice cultivation and accompanying changes in vector and parasite population dynamics have been associated with major epidemics (Carnevale et al., 1999; Ijumba & Lindsay, 2001). In highland Burundi, for example, vectorial capacity was 150 times higher in areas near a rice irrigation scheme compared to a nearby cotton-growing area, and parasite prevalence in rice growing communities ranged from 24.4 - 69.2% compared to 4 – 30% in control areas (Coosemans, 1985). Other studies from highland Kenya, Ethiopia, Tanzania, and Madagascar have observed higher malaria prevalence associated with rice cultivation (Carnevale et al., 1999; Kolsky, 1994; Rice et al., 2018; Rumisha et al., 2019). Dam construction and irrigation for other crops such as corn have been implicated in increased malaria transmission elsewhere in East Africa (el Gaddal et al., 1985; Ghebreyesus et al., 1999; Hunter et al., 1993; Keiser et al., 2005; Kibret et al., 2014; Yasuoka & Levins, 2007). In Rwanda, studies have observed associations between malaria infection and proximity to irrigated farmland and water-based agro-ecosystems (Rulisa, Kateera, Bizimana, Agaba, Dukuzumuremyi, Baas, de Dieu Harelimana, Mens, R Boer, et al., 2013; Tuyishimire, 2016). However, the longitudinal impacts of increased rice cultivation and dam construction in Rwanda have not been investigated.

Short and long-term temperature changes can also exert profound effects on vector and parasite populations and alter malaria transmission dynamics in the East African Highlands. Temperature is critical for regulating numerous vector and parasite biological processes, and slight fluctuations can dramatically alter transmission dynamics in regions at the margins of transmission suitability. *P. falciparum* parasites typically cannot develop within their insect hosts at ambient temperatures below 15-16° C, whereas temperatures above 30° C may also reduce vectorial capacity (Beck-Johnson et al., 2013; Mordecai et al., 2013). Surface water temperatures are a key driver of immature *Anopheles* population dynamics, and warmer temperatures generally increase productivity (Krijn P. Paaijmans et al., 2010). Diurnal temperature fluctuation and biting time are increasingly recognized as important determinants of parasite development (Blanford et al., 2013; Charlwood, 2017). Short-term fluctuations in temperature are widely associated with entomological and epidemiological parameters of malaria transmission throughout East Africa (Kweka et al., 2016; Nkurunziza et al., 2010).

Long-term warming as a result of climate change is widely thought to have contributed to increased malaria transmission in the East African highlands since the 1990s, although this subject has been hotly debated (Alonso et al., 2011; Chaves & Koenraadt, 2010; Hay et al., 2002; Loevinsohn, 1994; Omumbo et al., 2011; Pascual et al., 2006; Stern et al., 2011). Additionally, many models project that the East African Highlands will experience dramatic increases in malaria transmission as a result of future climate warming (Afrane et al., 2012; Caminade et al., 2014; Mordecai et al., 2020; Parham & Michael, 2010; Ryan et al., 2020; Ryan et al., 2015). This is a result of increased geographic distribution of A*nopheles* to higher altitudes, increased vectorial capacity due to temperature-sensitive accelerations of mosquito and parasite life history traits, exposure of immunologically naïve populations, human movement on a range of spatial scales, and high population density in highland East Africa. Long-term climate change may interact with finer-scale environmental changes such as deforestation or agricultural change, resulting in further increases in transmission suitability (Afrane et al., 2012; Himeidan & Kweka, 2012).

Short and long-term fluctuations in rainfall and humidity also have important impacts on *Anopheles* populations and malaria transmission. Rainfall events generally increase *Anopheles* populations via the creation of larval habitat sites and increased humidity, and rainy seasons drive malaria seasonality across sub-Saharan Africa (Abiodun et al., 2016; Krefis et al., 2011; Odongo-Aginya et al., 2005; Thomson et al., 1999). Periods of heightened rainfall, such as those associated with El Niño Southern Oscillation (ENSO) events, often result in malaria epidemics, particularly in areas of unstable transmission (Bouma & van der Kaay, 1996). In East Africa, ENSO events occur every 2-10 years and can result in large malaria epidemics (Lindsay & Martens, 1998). For example, high rainfall during an ENSO event in highland Uganda was associated with an increase increased indoor *An. gambiae* s.l. density and epidemic malaria transmission (Kilian et al., 1999; Lindblade et al., 1999). Periods of drought have been implicated in declining *An. funestus* populations and malaria transmission in arid West Africa (Diouf et al., 2017). On the other hand, failing rainfall and drought in wet areas can cause rivers to pool and increase *Anopheles* populations, leading to increased malaria transmission (Bouma & van der Kaay, 1996).

Long-term climate change is projected to alter the timing and length of East Africa's two rainy seasons, resulting in longer periods of drought as well as more frequent and intense heavy rainfall events (Nicholson, 2017). These changes can have both positive and negative effects on mosquito ecology and malaria transmission. For example, heavy rainfall events can actually flush larval habitats (Paaijmans et al., 2007), but increased rainfall variability has been associated with increased malaria transmission in the East African Highlands (Hammerich et al., 2002; Pascual et al., 2000; Zhou et al., 2004b). Variable rainfall and periodic droughts may alter vector species composition by selecting for more drought tolerant species, such as *An. arabiensis* (Thomson et al., 2017).

Insecticide resistance can result from widespread insecticide use for malaria control and agriculture. Resistance to the most commonly used insecticide class, pyrethroids, has dramatically increased across sub-Saharan Africa in the last twenty years (Hancock et al., 2020; Hancock et al., 2018). Insecticide resistance could lead to catastrophic failure of malaria control gains, 78% of which are attributable to insecticide-based vector control interventions (WHO, 2016). A number of studies have shown large reductions in malaria incidence when pyrethroid-treated nets are combined with a synergist such as PBO, or indoor residual spraying with another insecticide class, suggesting that insecticide resistance reduces the effectiveness of LLINs on their own (Kafy et al., 2017; Protopopoff et al., 2018; Sarah G. Staedke et al., 2020). Other studies in Kenya and South Africa have linked pyrethroid resistance to the reemergence of previously controlled vectors and resurgent malaria transmission (Hargreaves et al., 2000; McCann et al., 2014). However, multi-country cohort studies have found that insecticide resistance on its own was not associated with reduced LLIN effectiveness, infection prevalence, or incidence (Kleinschmidt et al., 2018; Lindblade et al., 2015; Ochomo et al., 2017).

Relatedly, changes in vector behavior such as outdoor or earlier biting can reduce the effectiveness of indoor interventions such as LLINs and IRS (Cooke et al., 2015; Reddy et al., 2011; Sherrard-Smith et al., 2019; Sougoufara et al., 2014; Steinhardt et al., 2017). Earlier biting may actually increase vectorial competence due to temperature sensitivity of malaria parasites (Suh et al., 2020). Selection pressure from vector control can also result in changes in the most dominant malaria vectors to more exophagic and exophilic species such as *An. arabiensis* and *An. coustani* (Bayoh et al., 2010; Cooke et al., 2015; Musiime et al., 2019). Evidence from Rwanda shows an increasing trend of pyrethroid and organochlorine insecticide resistance among *Anopheles* mosquitoes, increased outdoor biting, and a species composition shift towards *An. arabiensis* as the dominant vector (Hakizimana et al., 2018; Hakizimana et al., 2016; PMI, 2020). However, carbamates and organophosphates remain widely effective, and the NMCP has incorporated insecticide rotation as part of a broader strategy of insecticide resistance management since 2014 (Karema et al., 2020; PMI, 2020; RBC, 2020a).

Figure 2-3 shows a conceptual diagram of the key environmental changes considered in this analysis. The three distal changes are shown on the left, and include climate/meteorological change, land-use change, and vector control. Proximal environmental changes associated with the distal changes are shown in the next column, including changes in temperature, rainfall, humidity, larval habitat. Each of these proximal changes may influence vector and parasite life history traits and ecology (e.g. adult survival, biting rates, and external incubation periods, exophagy, host-preference, and species composition). These traits are key components of the malaria basic reproductive number, R_0, given by the classic Ross-McDonald formula (Macdonald, 1957):

$$R_0 = \left(\frac{Ma^2 bce^{-\mu EIP}}{Nr\mu} \right)^{1/2}$$

Where M stands for mosquito density, a is the mosquito biting rate, bc represents vector competence, μ is the mortality rate for adult mosquitoes, EIP is the external incubation period for the development of *Plasmodium* parasites within mosquitoes, N represents human density, and r is the rate at which humans recover from previous infections and acquire immunity (Dietz, 1993; Mordecai et al., 2013). Changes in these history traits above can have first-order, second-order, or further effects on R_0, which are reflected in changes in transmission parameters such as EIR and malaria incidence (Mordecai et al., 2013; Smith et al., 2007).

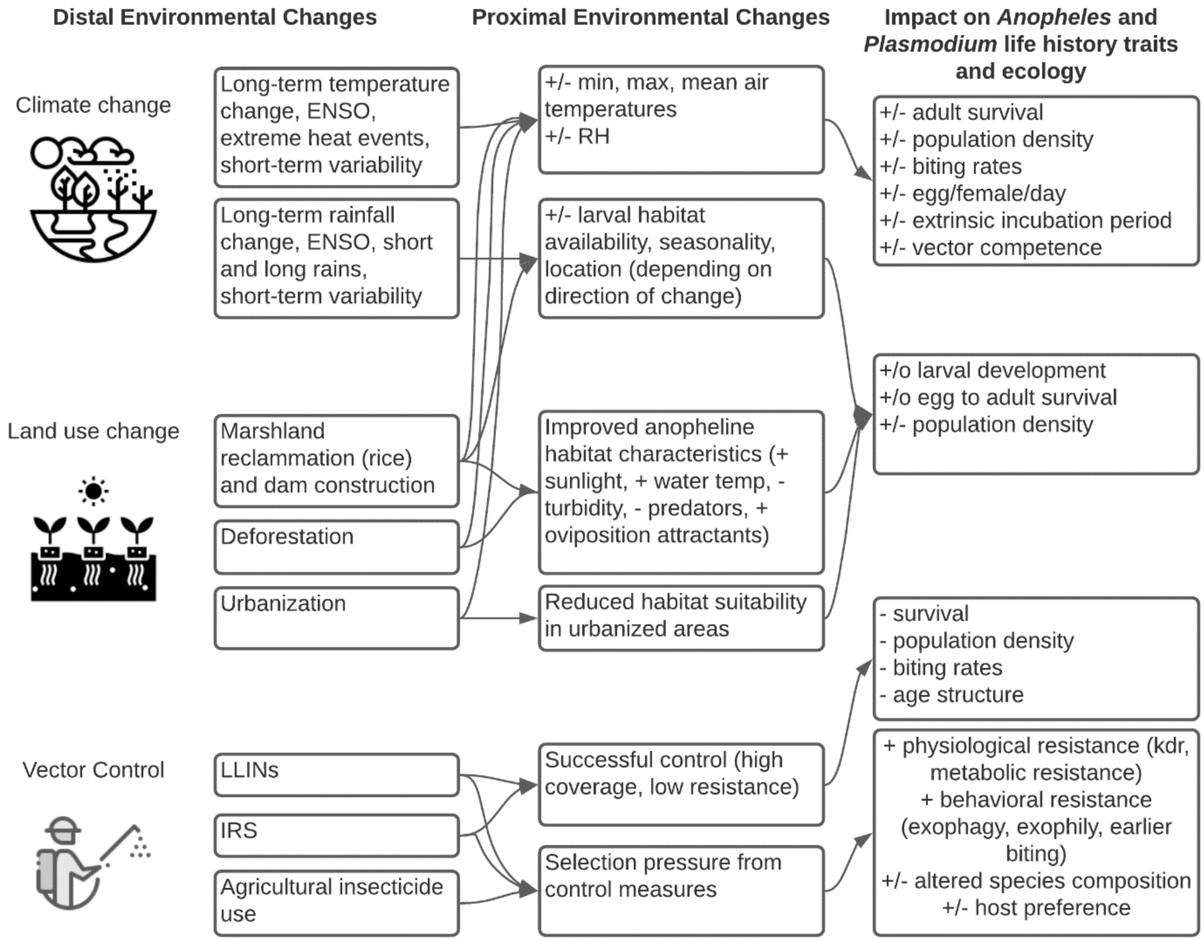

Figure 2-3 Conceptual model of impacts of multi-scale environmental change on *Anopheles* mosquito and *Plasmodium falciparum* life history traits. Distal environmental changes are shown on the left, with their relationship to proximal environmental drivers of vector-parasite dynamics in the center. Life history, behavioral, and ecological characteristics of *Anopheles* mosquitoes and Plasmodium parasite dynamics shown on the right, all of which are important components of malaria transmission dynamics.

3 Research Aim 1. Assessing the Effects of Cooking Fuels on *Anopheles* Mosquito Behavior: An Experimental Study in Rural Rwanda

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[ǂ]The views expressed here are those of the authors and do not represent official views or policies of the National Institutes of Health or the U.S. Government.

3.1 Introduction

Cleaner cooking fuels such as liquid petroleum gas are increasingly promoted to reduce household air pollution (HAP), which is responsible for more than 2.3 million deaths per year (Naghavi et al., 2017; WHO, 2018a). However, there is some concern that reductions in smoke or other volatiles from traditional fuels could affect mosquito behavior and transmission of malaria or other vector-borne diseases (Biran et al., 2007). Smoke has been used as an insect repellent for centuries,(Torr et al., 2011) and components of biomass combustion such as carbon dioxide (CO_2), heat, and chemical volatiles are known to influence mosquito behavior (Dube et al., 2011; Gillies, 1980; Hawkes et al., 2017).

Numerous entomological studies have reported negative associations between biomass combustion and density and household entry of *Anopheles* mosquitoes (Hiscox et al., 2013; Kweka et al., 2008; McCann et al., 2017; K. Pålsson & T. G. Jaenson, 1999b). Biomass combustion is also associated with reduced blood feeding success, altered resting behavior, and higher exit rates of mosquitoes in experimental and observational field settings (Bockarie et al., 1994; Charlwood et al., 2018; Kweka et al., 2008; Lindsay, 1995; Maia et al., 2016; Paru et al., 1995; Vernède et al., 1994). However, these studies were not designed to measure the effects of biomass fuel combustion for cooking or domestic heating, or compare the effects of different cooking fuels.

Although epidemiological evidence is limited, a cluster randomized controlled trial of cleaner-burning biomass stoves in Malawi reported a significant increase in malaria incidence among children in houses that received the intervention (Mortimer et al., 2017). A recent case-control study in Guatemala also found that individuals from houses which cooked with fuels other than firewood had an increased risk of arbovirus infection compared to houses that cooked with firewood in the main house or on open hearths (Madewell et al., 2020). In both cases, however, these were secondary outcomes of health impact evaluations. Other observational studies have reported mixed associations between biomass fuel use and malaria incidence (Chaveepojnkamjorn & Pichainarong, 2005; Danis-Lozano et al., 1999; Ghebreyesus et al., 2000; Jaenson et al., 1994; Snow et al., 1987; van der Hoek et al., 1998; Yamamoto et al., 2011; Yamamoto et al., 2010).

Despite this entomological and epidemiological evidence, no studies have directly investigated the impacts of the adoption of clean-burning cooking fuels on mosquito behavior or vector-borne disease transmission (Biran et al., 2007). This information is critical for understanding potential effects of clean fuel adoption and, if necessary, recommending the promotion of vector control measures in tandem with clean cooking interventions. As a preliminary step in addressing this research gap, we undertook an experimental evaluation of the impacts of traditional and clean cooking fuels on the behavior of the most important malaria vector in Rwanda, *Anopheles gambiae.*

3.2 Materials & Methods

3.2.1 Research objectives

The primary objective of this study was to evaluate if, and to what extent, the adoption of clean-burning fuels could affect *Anopheles* mosquito behavior. We employed a series of controlled, semi-field experiments to measure differences in household entry, host-seeking, household exiting, and mortality among *Anopheles* mosquitoes across three commonly used fuel types: wood, charcoal, and liquid petroleum gas (LPG).

3.2.2 Study location

This study was conducted in Eastern Province, Rwanda. The area was selected in part because of its proximity to a large randomized controlled trial to assess the health effects of cooking with LPG in a population traditionally relying on solid biomass fuels (Clasen et al., 2020). Eastern Province has the highest malaria burden of any part of the country (ICF, 2017), and malaria prevalence among children under five increased from 3.4% to 18.4% between 2010 and 2017 (PMI, 2019). The *An. gambiae* species complex are the principal malaria vectors in Eastern Province and elsewhere in Rwanda (Hakizimana et al., 2018).

3.2.3 Experimental huts

The Rwanda Biomedical Center (RBC) maintains a group of six experimental huts near the town of Ruhuha in Bugesera District, Eastern Province. Experimental huts are typically used to test vector control methods, and are identically constructed and situated close together in order to reduce potential confounding from environmental conditions such as ambient temperature or humidity (WHO, 2013). The Rwanda experimental huts are constructed in the West Africa style with concrete block walls, corrugated metal roofs, and a water-filled moat around the perimeter to deter ants. They have four small windows around the outside, and a screened veranda in the upper section of the back wall. They do not have chimneys, but emissions from indoor cooking can escape from windows, a screened veranda, and gaps between the walls and roof. Three of the RBC huts had cement walls and three had mud walls. The main room dimensions were 1.75 x 2.5 meters, with a ceiling height of approximately 2 meters. The adjacent verandas were approximately 1.5 x 1.5 meters.

3.2.4 Trap design and hut modifications

This study necessitated slight modifications to the experimental huts, which are typically designed to allow entry of wild mosquitoes, but do not have compartments for introducing lab-raised mosquitoes or trapping mosquitoes which exit the huts. We worked with a local team of tailors and welders to build and install entry compartments and window exit traps. Designs for both entry compartments and exit traps were adapted from window entry and exit traps, as described by Okumu et al. (2012), Diabaté et al. (2013), and the World Health Organization (WHO, 2013) for use in *Anopheles* mosquito sampling and trapping in experimental huts (Diabaté et al., 2013; Okumu et al., 2012; WHO, 2013).

Entry compartments were constructed of 1m x 1m x 1m rigid steel frames wrapped in untreated mosquito netting. A convex trapezoidal prism extended 15cm into the interior of the hut, with a 1cm high x 50 cm wide opening slit (Figure 3-1a). Collectors used manual aspirators to introduce mosquitoes through a 10cm diameter baffle on the outside of each compartment, which otherwise was tied off to prevent mosquitoes from escaping. Mosquitoes could then pass through the opening slit into the hut, but once inside the hut, the convex design prohibited them from returning back into the entry compartment. This entry compartment design has been shown to be effective in ensuring that mosquitoes can enter a space through the opening at the end of the convex prism, but cannot return back the same way (Diabaté et al., 2013; Khattab et al., 2017). Window exit traps were almost identical to the entry compartments, but were fitted with a concave trapezoidal prism instead of a convex prism (Figure 3-1c). The concave side of the window traps were fixed to the windows of the hut such that mosquitoes could fly out of the hut and into the window exit trap, but could not return the other direction.

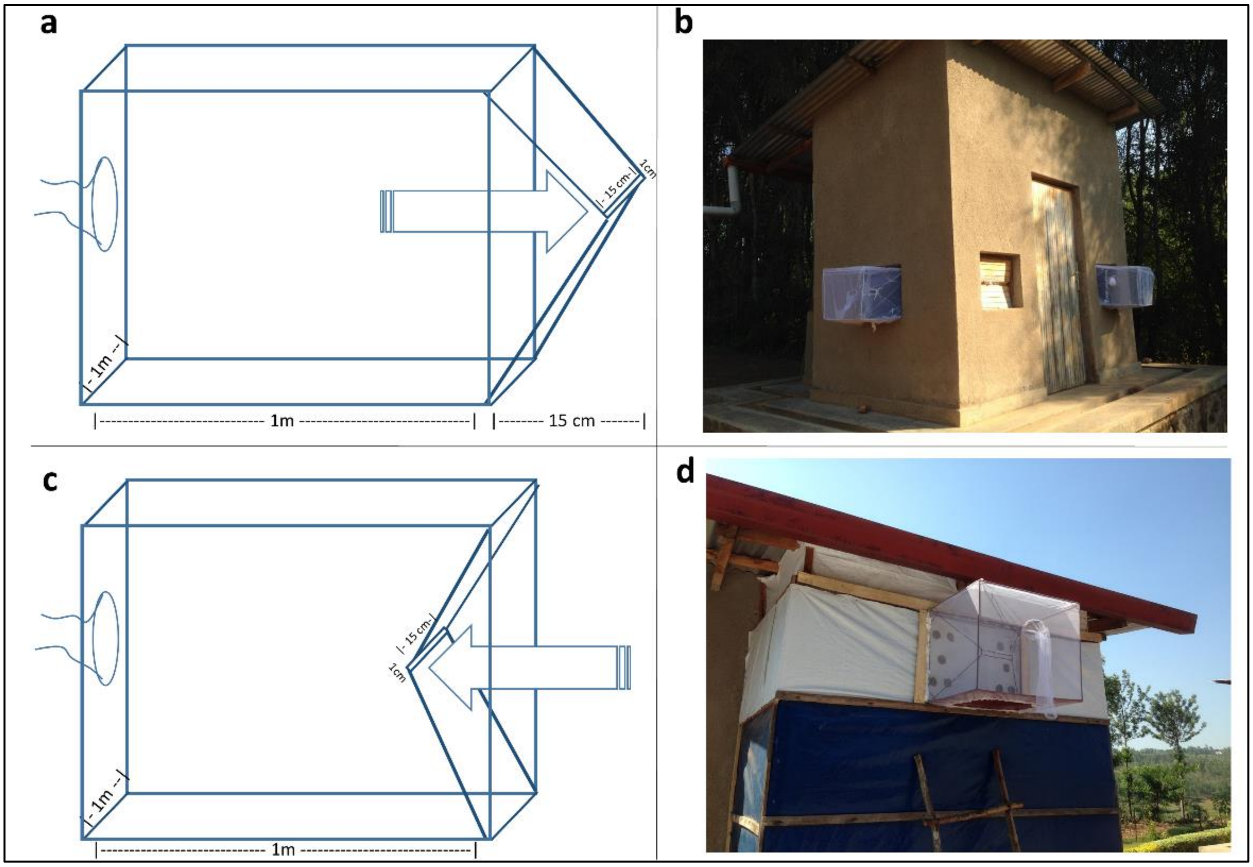

Figure 3-1 Design and layout of entry compartments and exit traps. Design of entry compartment (a), and window exit traps (c). Entry compartments were mounted to the right of the front door of each hut, which faced east (b). Exit traps were mounted to the south wall of each hut (b), as well as the upper veranda area in the back of each hut (d).

One entry compartment was installed on each hut via an open window to the right of the front door (Figure 3-1b). Any remaining gaps between the frame of the compartment and the window frame were sealed with cotton to prevent escape. Two window exit traps were installed on each hut. One was installed on a lower window on the left, south-facing wall of each hut (Figure 3-1b). Another window exit trap was installed on the uppermost section of the veranda, in the back wall of each experimental hut (Figure 3-1d). Typically the veranda section of the experimental huts is screened to mimic a semi-outdoor setting (Djènontin et al., 2009). However, because our experiment was designed to investigate the effects of indoor cooking on mosquito behavior, we sealed the screening with plastic tarp so the whole hut was more representative of indoor conditions. We then installed ten 5cm diameter holes in the tarp to mimic ventilation blocks, known locally as *claustras*, which are commonly used in Rwandan concrete block and mud-brick houses for ventilation. The veranda exit traps were placed over the ventilation holes (Figure 3-1d).

3.2.5 Laboratory methods for raising mosquitoes

An. gambiae s.s., Kisumu strain mosquitoes were raised in Kigali at the entomology laboratory of the RBC – Malaria and Other Parasitic Diseases Division (MOPDD). Larvae were reared in distilled water and fed with a 10% liver powder solution, and emerging adults were kept in holding cages at 26-28°C and 70-80% relative humidity. Non blood-fed, 3-5 day old females were collected using manual aspirators and transported to the study site in mesh-covered cups with a 10% sugar solution on cotton wool pads. Each cup was held in a cooler prior to use.

3.2.6 Fuels tested

We tested three different cooking fuels: wood, charcoal, and LPG. Wood is the most commonly used domestic cooking fuel in Rwanda, and is used by 63% of households as their primary cooking fuel. Charcoal is the second most commonly used cooking fuel Rwanda, and is used by more than 17% of households (ICF, 2017). We used locally sourced, dried eucalyptus firewood, which is the most widely cultivated fuel-wood species (Ndayambaje & Mohren, 2011). We sourced local charcoal made from eucalyptus wood. Finally, while LPG is not yet widely used in Rwanda, it is increasingly promoted to reduce the harmful health effects of household air pollution from cooking with solid biomass fuels (Das et al., 2018; Kirby et al., 2019; Rosa et al., 2014). Its use for cooking has increased from 0.1% of Rwandan houses in 2010 to 1.6% in 2017, and it is slated to become a major source of cooking fuel in the next decade (ICF, 2017; Infrastructure, 2018; NISR & Macro, 2012). Globally, nearly 3 billion people still rely on traditional biomass fuels such as wood and charcoal for cooking and heating. However, LPG use is expanding rapidly in many low and middle income countries (IEA, 2020; Van Leeuwen et al., 2017).

3.2.7 Schedule and timeline of experiments

We conducted three phases of experiments. In phase 1 we conducted six days of baseline testing in which collections were performed in the absence of any cooking fuel. This phase served as a baseline metric for household entry, host-seeking, and exiting, and to ensure that there were no systematic differences between the huts.

In phase 2 we employed a modified Latin Square design in which collectors rotated between each experimental hut each night, but fuels were held constant for each hut. This was to address the potential residual effects from the combustion of certain fuels, especially wood fires (Kweka et al., 2008). Collectors rotated huts each night in order to address the potential for differing biting attractiveness or different practices between collectors. Phase two consisted of three full rotations of the collectors across the six huts, for 18 total days of collection.

In phase 3 we employed a true Latin Square design, in which LPG and wood fuels were rotated each night to reduce the potential effects of ambient environmental differences (Okumu et al., 2012). Collectors remained in the same hut each night. Phase 3 lasted 6 days and employed two iterations of a 2x3 Latin Square layout to obtain a fully balanced sample. Otherwise, all methods as described below were identical for each phase.

3.2.8 Experimental procedures

Prior to the study, the huts were randomly assigned to either LPG, wood, or charcoal fuels, so that there were two huts for each fuel type (Figure 3-2a). These fuel assignments were held constant throughout phase 2. For phase 3, only LPG and wood fuels were used. They were randomly assigned to each hut for the first night, and rotated nightly thereafter. This decision was made to maximize our ability to compare the effects of LPG with wood, which is the most commonly used cooking fuel in Rwanda. Wood fires were lit in traditional three-stone stoves. Charcoal fires were lit in locally made, unimproved stoves call *imbaburas* (Barstow et al., 2014). Ten kg LPG fuel cylinders were purchased locally, and fitted with a simple burner and an attachment for cooking on top of the cylinder.

Each sampling round lasted from approximately 6pm to 6am the next morning. No fuels were lit during phase 1. During phase 2 and 3, each fuel was lit in the veranda area of the experimental huts at approximately 5:45pm each night, and a two-liter pot of water placed above the flame and brought to a low rolling boil to standardize combustion intensity across fuels. Four kilograms of dried eucalyptus wood and 200 grams of charcoal were pre-measured and the wood chopped into 2-5cm diameter pieces so it could be routinely replenished during cooking. The LPG stoves were lit and maintained at medium intensity. Each fuel source remained burning for one hour and was extinguished at 7pm.

At 6pm, trained entomology officers employed by the Rwanda Biomedical Center used manual aspirators to release 25 *An. gambiae* s.s. mosquitoes in entry compartments attached to the windows of each hut. Trained collectors sat on a small stool approximately one meter from the stove and wore long-sleeved clothes with one pant-leg rolled up. They then conducted human landing catch using a flashlight and glass collection tubes to catch all mosquitoes which landed on them (Hakizimana et al., 2018). Human landing catch was used to measure host-seeking behavior during cooking (6pm – 7pm) and for 2 hours afterwards (7pm – 8pm & 8pm – 9pm) (Figure 3-2b, Intervals 1-3). Mosquitoes caught during each of the three hourly intervals were placed in pre-labeled envelopes for counting the next day. At the end of each hour, entomology officers used flashlights to count and record the number of mosquitoes remaining in the entry traps, as well as in the two window exit traps outside each hut. Because all mosquitoes were laboratory-reared, there was no potential for pathogen transmission if collectors were incidentally bitten while conducting human landing catch.

At 9pm the collectors were asked to retire under untreated bed nets. Twenty-five more mosquitoes were then released into the entry compartments of each hut in order to simulate the arrival of host-seeking mosquitoes long after cooking was completed. Host-seeking was approximated during the fourth interval (9pm – 6am) via Miniature CDC light traps (Model 512, John W. Hock Company, Gainesville, FL). CDC light traps are frequently used as a proxy for host-seeking in settings where human landing catch is not ethical or feasible (Lima et al., 2014; Pinder et al., 2015; Protopopoff et al., 2018). Light traps were hung at a height of approximately 1.5 meters at the foot end of the bed, a height that has been shown to maximize catches of host-seeking *An. gambiae* s.l. mosquitoes (Mboera et al., 1998). The light traps were illuminated from 9pm – 6am the next morning to measure host-seeking during the night (Figure 3-2b, Interval 4). At 6am the next morning the entomology officers made final counts of the number of mosquitoes remaining in the entry compartments and window traps outside each hut. They also recorded the number of mosquitoes found in CDC light traps, as well as all dead mosquitoes found in huts or entry traps.

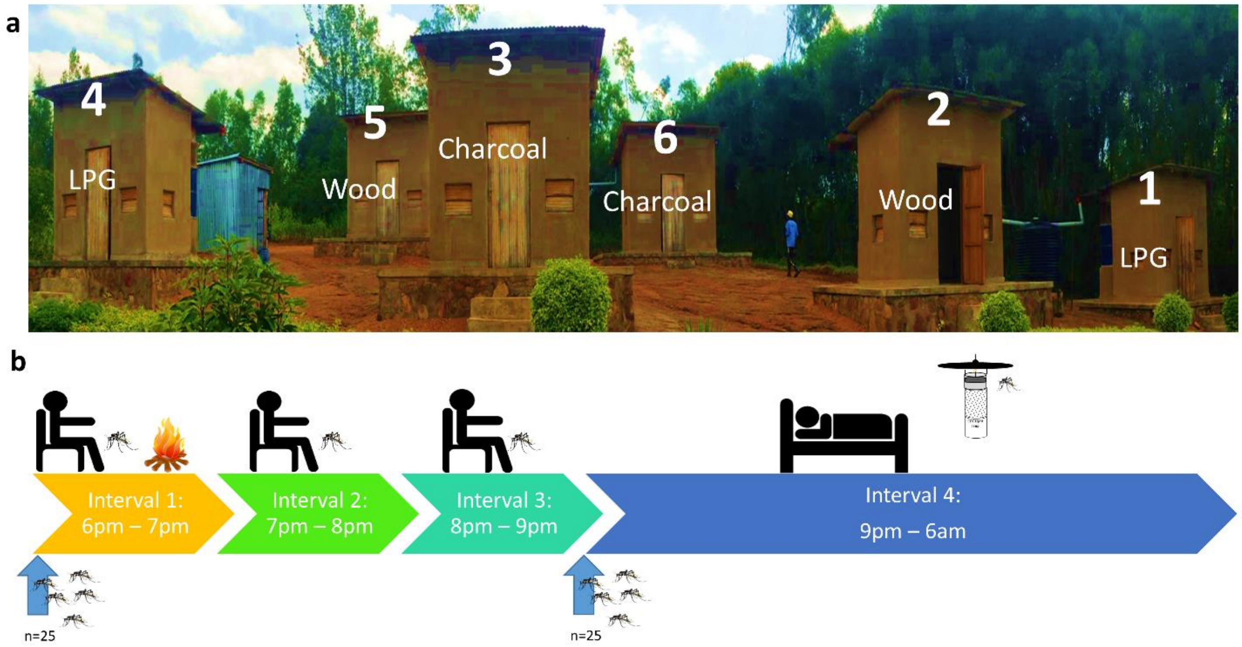

Figure 3-2. Experimental hut layout and experimental design. Figure 3-2a shows the layout of the experimental huts in Bugesera District, Rwanda, with example cooking fuel designations. Figure 3-2b depicts the experimental procedures. Collectors cooked and conducted human landing catch for one hour between 6pm and 7pm (Interval 1). They then extinguished cooking fires and conducted human landing catch for two more hours until 9pm (Intervals 2 & 3). They then retired under an untreated bed net, and CDC Light traps were used for the rest of the night until 6am the following morning (Interval 4). Twenty-five An. gambiae mosquitoes were released at 6pm, and another 25 were released at 9pm. Household entry, host-seeking, and household exit were recorded at the end of each interval. Mortality was recorded only at the end of each complete sampling round.

Real-time fine particulate matter ($PM_{2.5}$) concentrations, temperature (°C), and percent relative humidity (RH) were measured inside of each experimental hut with Particle And Temperature Sensors (PATS+) devices, Berkeley Air Monitoring Group, Berkeley CA, USA (Group, 2020; Pillarisetti et al., 2017). Devices were clean air zeroed according to manufacturing instructions and set to provide PM2.5

readings every minute for the 12-hr duration of each study round. Devices have a $PM_{2.5}$ lower detection limit of 10 - 50 μg/m^3, and values below the lower end of this limit were recorded as 10 μg/m^3. Likewise, values above the upper limit of detection of 25,000 – 50,000 μg/m^3 were recorded as 25,000 μg/m^3.

PATS+ devices were suspended on one wall in each hut next to the CDC light traps at 1.5 meters, approximately 2 meters from the cooking fuels. Devices were hung approximately 1.5 meters from the closest window where the entry compartments were installed. 12-hour means, medians, and IQRs of $PM_{2.5}$, temperature, and RH were calculated to represent average levels during each sampling round, and interval-specific means were also calculated.

3.2.9 Primary outcomes

Primary outcomes included percent household entry, host-seeking, household exit, and mortality. These were measured cumulatively during each sampling round, as well as during individual sampling intervals (Interval 1 = 6pm – 7pm, Interval 2 = 7pm – 8pm, Interval 3 = 8pm – 9pm, & Interval 4 = 9pm – 6am).

Household entry: cumulative household entry (HE_{cumu}) was defined as the proportion of mosquitoes that entered each hut out of the total number of mosquitoes released (n=50). Interval-specific household entry HE_i was calculated as the proportion of mosquitoes that entered each hut by the end of each interval, *i* (*i* = 1, 2, … 4), out of the number remaining in entry compartments at the start of that interval.

$$HE_{cumu} = \frac{total\ entered}{50}$$

$$HE_i = \frac{entered\ during\ interval_i}{remaining\ at\ start\ of interval_i}$$

Host seeking: cumulative host seeking (HS_{cumu}) was measured as the proportion of mosquitoes that sought a host during each sampling round over the total number of mosquitoes released (n=50). Interval-specific host-seeking (HS_i) was calculated as the proportion of mosquitoes that sought a host during each interval over the number that had not sought a host prior to the start of that interval (including mosquitoes remaining in entry compartments at the start of the interval, as well as those that had entered huts but not sought a host during prior interval(s)). During the first three intervals, host-seeking was measured directly via human landing catch; light traps were used to approximate host-seeking during the fourth interval in order to reduce collector fatigue.

$$HS_{cumu} = \frac{total\ sought\ host}{50}$$

$$HS_i = \frac{sought\ host\ during\ interval_i}{\begin{array}{c} remaining\ in\ entry\ compartment\ at\ start\ of\ interval_i + \\ (\ entered\ during\ prior\ intervals -\ sought\ host\ during\ prior\ intervals) \end{array}}$$

Household exit: cumulative household exit (HEX_{cumu}) was defined as the proportion of mosquitoes that exited the hut via the two window exit traps during each sampling round over the total number released (n=50). Interval-specific household exit (HEX_i) was defined as the ratio of mosquitoes that exited huts into exit traps during each sampling interval divided by the sum of the number that entered huts over the course of the interval plus those that had entered and not exited during the prior interval(s).

$$HEX_{cumu} = \frac{total\ exited}{50}$$

$$HEX_i = \frac{exited\ during\ interval\ i}{\begin{array}{c} enterted\ during\ \mathrm{interval}_i + \\ (entered\ during\ prior\ intervals - exited\ during\ prior\ intervals) \end{array}}$$

Mortality: Cumulative mortality ($Mort_{cumu}$) was calculated by counting the number of dead mosquitoes inside the hut as well as in entry compartments and window traps. Mortality was only measured at the end of each sampling round, instead of at each interval due to challenges with locating dead mosquitoes in the dark. Percent mortality was calculated as the number of dead mosquitoes divided by the total number of mosquitoes released in each hut (n = 50).

$$(Mort_{cumu}) = \frac{total\ dead}{50}$$

3.2.10 Statistical analysis

We conducted all statistical analyses using R version 4.0.2 (R Core Team, Vienna Austria) (Team, 2013) and SAS version 9.4 (SAS Institute, Cary NC). We fit generalized linear mixed effect models (GLMMs) with a logit link and a binomial distribution to estimate the impacts of each fuel type on the odds of household entry, host-seeking, household exit and mortality. Because wood is the most commonly used cooking fuel in Rwanda (ICF, 2017), we treated wood as the reference variable and included charcoal and LPG as separate dummy variables. In order to account for non-independence of observations, we included random effects for hut, collector, and day (WHO, 2013). We also included a fixed effect for wall-type using a dummy value for cement versus mud, with mud as the reference category. Primary analyses were first conducted for cumulative outcomes. Interactions between sampling interval and each outcome were assessed, and, if significant, effect measures were reported separately for each sampling interval. As a secondary analysis, we included the baseline results in the model and conducted pairwise comparisons across all groups using Tukey's tests to account for multiple comparisons.

We then assessed potential dose-response effects of $PM_{2.5}$, indoor temperature and RH on household entry, host-seeking, household exit, and mortality. We first fit linear mixed effect models with random effects for hut, collector, and day in order to model the change in each outcome per standard deviation increase in each predictor variable. We assessed the relative importance of $PM_{2.5}$, temperature and RH via the change in adjusted R^2 and Akaike Information Criteria (AIC) values when each variable was added last to the full model. We also analyzed potential non-linear associations between the predictors and each outcome using Generalized Additive Mixed Effect Models (GAMMs). Analyses were conducted separately for phase 1, 2 and 3. However, the results for phase 2 and 3 were nearly identical, and were therefore pooled for the final analysis.

3.2.11 Ethics

The study was reviewed and approved by the Rwanda National Ethics Committee under IRB 00001497, No.194/RNEC/2019. The Emory Institutional Review Board (IRB) reviewed this study and determined it was exempt from IRB clearance because it did not involve research on human subjects.

3.3 Results

3.3.1 Baseline

During phase 1, 6 days of collections were conducted in the absence of any fuel use in order to estimate baseline parameters for each outcome and to ensure comparability across each of the six huts. Cumulatively, a mean of 67.9% released mosquitoes entered huts and 41.3% sought a host (Table 3-1). Of the mosquitoes which entered huts, a mean of 11.9% exited. Household entry and host-seeking were generally highest during the first and fourth sampling intervals, and household exit peaked during the fourth sampling interval. Mortality was low, averaging 4.2% of all mosquitoes released. One-way ANOVA tests showed no significant differences in any of the four outcomes across the six experimental huts ($p > 0.05$). Wall type (cement vs. mud) was not a significant predictor of any outcome.

Table 3-1: Mean and standard deviation of percent household entry, host-seeking, exit, and mortality during baseline with no fuels

		Sampling intervals			
	Cumulative	**6pm - 7pm**	**7pm - 8pm**	**8pm - 9pm**	**9pm - 6am**
Household entry	67.9 (23.5)	43.1 (26.7)	30.8 (23.4)	27.2 (29.2)	56.3 (27.2)
Host-seeking	41.3 (19.4)	23.2 (23.9)	21.4 (21.6)	16.7 (16.5)	21.6 (14.3)
Household exit	11.9 (12.7)	2.3 (4.9)	8.6 (24.7)	27.8 (40.0)	19.7 (21.7)
Mortality	4.2 (4.4)	-	-	-	-

Mortality was only measured once at the end of each sampling round, so interval-specific results are not reported.

3.3.2 Household entry

When fuels were introduced, the cumulative proportion of mosquitoes that entered houses ranged from 65.1% in huts cooking with wood to 71.1% in charcoal and 87.3% in LPG huts. Overall, the odds of household entry were 2.7 (95% CI: 2.1 – 3.3) times higher in LPG-burning huts compared to wood, and 1.7 (95% CI: 1.2 – 2.4) times higher in charcoal-burning huts compared to wood (Table 3-2). After applying Tukey's test for multiple comparisons, household entry in LPG huts was also significantly higher than in charcoal huts and baseline huts where no fuel was used (Figure 3-3a).

Table 3-2: Cumulative household entry, host-seeking, household exit, and mortality as a percent of all mosquitoes released

	Fuel	**Mean (SD)**	**OR (95% CI)**	**P value**
Household entry	*LPG*	87.3 (8.6)	2.7 (2.1, 3.3)	<0.001***
	Charcoal	71.1 (18.6)	1.7 (1.2, 2.4)	0.002**
	Wood (ref)	65.1 (23.9)	1 (NA, NA)	NA
Host seeking	*LPG*	54 (14.9)	2.5 (2.1, 2.9)	<0.001***
	Charcoal	41.1 (16.8)	1.7 (1.3, 2.3)	<0.001***
	Wood (ref)	29.7 (17.9)	1 (NA, NA)	NA
Household exit	*LPG*	8.9 (6.5)	0.8 (0.6, 1.1)	0.246
	Charcoal	13.3 (7.7)	1.4 (0.9, 2.1)	0.088
	Wood (ref)	10.3 (7.3)	1 (NA, NA)	NA
Mortality	*LPG*	3.8 (4.6)	0.6 (0.4, 0.8)	0.001**
	Charcoal	8.1 (8.8)	0.9 (0.6, 1.4)	0.607
	Wood (ref)	8.7 (7.5)	1 (NA, NA)	NA

LPG= Liquid petroleum gas; SD = standard deviation; OR = odds ratio; ref =reference variable. For all regressions, wood was treated as the reference variable and LPG and charcoal were included as separate dummy variables.

Sampling interval was a significant effect modifier of the relationship between household entry and fuel type ($p < 0.01$). The difference was particularly pronounced during cooking (6pm – 7pm), when the odds of household entry were 3.3 (95% CI: 2.6 – 4.0) times higher in LPG and 3.5 (95% CI: 3.4 – 4.6) times higher in charcoal huts, compared to wood (Table 3-3). These differences were less pronounced in later intervals, although household entry remained higher in LPG huts compared to wood and charcoal huts during all subsequent intervals (Figure 3-4a).

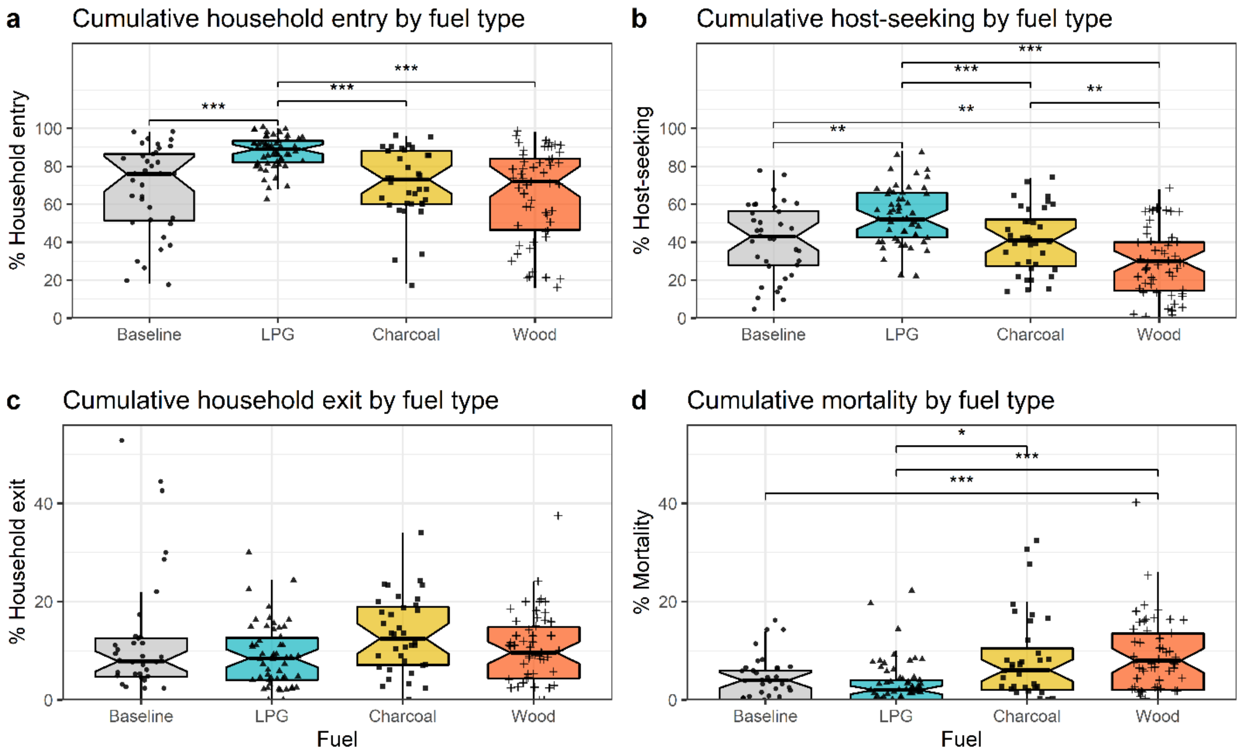

Figure 3-3:. Cumulative household entry, host-seeking, household exit, and mortality by fuel type. Box plots show each outcome as a percent of all mosquitoes released. Points overlaid on boxplots are individual measurements for each sampling round. Asterisks depict significant differences between fuel-types after adjusting for multiple comparisons, where * corresponds to $p < 0.05$, ** corresponds to $p < 0.01$, and *** corresponds to $p < 0.001$.

Table 3-3: Household entry, host-seeking, and household exit during each sampling interval

		Interval 1: 6pm - 7pm		**Interval 2: 7pm - 8pm**	
		Mean (SD)	*OR (95% CI)*	*Mean (SD)*	*OR (95% CI)*
Household entry	*LPG*	49 (20.8)	3.2 (2.6, 4.0)***	42.4 (24.6)	2.8 (2.1, 3.6)***
	Charcoal	50 (19.6)	3.5 (2.4, 4.6)***	23.7 (16)	1.4 (0.9, 2.2)
	Wood (ref)	23 (13.5)	-	21.8 (19.4)	-
Host-seeking	*LPG*	20.1 (13.6)	8.7 (5.7, 13.3)***	30.9 (16)	3.9 (2.9, 5.3)
	Charcoal	23.9 (13.9)	13.0 (7.8, 21.5)***	21.1 (15.4)	2.36 (1.48, 3.75)
	Wood (ref)	2.7 (5.3)	-	10 (11.6)	-
Household exit	*LPG*	2.1 (5)	0.5 (0.2, 1.1)	9.6 (22.7)	4.8 (1.4, 17.4)*
	Charcoal	3.9 (6)	0.9 (0.3, 2.3)	21.9 (35.7)	4.7 (0.8, 19.7)*
	Wood (ref)	2.3 (5.5)	-	5.1 (16.8)	-

		Interval 3: 8pm - 9pm		**Interval 4: 9pm - 6am**	
		Mean (SD)	*OR (95% CI)*	*Mean (SD)*	*OR (95% CI)*
Household entry	*LPG*	33 (27.4)	1.1 (0.7, 1.7)	79.3 (13.3)	2.4 (1.9, 3.1)***
	Charcoal	24 (20.9)	0.6 (0.3, 1.0)	57.1 (24.3)	1.5 (1.0, 2.2)*
	Wood (ref)	22.4 (18.2)	-	54.9 (27.4)	-
Host-seeking	*LPG*	26.2 (18.4)	2.1 (1.5, 3.1)***	28.8 (19)	2.0 (1.6, 2.5)***
	Charcoal	17.8 (17.4)	1.2 (0.7, 2.0)	19.6 (13.8)	1.2 (0.8, 1.7)
	Wood (ref)	12.5 (13.5)	-	16.6 (14.3)	-
Household exit	*LPG*	28.9 (42.1)	1.8 (0.9, 4.2)	14.7 (11.8)	0.9 (0.6, 1.2)
	Charcoal	41.9 (46.3)	5.3 (2.4, 13.7)***	24 (19.5)	1.5 (0.9, 2.4)
	Wood (ref)	16.2 (34.1)	-	16.8 (15.2)	-

LPG= Liquid petroleum gas; SD = standard deviation; OR = odds ratio; ref =reference. For all regressions, wood was treated as the reference variable and LPG and Charcoal were included as separate dummy variables. * signifies p-value <0.05; ** p-value < 0.01, *** p-value < 0.001

3.3.3 Host-seeking

Cumulative host-seeking as a percent of all mosquitoes released each sampling round averaged 29.7% in wood, 41.1% in charcoal, and 54.0% in LPG-burning huts. Cooking with LPG was associated with 2.5 (95% CI: 2.1 – 2.9) times higher odds of host-seeking compared to wood, and the odds of host-seeking were 1.7 (95% CI: 1.3 – 2.3) times higher in charcoal compared to wood-burning huts (Table 3-2). Compared to baseline conditions, host-seeking was significantly higher in LPG and lower in wood-burning huts (Figure 3-3b).

Again, we observed a significant interaction between fuel-type and sampling interval (*p* < 0.001). The odds of host-seeking during cooking (6pm – 7pm) were 8.7 (95%CI: 5.7 – 13.3) times higher in LPG and 13.0 (95% CI: 7.8 – 21.5) in charcoal compared to wood-burning huts (Table 3-3, Figure 3-4b). Host-seeking remained significantly higher in LPG-burning huts compared to wood for every subsequent sampling interval, whereas the difference between charcoal and wood declined in later intervals and was no longer significant during the third and fourth intervals.

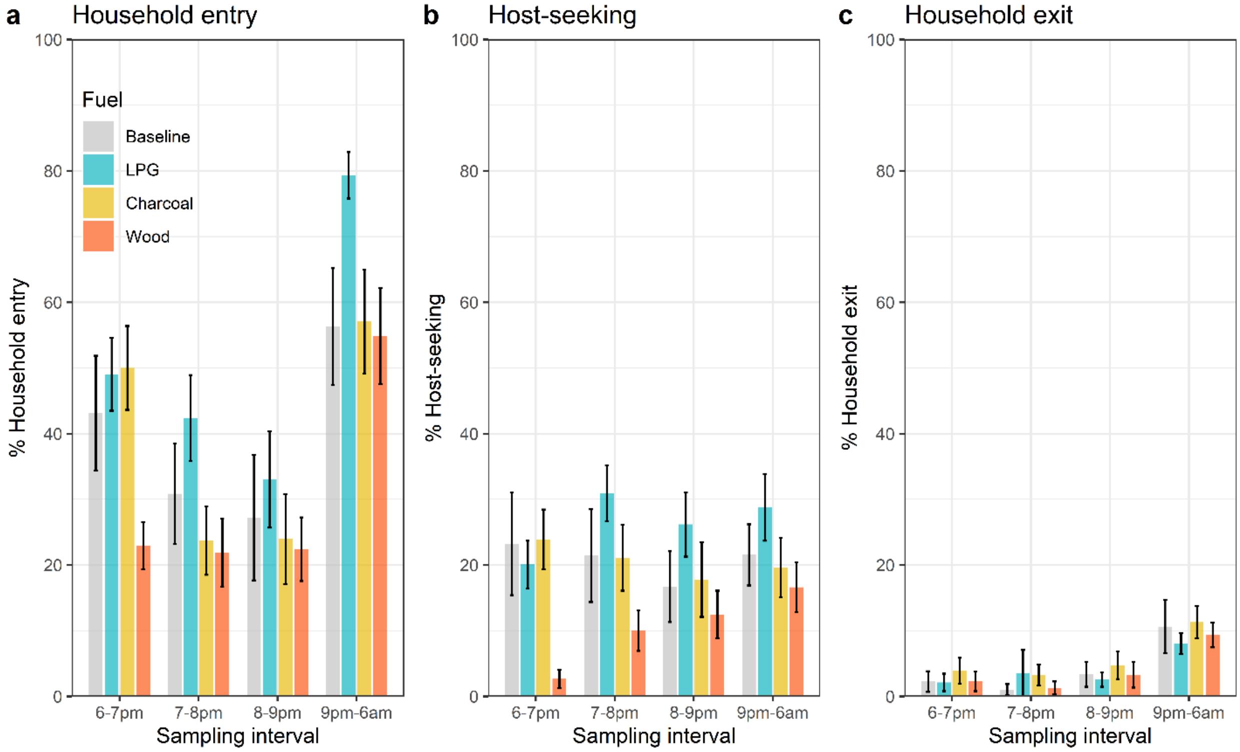

Figure 3-4. **Interval-specific household entry, host-seeking, and household exit by fuel type.** The first plot (a) shows mean household entry during each sampling interval as a percent of all mosquitoes remaining in entrance compartments at the start of each interval. The second plot (b) shows mean host-seeking during each sampling interval as a percent of all mosquitoes which had not sought a host by the start of each interval. The third plot (c) shows mean household-exit during each sampling interval as a percent of all mosquitoes which entered houses and did not seek a host by the end of each interval. Error bars represent 95% confidence intervals for each mean.

3.3.4 Household exit

Mean household exit was low (8.9% in LPG, 13.3% in charcoal, and 10.3% in wood burning huts), and was not significantly different across all three fuel types *(Table 3-2).* After adjusting for multiple comparisons, none of the fuels were significantly different from baseline conditions (Figure 3-3c).

Sampling interval was not a significant effect modifier, although exiting was higher in LPG than in wood during interval 2, and higher in charcoal than in wood huts during intervals 2 and 3 (Table 3-3, Figure 3-4c).

3.3.5 Mortality

Mortality was also low across each fuel type, ranging from 3.8% in LPG huts, to 8.1% in charcoal and 8.7% in wood. This translated to a 40% lower odds of mortality in LPG huts compared to wood (OR = 0.6, 95% CI: 0.4 – 0.8), whereas charcoal and wood were not significantly different (Table 3-2). Compared to baseline conditions where no fuel was burned, mortality was higher in wood-burning huts (Figure 3-3d).

3.3.6 Effects of $PM_{2.5}$, temperature, and relative humidity

Average $PM_{2.5}$ concentrations ranged from 29 $\mu g/m^3$ in LPG, 223 $\mu g/m^3$ in charcoal, and 1,672 $\mu g/m^3$ in wood huts (Table 3-4). $PM_{2.5}$ concentrations in charcoal and wood huts were higher than baseline conditions in which no fuels were burned, whereas LPG was not significantly different. Indoor temperatures were elevated in huts cooking with all three fuels relative to baseline conditions, with the highest temperatures recorded in wood huts (mean = 27° C). Average relative humidity ranged from 56% in charcoal and wood huts to 61% in LPG huts, all of which were significantly higher than the baseline mean of 46% RH.

Table 3-4: Twelve-hour averages of PM2.5, temperature, and relative humidity by fuel type

	Fuel	Mean (SD)	Median (IQR)
$PM_{2.5}$ ($\mu g/m^3$)	*LPG*	29.2 (16.2)	25.1 (16.7)
	Charcoal	149.4 (350.3)***	73.7 (41)
	Wood	1672.3 (511.5)***	1759.7 (631.5)
	Baseline (Intercept)	16.1 (5.8)	14.4 (9.3)
Temperature (°C)	*LPG*	25.7 (1.1)*	25.8 (1.6)
	Charcoal	26.6 (1)*	26.8 (1.1)
	Wood	27.2 (0.9)***	27.2 (1.4)
	Baseline (Intercept)	24.8 (0.8)	25 (0.5)
Relative humidity (%)	*LPG*	60.6 (5.5)***	60.6 (9.3)
	Charcoal	56.6 (4.2)***	56.2 (4)
	Wood	56.3 (4.5)***	56.1 (6.2)
	Baseline (Intercept)	46.2 (3.7)	45.6 (3.6)

Mean and standard deviation (SD) of 12-hr averages for each fuel presented with median and inter-quartile ranges (IQR). Differences in means for each fuel were compared to baseline values using linear mixed effect regression. * signifies p-value <0.05; *** signifies p-value < 0.001

Indoor temperature was the most important predictor of household entry, explaining 9% of variance in linear mixed effect models which included $PM_{2.5}$ and RH (Table 3-5). Each standard deviation increase in temperature was associated with a 13.1 point (95% CI: 10.0 – 16.2) decrease in the percentage of mosquitoes that entered huts. In contrast, host-seeking appeared to be most influenced by $PM_{2.5}$; $PM_{2.5}$ accounted for 4% of model variance and each standard deviation increase was associated with a 5.4 percentage point (95% CI: 3.4 – 7.4) decline in host-seeking. Temperature was the best predictor of household exit, accounting for 16% of model variance when added last to the full model. Each standard deviation increase in temperature was associated with a 3.3 point (95% CI: 2.3 – 4.4) decline in the percentage of mosquitoes which exited huts. Conversely, higher $PM_{2.5}$ levels were associated with marginal increases in household exit rates. Finally, $PM_{2.5}$ was positively associated with mortality and explained 4% of model variance.

Table 3-5: Effects of PM2.5, temperature, and relative humidity on household entry, host-seeking, household exit, and mortality

	Parameter	**β (95% CI)**	**Δ in AIC**	**Δ in R^2**
Household entry	*$PM_{2.5}$ ($\mu g/m^3$)*	1.2 (-1.8, 4.1)	-1.2	0.00
	Temperature	-13.1 (-16.2, -10.0)***	-62.7	0.09
	Relative humidity	-0.7 (-3.9, 2.5)	-0.9	0.00
	Intercept	44.2 (38.9, 49.4)		
Host-seeking	*$PM_{2.5}$*	-5.4 (-7.4, -3.4)***	-27.5	0.04
	Temperature	0.9 (-1.3, 3.1)	-0.7	0.01
	Relative humidity	-0.4 (-2.9, 2.2)	-0.3	0.00
	Intercept	21.6 (16.0, 27.2)		
Household exit	*$PM_{2.5}$*	1.1 (0.2, 2.0)*	-4.3	0.03
	Temperature	-3.3 (-4.2, -2.3)***	-40.6	0.16
	Relative humidity	0.3 (-0.6, 1.0)	1.5	0.01
	Intercept	4.5 (2.4, 6.7)		
Mortality	*$PM_{2.5}$*	1.3 (0.0, 2.6)*	-1.0	0.04
	Temperature	0.8 (-0.5, 2.2)	1.5	-0.01
	Relative humidity	-0.3 (-1.5, 0.8)	2.9	0.00
	Intercept	6.6 (2.6, 10.5)		

β values represent the linear change in each outcome for a one standard deviation increase in each predictor variable, controlling for all other independent variables. Variables are scaled for comparison. Δ in AIC shows relative change in model fit when each variable is added last to a full model. Negative values indicate improved model fit. Δ in R2 is the proportion of variance explained by each variable, calculated as the change in the conditional R2 value when each variable is added last to the full model. * signifies p-value <0.05; ** p-value < 0.01, *** p-value < 0.001

Generally, $PM_{2.5}$ and temperature showed linear associations with each outcome. However, the effects of RH on each outcome appeared non-linear when fitted with GAMM models (Figure 3-5). Household entry and host-seeking appeared to decline between 50 and 60% RH but then increased above 60% RH. In contrast, household exit and mortality peaked between 50 and 60% RH and declined at lower and higher RH values. After accounting for these non-linear associations via GAMM models, RH was a significant predictor of host-seeking and household exit, accounting for 5% and 2% of model variance, respectively. Temperature remained the most important predictor of household entry and household exit, and $PM_{2.5}$ was a significant predictor of household entry, host-seeking, and mortality (data not shown).

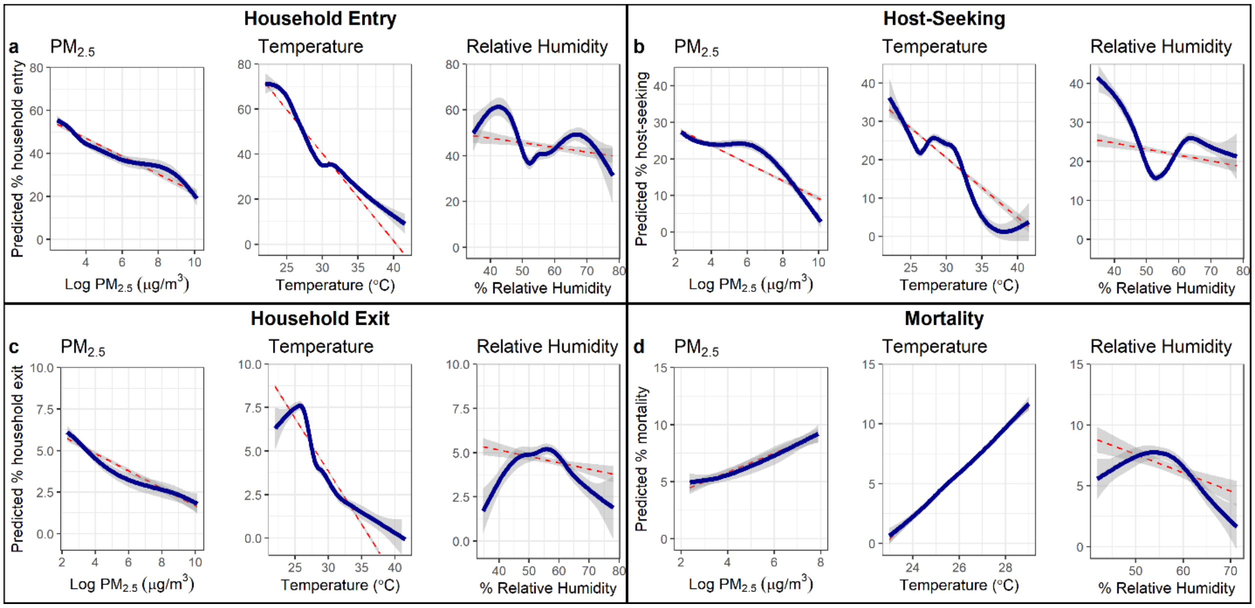

Figure 3-5. Dose-response effects of PM2.5, temperature, and relative humidity on household-entry, host-seeking, household exit, and mortality. Red dotted lines represent predicted values of linear mixed effect models, whereas solid blue lines represent generalized additive mixed effect model predictions. Grey bands indicated 95% confidence intervals of each model.

3.4 Discussion

Under these experimental conditions, the combustion of LPG resulted in increased household entry and host-seeking and reduced mortality, compared to wood. Charcoal showed a similar pattern when compared to wood, although the differences were less dramatic. Other experimental and observational studies have reported similar effects of biomass fuel combustion on *Anopheles* mosquito behavior and mortality compared to conditions in which no fuels were burned (Denloye et al., 2006; Hiscox et al., 2013; Kweka et al., 2008; Maia et al., 2016; K. Pålsson & T. G. Jaenson, 1999b; Paru et al., 1995; Vernède et al., 1994). However, this was the first study to explicitly investigate and compare the effects of three commonly used cooking fuels on *Anopheles* mosquito behavior. This information is needed for accurately characterizing potential effects of clean fuel adoption, particularly as LPG is projected to become a dominant fuel in Rwanda and many malaria endemic countries (Van Leeuwen et al., 2017).

Given the controlled nature of the experiments, the results are not directly generalizable to field conditions. However, they indicate a potential for clean fuel adoption to result in higher exposure to

Anopheles mosquitoes via increased household entry and host-seeking compared to houses that cook with biomass fuels. Higher vector density and biting rates are important determinants of malaria and other mosquito-borne disease transmission risk (Churcher et al., 2015; Dietz, 1993; Killeen et al., 2000; Trape et al., 1992). Reduced mosquito mortality could also facilitate parasite development and malaria transmission.(Smith et al., 2007) Previous investigations have suggested that reductions in indoor *Anopheles* density in wood-burning houses may be due to higher exiting rates after entry rather than a direct repellent effect of wood fuel combustion (Bockarie et al., 1994). However, we observed no differences in household exiting rates across fuel types.

To our knowledge, no other studies have investigated the dose-response effects of $PM_{2.5}$ or other components of fuel combustion on *Anopheles* mosquito behavior. After adjusting for the effects of temperature and RH, $PM_{2.5}$ was a significant predictor of host-seeking and mortality. As has been observed elsewhere (Mordecai et al., 2013), temperature was an important determinant of mosquito behavior, particularly household-entry and exit. While RH was generally less important than temperature and $PM_{2.5}$, it showed significant non-linear associations with household entry and host-seeking, which both increased when RH levels exceeded 60%. Other studies have also reported increased longevity and fitness of *An. gambiae* at RH levels above 60% (Lindsay et al., 2019; Yamana & Eltahir, 2013).

We observed higher household entry and host-seeking among houses that cooked with LPG compared to baseline conditions where no fuel was used, suggesting a potential attractant effect of LPG fuel. However, $PM_{2.5}$, temperature, and RH explained a relatively low proportion of overall variance in entry and host-seeking. This indicates a potentially important role of other unmeasured components of fuel combustion, such as CO_2. CO_2 is primary component of fuel combustion and is the most important attractant for host-seeking mosquitoes (Gillies, 1980; Takken & Knols, 1999). Increases in ambient CO_2 levels as little as 0.01% above baseline levels can stimulate female mosquitoes to search for blood meals (Healy & Copland, 1995; Webster et al., 2015). LPG produces more CO_2 per kilogram of fuel burned than wood or charcoal due to improved combustion efficiency (Edwards, 2014). It is conceivable that CO_2 emissions from LPG attract mosquitoes, whereas components of biomass fuel combustion such as high heat and particulate matter counteract similar effects in wood and charcoal. Average relative humidity was also 14 percentage points higher in LPG huts than in baseline conditions, which could have further attracted host-seeking mosquitoes. Additional experiments are needed to investigate these potential attractant effects of LPG combustion.

A number of factors limit the generalizability of these findings to field settings. In Rwanda, for example, cooking outdoors or in separate kitchen structures is common (NISR & Macro, 2012), and could have very different effects on mosquito behavior. Other fuelwood species and biomass fuels are also used for cooking (Ndayambaje & Mohren, 2011), each of which could have different effects on mosquitoes (Kweka et al., 2008; Vernède et al., 1994). The smoke exposures measured in this experiment could also exaggerate true exposures under field conditions; individuals may not spend as much time in smoky kitchens as they did in this experiment, and bedrooms are often much further away from kitchens than they were in the experimental huts. Finally, other factors such as proximity to breeding sites, housing characteristics, or use of mosquito control interventions could be more important than cooking fuels in influencing vector density and human exposure (Bizimana et al., 2015; Hakizimana et al., 2018; Murindahabi et al., 2021; Rulisa, Kateera, Bizimana, Agaba, Dukuzumuremyi, Baas, de Dieu Harelimana, Mens, Boer, et al., 2013). Further experimental studies could explore the impacts of cooking characteristics (e.g. cooking location and fuelwood types) on the behavior of mosquitoes. Well-designed field studies are also needed to measure possible impacts under real-world conditions where other determinants of vector bionomics could vary widely.

We measured $PM_{2.5}$ served as a proxy for smoke. However, our ability to draw conclusions about direct effects of $PM_{2.5}$ is limited because we were unable to measure other elements of fuel combustion such as CO_2, CO, black carbon, or chemical volatiles. Additionally, we were not able to calibrate the nephalometric PATS+ devices against gravimetric readings, and some readings were outside the limits of detection set by the manufacturer. $PM_{2.5}$ measurements should be therefore being interpreted as relative concentrations between each fuel type rather than exact values.

We employed lab-reared *An. gambiae* s.s., Kisumu strain mosquitoes in order to eliminate potential health risks and confounding associated with conducting the experiment with wild mosquitoes. However, lab-reared insects may be less robust than their wild counterparts (Hoffmann et al., 2001), and it is unknown whether wild *Anopheles* mosquitoes would display the same behaviors as those used in this study. We are also unable to generalize to other *Anopheles* species, nor to other important vector genera such as *Culex* and *Aedes* mosquitoes. However, other studies have reported repellant and deterrent effects of biomass combustion on species within these genera (Lindsay & Janneh, 1989; Moore et al., 2007; K. Pålsson & T. G. Jaenson, 1999b; Paru et al., 1995).

Changes in cooking fuels could also indirectly influence malaria risk independently of their direct effects on vector behavior. For example, less smoky fuels could reduce the need for frequent net washing, which could reduce net deterioration and prolong insecticidal activity (Atieli et al., 2010; Birhanu et al., 2019). LPG adoption can lead to changes in cooking behavior or time spent indoors (Kirby et al., 2019; Seguin et al., 2018), which could affect exposure to insect vectors independently of the actual type of fuel used. Reductions in HAP exposure could also improve innate immune function (Gordon et al., 2014; Lee et al., 2015), and reduce susceptibility to malaria or other infectious diseases. Conversely, a recent cohort study from Ghana showed that malaria can attenuate the health benefits of reduced HAP exposure; reduced CO resulted in improved growth for infants born to mothers with no evidence of placental malaria, whereas the same effect was not observed among mothers who had placental malaria (A. K. Quinn et al., 2021). Epidemiological studies should accompany entomological efforts to better characterize the overall effects of clean fuel adoption on risk of malaria and other vector-borne diseases.

3.5 Conclusion

Our study suggests that cooking fuels can have important impacts on mosquito behavior. Huts cooking with LPG saw higher household entry and host-seeking, coupled with reduced mortality. This implies that, at least in highly controlled conditions, the adoption of cleaner fuels could reduce or reverse repellant and deterrent effects from biomass fuels, potentially altering human exposure to *Anopheles* mosquitoes and the pathogens they can transmit. If these findings are confirmed in larger studies under field conditions, these implications would make it incumbent on program implementers to address the increased exposure to disease vectors that may be associated with adoption of cleaner cooking fuels.

Despite these findings, the benefits of clean cooking fuels almost certainly outweigh potential risks from potentially associated changes in vector behavior. Household air pollution is responsible for 1.8 million deaths each year, and the promotion of HAP reduction interventions should remain a public health priority (Lee et al., 2020; WHO, 2018a). At the same time, further entomological and epidemiological studies should be conducted to better characterize changes associated with clean fuel adoption and their potential to affect incidence of malaria or other vector-borne diseases. If the risk is indeed elevated, enhanced vector control interventions could be promoted in tandem with cleaner cooking fuels. For example, a growing line of research is investigating built-environment solutions such as house screening for reducing exposure to disease vectors (BOVA, 2020). These strategies could be paired with clean cooking interventions as part of an overall approach to improving household environmental health (Clasen & Smith, 2019).

4 Research Aim 2. Effects of cooking with liquid petroleum gas fuel versus solid biomass on mosquito and fly density in the home: a randomized controlled trial in Eastern Province, Rwanda

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[ǂ]The views expressed here are those of the authors and do not represent official views or policies of the National Institutes of Health or the U.S. Government.

4.1 Introduction

4.1.1 Background

Clean cooking fuels such as liquified petroleum gas (LPG) are increasingly promoted to reduce household air pollution (HAP), which is responsible for more than 2.3 million deaths per year (Naghavi et al., 2017; WHO, 2018a). However, traditional biomass fuels are often used as insect repellents (Torr et al., 2011), and components of biomass combustion such as carbon dioxide (CO_2), heat, and chemical volatiles are known to influence insect behavior (Dube et al., 2011; Gillies, 1980; Hawkes et al., 2017). There is therefore some concern that the replacement of traditional biomass fuels with cleaner-burning fuels could affect mosquito or fly behavior, and potentially alter exposure to vector-borne pathogens (Biran et al., 2007).

A variety of experimental and observational studies have shown that smoke from biomass combustion can repel *Anopheles* mosquitoes, the vectors of malaria (Bockarie et al., 1994; Charlwood et al., 2018; Hiscox et al., 2013; Kweka et al., 2008; Lindsay, 1995; Maia et al., 2016; McCann et al., 2017; K. Pålsson & T. G. Jaenson, 1999b; Paru et al., 1995; Vernède et al., 1994). Similar effects have been reported for *Culex* and *Aedes* mosquitoes, which are vectors of lymphatic filariasis, Dengue, Yellow Fever, Chikungunya, Zika, and numerous other arboviruses (Jantan et al., 1999; Lindsay & Janneh, 1989; Nagao et al., 2003; K. Pålsson & T. G. T. Jaenson, 1999b; Vernède et al., 1994). However, until recently no studies had directly investigated the effects of different cooking fuels on mosquito behavior.

We recently conducted an experimental hut study in rural Rwanda to compare the effects of cooking with liquified petroleum gas (LPG) and traditional biomass fuels on mosquito behavior. Cooking with LPG compared to biomass fuels was associated with substantial increases in household entry and host-seeking by lab-reared *Anopheles gambiae* mosquitoes, whereas mortality was reduced. Host-seeking was also higher in LPG houses compared to houses where no fuels were used (Hennessee et al., 2022). These results indicate a potential for clean fuel adoption to reduce or even reverse the repellent effects of biomass fuel use and potentially alter human exposure to *Anopheles*-transmitted pathogens. However, this study was conducted in highly controlled experimental conditions with lab-reared mosquitoes, and additional entomological and epidemiological studies are needed to investigate changes in disease vector exposure associated with cleaner fuel adoption in real-world conditions.

Changes in fuel use may also impact synanthropic flies, also known as filth flies, which mechanically vector *Shigella, E. coli, Salmonella,* and other enteric pathogens (Chavasse et al., 1999; Greenberg, 1973). Burning pelletized wood has been experimentally shown to deter *Musca domestica* (Denloye et al., 2006), and smoke from burning plant parts is widely used to repel flies (Baana et al., 2018). Woodfire smoke can also reduce Tsetse fly density in field conditions (Torr et al., 2011). Conversely, smoky wood fires may be an attractant for other medically important fly species such as *Chrysops silacea,* which transmit Loa loa (Caubere & Noireau, 1991).

Changes in cooking practices from the shift to cleaner cooking fuels could also indirectly affect exposure to mosquitoes and flies. For example, indoor cooking with biomass fuel can lead to soot buildup on long-lasting insecticide treated nets (LLINS). Cleaner cooking fuels could reduce soot and promote net integrity if users do not need to wash the net as frequently (Bhatt et al., 2012; Miller et al., 1999). Users of LPG stoves also may be more likely to cook indoors (Seguin et al., 2018), which could alter the kitchen’s proximity to latrines or other fly breeding sites. Latrine proximity is positively associated with synanthropic fly density and food contamination in kitchens (S. W. Lindsay et al., 2012; T. Lindsay et al., 2012; Sharma & Jain, 2019; Wolfe et al., 2017).

Although epidemiological evidence is limited, a cluster randomized controlled trial of cleaner-burning biomass stoves in Malawi reported a statistically significant increase in malaria incidence among children in houses that received cleaner burning stoves (Mortimer et al., 2017). A recent case-control study in Guatemala also found that individuals from houses which cooked with fuels other than firewood had an increased risk of arbovirus infection compared to houses that cooked with firewood in the main house or on open hearths (Madewell et al., 2020). In both cases, however, these were secondary outcomes of health impact evaluations. In contrast, reduced air pollution exposure could improve innate immune function and reduce susceptibility to malaria or other infectious diseases (Lee et al., 2015). No studies have investigated potential effects of cooking fuel changes on enteric diseases which are mechanically vectored by synanthropic flies.

As cleaner cooking is increasingly promoted in order to reduce the health burden of household air pollution, rigorous studies are needed to characterize potential effects of clean fuel adoption on mosquito and flies and the diseases they transmit (Hennessee et al., 2022). We therefore conducted a study to examine the impact of LPG adoption on vector density and reported malaria and diarrhea prevalence in houses participating in a household air pollution intervention trial in Eastern Province, Rwanda.

4.1.2 Research objective

The primary objective of this study was to understand if, and to what extent, the provision of an intervention consisting of LPG stoves and fuel could impact mosquito and fly density among households using biomass at enrollment. A secondary objective was to determine whether the intervention affects reported malaria and diarrhea longitudinal prevalence among household residents.

4.2 Materials and methods

4.2.1 Study design

This study was nested within the Rwanda center of the multi-country Household Air Pollution Intervention Network (HAPIN) study, a randomized controlled trial to assess the health effects of an LPG stove and fuel intervention among populations which traditionally rely on solid biomass fuels for cooking (Clasen et al., 2020). The HAPIN trial has reported high fidelity and adherence of the intervention (Ashlinn K. Quinn et al., 2021). The intervention also achieved substantial reductions in PM2.5, black carbon and carbon monoxide (Liao et al., 2021; M. Johnson, 2022). The current study employed a random sample of houses participating in the HAPIN trial in Rwanda, and maintained the randomized trial design of the larger study. The eligibility criteria for inclusion in the HAPIN trial are reported elsewhere (Clasen et al., 2020). Briefly, women between 18-35 years of age with viable singleton pregnancies between 9 < 20wk gestation who cooked primarily with biomass stoves were considered eligible and were enrolled at antenatal care clinics.

4.2.2 Sample size and eligibility

The sample size was calculated in order to observe a 25% change in *Anopheles* spp. density, based on previously published estimates of *Anopheles gambiae* s.l. bites/person/night from Kayonza district (Hakizimana et al., 2018). With 80% power, $\alpha=0.05$, and a correlation coefficient among repeated measurements of 0.6, we estimated a required sample size of 100 households per arm, and sought to enroll 110 from each arm in order to account for 10% refusal or loss to follow-up.

Houses which were participating in the HAPIN trial and had been randomized after August 1, 2018 were eligible to be selected such that houses would have at least five remaining months of follow-up by the first round of vector sampling. Houses which had completed the trial, voluntarily exited the trial, or had been enrolled prior to August 1, 2018 were not eligible. 110 intervention and 110 control houses were randomly selected from eligible households (group 1). Selected houses were visited by a study team who administered information and consent forms to the primary HAPIN participants. Houses were enrolled in the study after providing written informed consent.

Burning biomass indoors may be more likely to repel indoor-biting mosquitoes such as *Anopheles gambiae* than burning biomass outdoors or in a separate outdoor kitchen (Hennessee et al., 2022; McCann et al., 2017). We therefore hypothesized that the effect of the intervention may be modified by cooking location, although preliminary HAPIN baseline data suggested that cooking inside the main house is relatively uncommon in the study area. We therefore purposefully selected 40 additional households (20 intervention and 20 control) that reported cooking indoors during HAPIN baseline visits. These houses were enrolled as described above. We conducted and report all primary analyses with the randomly selected group (group 1), but included this purposefully selected group (group 2) in analyses of effect modification between cooking location and the intervention.

4.2.3 Study setting

This study took place in Kayonza District, Eastern Province, Rwanda, which is where the HAPIN trial was conducted. The majority of the population in the district lives in rural settings (Rwanda, 2012). Malaria prevalence among children 6-59 months was 18% in 2017. Approximately 75% of households owned at least one insecticide-treated net, but net use was lower at 51% (ICF, 2017). *An. gambiae* s.l. is the dominant *Anopheles* species. Culicine mosquitoes are common as well, particularly *Culex quinquefasciatus* (Hakizimana et al., 2018). The district received a round of indoor residual spraying (IRS) with a neonicotinoid/pyrethroid insecticide combination in April and May 2019 (PMI, 2020).

4.2.4 Procedures

Household visits

Three rounds of entomological sampling were conducted during the rainy seasons in the study area during which mosquito abundance was expected to be elevated. Rounds 1 and 2 were conducted during the short rains in October-November 2019 and November-December 2019, and Round 3 was conducted during the long rains in March 2020. Study teams visited each house over two consecutive days during each round. Visits were conducted in the morning between 07:00 and 11:00. During the first day study teams administered a brief survey and placed insect traps. They then returned to each house the following morning to collect the traps and complete the survey.

Baseline and visit characteristics

Baseline characteristics of study participants were collected upon enrollment in the HAPIN trial and included maternal age at baseline, gestational age at baseline, and education. We also assessed baseline cooking practices including the primary type of fuel used and the location of the primary cooking stove. Baseline housing characteristics included wall, floor, and roofing materials. Baseline environmental characteristics for each household were extracted from geospatial datasets. Elevation was extracted using a Shuttle Radar Topography Mission 30-meter (SRTM30) digital elevation model from the Regional Center for Mapping of Resources for Development (RCMRD, 2015). Population density was derived from WorldPop UN-adjusted 1km population estimates (Tatem, 2017). Supervised image classification of a cloud-free LANDSAT-8 retrieval on March 4 2020 was used to classify rice fields in the study area (Roy et al., 2014). Proximity of rice fields within 2km of each household was assessed using the *rgeos* package in R version 4.0.2 (Bivand et al., 2017) (see Supplementary Materials for detailed methods).

Questionnaires were administered to HAPIN participants at each entomological sampling visit. Questionnaires included information about cooking practices at follow-up such as types of fuel used for cooking in the last 24 hours and the primary cooking location. Participants were asked about the number of people that slept in the house the night before. Study staff visually assessed the presence of >1cm-wide cracks or openings in exterior windows, doors, or walls through which mosquitoes or flies could enter, and checked for the presence of open, water-holding containers which could be larval sites. They also assessed whether toilets or latrines were covered with a tight-fitting lid and measured the distance from toilets and rubbish piles to the primary cooking location. Staff also visually assessed the presence of domestic animals in compounds, the presence of animal or human feces in compounds, and whether uncovered, cooked food was stored in kitchens.

Questionnaires included questions about fly and mosquito control practices including whether occupants had slept under a LLIN the night before the survey, whether the household received IRS in the last 12 months, and whether participants had used insecticides or burned any materials to repel mosquitoes or flies in the prior 24 hours. Current and one-month lagged land surface temperature (LST) and rainfall at each household were estimated using monthly gridded LST and rainfall measurements from MODIS MOD11C3 retrievals (Wan et al., 2015) and Climate Hazards Infrared Precipitation with Stations (CHIRPS) rainfall products (Funk et al., 2015), respectively (see Supplementary Materials for detailed methods).

Entomological sampling methods

CDC light traps: Miniature CDC light traps (Model 512; John W. Hock Company, Gainesville, FL) were placed at approximately 1.5 meters above the foot end of the respondent's bed in order to collect host-seeking *Anopheles* and culicine mosquitoes (Mboera et al., 1998). Participants were asked to plug in the battery at 18:00, and leave it running throughout the night until the return of the study team the following morning. At that point mosquitoes were transported in coolers to the entomology laboratory for processing and identification.

Prokopacks: Prokopacks are battery-powered vacuum aspirators which are used to sample resting mosquitoes in and around houses (Maia et al., 2011; Ndenga et al., 2017). Prokopacks were used to sample resting mosquitoes in bedrooms, kitchens, and outdoors around the perimeter of each house. Collections from each location were kept in separate containers and transported to the field laboratory for processing and identification.

Fly traps: Extra-large blue sticky fly traps (Product code 10303, Suterra Ltd, UK) (Agrisense.co.uk, 2017) were used to sample domestic synanthropic flies in cooking areas during each round. Blue traps have been shown to be more effective than yellow cards or other common methods for sampling *Musca domestica* and other synanthropic flies (Bell et al., 2019). Each card was cut in half, and each half was placed at a 45-degree angle 1-2 meters from the primary stove location. Traps were retrieved on the following day and transported to a field entomology laboratory for identification.

Entomological sample processing and identification

All mosquitoes were counted and morphologically identified using standard keys in a field entomology laboratory (Gillies & Coetzee, 1987). All *Anopheles* mosquitoes and bloodfed *Cx. quinquefasciatus* mosquitoes were stored in Eppendorf tubes with cotton and silica gel and transported in coolers to the Rwanda Biomedical Center entomology laboratory in Kigali for further processing. All *An. gambiae s.l.* were speciated with polymerase chain reaction (PCR), and all *Anopheles* were tested for *Plasmodium falciparum* infection using enzyme-linked immunosorbent assay (ELISA) (Hakizimana et al., 2018; Scott et al., 1993). ELISA was also used for bloodmeal analysis of bloodfed mosquitoes (Mwangangi et al., 2003).

HAP sampling

Particulate matter 2.5 ($PM_{2.5}$) concentrations were measured in a subset of 144 houses using Particulate and Temperature Sensors (PATS+) (Group, 2020). These were placed adjacent to CDC light traps at approximately 1.5 meters above the ground at the foot of participant's beds. Devices were clean air zeroed according to manufacturing instructions and set to provide $PM_{2.5}$ concentrations ($\mu g/m^3$), temperature (°C), and percent relative humidity (RH) readings every minute from 4pm on the day the CDC light traps were installed until 10am the next morning. This time period was chosen in order to detect PM peaks associated with fuel usage for evening and morning meal preparation. The devices have a $PM_{2.5}$ lower detection limit of 10 - 50 $\mu g/m^3$, and values below the lower end of this limit were recorded as 10 $\mu g/m^3$.

4.2.5 Outcomes

Entomological Outcomes

We analyzed three primary entomological outcomes. The first outcome, *Anopheles* density, was defined as the total number of *Anopheles* sp. mosquitoes collected in CDC light traps and Prokopacks per house per sampling round. The second outcome, culicine density, was the total number of culicine mosquitoes sampled in CDC light traps and Prokopacks per house per sampling round. Culicine mosquitoes included *Culex* sp., *Aedes* sp., *and Mansonia* sp. mosquitoes. The third outcome, synanthropic fly density, was measured as the number of synanthropic flies collected in primary cooking areas per household per sampling round. Synanthropic flies included all flies in the Muscidae, Calliphoridae, Fanniidae, and Sarcophagidae families. Flies in these families are the most common synanthropic flies and are widely implicated as mechanical vectors of enteric pathogens (Greenberg, 1973).

We also assessed *P. falciparum* infection among *Anopheles* mosquitoes and blood-meal composition of blood-fed *Anopheles* sp. and *Culex quinquefasciatus* mosquitoes as secondary entomological outcomes.

Epidemiological outcomes

Secondary outcomes included self-reported malaria and diarrhea prevalence among mothers and their infants at whose houses we conducted vector sampling. Malaria and diarrhea outcomes were assessed using data collected as part of the HAPIN trial. After a baseline assessment and randomization into the HAPIN trial, assessments were conducted for pregnant women between 24-28 weeks gestation (visit P1) and again between 32-36 weeks gestations (visit P2). Three assessments were then conducted after birth when children were approximately 3, 6, 9, and 12 months old (visits B1, B2, B3, and B4). At each visit except for B3, mothers were asked whether they were tested for malaria in the period since the prior visit and the result of the test. We defined longitudinal prevalence of malaria in mothers as the number of periods in which mothers reported having one or more positive malaria tests divided by the number of periods during which mothers were observed. In order to assess potential reporting bias, we also conducted a sensitivity analysis to include only positive malaria tests which study staff were able to verify via clinic cards. Study staff were able to confirm approximately 60/81 (74%) of reported positive malaria tests via clinic cards. Longitudinal prevalence is often used to assess infectious disease morbidity in longitudinal intervention studies (Schmidt et al., 2010; Schmidt et al., 2007; Wolf et al., 2018).

At visits B1 through B4 mothers were asked to report any diagnostically confirmed cases of malaria in their newborns since the prior visit, or since birth for the B1 visit. We defined longitudinal prevalence of malaria in children as the number of periods in which children had one or more positive reported malaria test divided by the number of periods during which children were observed. Again, we conducted a sensitivity analysis to only include positive tests which study staff were able to verify via clinic cards.

Finally, during visits B1 to B4 mothers were asked to report if their children had diarrhea at any point the past seven days, defined as passage of three or more loose stools within a 24-hour period.

Longitudinal prevalence of diarrhea in children was defined as the number of weeks in which children had one or more reported episodes of diarrhea divided by the number of weeks of observation.

4.2.6 Statistical analysis

All statistical analyses were conducted with R version 4.0.2 (R Core Team, Vienna Austria) (Team, 2013) and SAS version 9.4 (SAS Institute, Cary NC). We fit generalized linear mixed effect models to assess the impact of the intervention on rate ratios (RR) of *Anopheles* and culicine mosquitoes, with the control arm as the reference group. Vector counts were over-dispersed and were therefore modeled using a negative binomial distribution, and we included random effects to account for repeated observations at the household level (Kirby et al., 2008; Zhou et al., 2004a). We fit log-binomial models to assess the effect of the intervention on longitudinal prevalence ratios (LPR) of reported malaria and diarrhea in mothers and children (Schmidt et al., 2010) .

We first fit unadjusted models to measure the independent effects of the HAPIN intervention status as well as other independent variables. We then fit multivariate models adjusting for covariates which were imbalanced between study arms and potential confounders. For *Anopheles* and culicine mosquitoes and reported malaria these included maternal education, the number of occupants that slept in the house the night before, presence of openings in houses, household floor material, bed net usage, elevation, proximity to rice fields, and population density. Potential confounders assessed for flies and reported diarrhea were drawn from previous research and included maternal education, presence of openings in houses, floor material, elevation, and population density. We assessed potential effect modification of the association between the intervention and each outcome based on the primary cooking location (indoor vs. outdoor/ in a separate cooking structure) on the day that vector sampling was conducted.

4.2.7 Ethics

This study was reviewed and approved by the Emory Institutional Review Board (IRB 00110407) and the Rwanda National Ethics Committee (IRB 00001497, No.194/RNEC/2019).

4.3 Results

4.3.1 Study population characteristics

Of 220 group 1 houses that were randomly selected, 101 control and 109 intervention households were enrolled. An additional 38 (19 int and 19 control) of the group 2 households that were purposefully selected based on their cooking location at baseline were also enrolled. Inability to contact households in order to schedule sampling visits was the primary reason for non-enrollment. All enrolled houses received at least one round of entomological sampling. Five intervention and six control houses did not receive a second round of sampling because they completed and exited the HAPIN trial before the second visit. Sampling was interrupted during the third round due to the COVID-19 pandemic and 26 intervention houses and 29 control houses did not receive a third visit. A total of 567 sampling visits were conducted among group 1 houses, and 103 visits were conducted among group 2 houses (Figure 4-1).

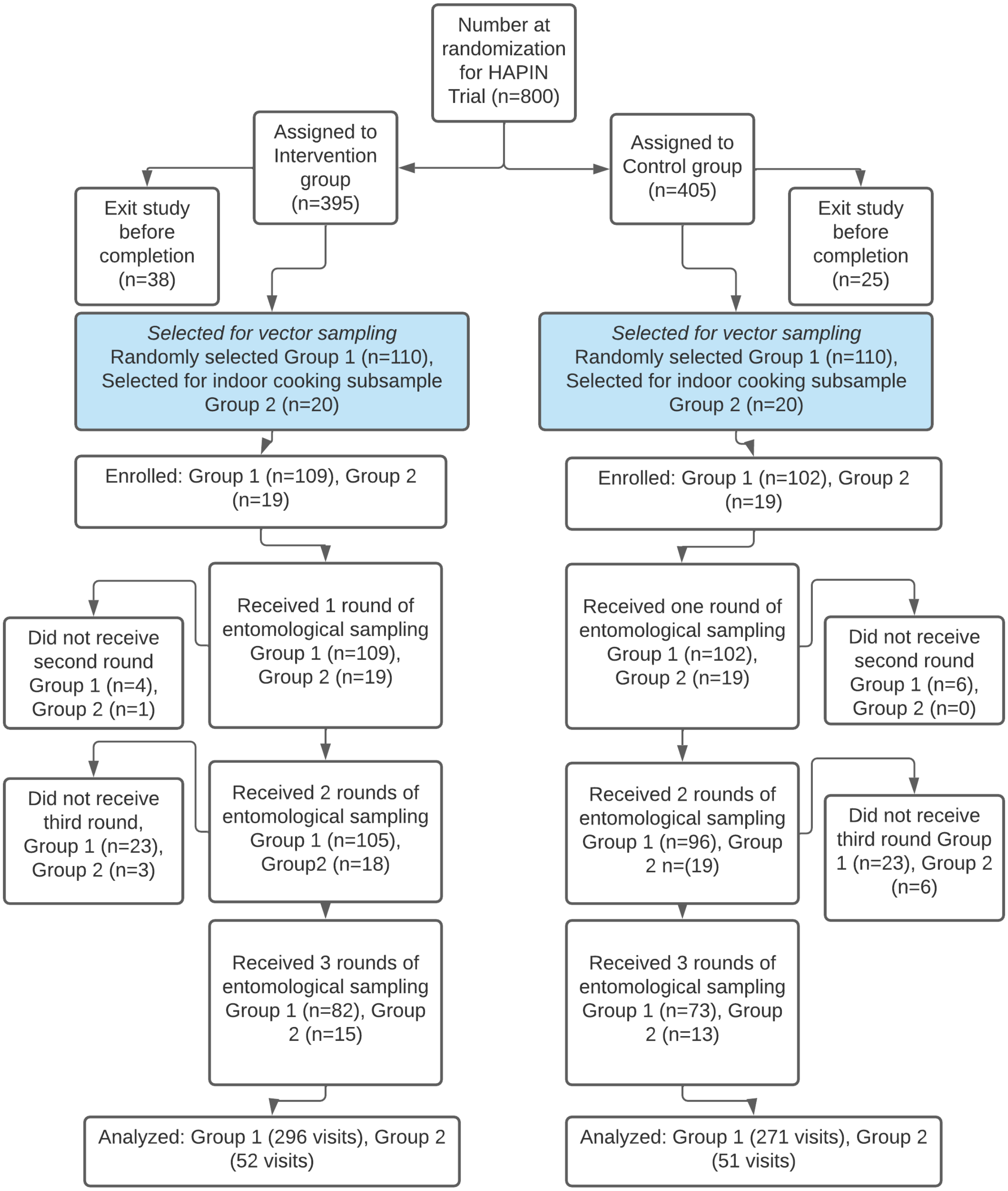

Figure 4-1: Flow diagram of household selection from HAPIN trial population

Fidelity was high; intervention houses received LPG stoves and fuels a median of 9.5 days after randomization (IQR = 6 – 16 days), and all houses received the intervention prior to the start of entomological sampling. Maternal age and gestational age at baseline were similar between participants in each arm, whereas mothers in the control arm were less likely to have completed secondary or further education. Wood was the most common fuel source among control and intervention groups at baseline, followed by charcoal, which was more commonly used in intervention houses compared to control houses. A majority of participants in both groups reported cooking outdoors or in a separate cooking structure at baseline. Mud floors were somewhat more common in control houses. Control houses were located in areas with lower average elevation and lower average population density than intervention houses, whereas slightly more intervention houses were located within 2km of rice fields (Table 4-1).

Table 4-1. Baseline characteristics of study participants and households

	Control (n = 102)	Intervention (n = 109)
Characteristics of women participants		
Maternal age at baseline, mean (SD)	27.3 (4.3)	27.4 (4.2)
Gestational age at baseline, mean (SD)	15.4 (2.9)	15.5 (2.9)
Maternal education, n (%)		
No formal education	5 (4.9)	9 (8.3)
Primary	68 (66.7)	52 (47.7)
Secondary or college	29 (28.4)	48 (44.0)
Number of children under 12 years, mean (SD)	1.52 (0.82)	1.55 (1.02)
Cooking practices at baseline		
Primary fuel used, n (%)		
Wood	86 (84.3)	71 (65.1)
Charcoal	15 (14.7)	37 (33.9)
Other	1 (1.0)	1 (0.9)
Location of primary cooking stove, n (%)		
Outdoors or separate cooking structure	97 (95.1)	99 (90.8)
Indoors in main house	5 (4.9)	10 (9.2)
Characteristics of households		
Wall materials, n (%)		
Mud	53 (52.0)	56 (51.4)
Concrete	15 (14.7)	35 (32.1)
Floor materials, n (%)		
Mud	79 (77.5)	62 (56.9)
Concrete	26 (25.5)	47 (43.1)
Roof materials, n (%)		
Corrugated metal	102 (100.0)	109 (100.0)
Environmental characteristics		
Elevation (m), mean (SD)	1549.62 (100.15)	1584.57 (97.11)
Rice fields within 2km, n (%)	19 (18.6)	22 (20.2)
Population density / km^2, mean (SD)	583.60 (385.44)	815.41 (593.71)
Follow-up periods:		
Mothers, mean (SD)	4.6 (0.6)	4.62 (0.62)
Children, mean (SD)	3.6 (0.8)	3.6 (0.7)

Intervention adherence was high during the three rounds of entomological sampling visits; 97% of intervention houses reported using LPG as their primary cooking fuel at follow-up (the day of each visit) and 99% of control houses reported using biomass as their primary cooking fuel at follow-up. Nearly 90%

of control houses reported cooking outdoors or in a separate cooking structure at follow-up, whereas 91% of intervention houses reported cooking inside the main house (Table 4-2).

Table 4-2: Characteristics of study participants and households during entomological sampling visits

			Control (visits = 271)	Intervention (visits = 296)
Cooking practices the night before sampling visit				
	Fuel used in primary stove, n (%)			
		Biomass	269 (99.6)	9 (3.1)
		LPG	1 (0.4)	283 (96.9)
	Cooking location for primary stove, n (%)			
		Outdoors or separate cooking structure	239 (88.8)	27 (9.3)
		Indoors in main house	30 (11.2)	264 (90.7)
Housing characteristics				
	People slept in house night before, mean (SD)		3.96 (1.30)	4.02 (1.43)
	Cracks or openings in windows, doors, and/or walls, n (%)		199 (73.4)	199 (67.2)
	Open, water-holding containers, n (%)		97 (35.8)	102 (34.5)
	Toilet/ latrine is covered, n (%)		16 (9.6)	25 (17.0)
	Distance (m) from latrine to kitchen, mean (SD)		10.38 (8.49)	13.48 (7.54)
	Distance (m) from rubbish pile to kitchen, mean (SD)		8.77 (5.63)	11.61 (9.39)
	Domestic animals in compound, n (%)		46 (59.0)	47 (55.3)
	Animal/human feces in compound, n (%)		33 (42.3)	26 (30.6)
	Uncovered, cooked food in kitchen, n (%)		26 (52.0)	30 (41.7)
Vector control activities				
	% of occupants that slept under net night before, mean (SD)		0.65 (0.41)	0.78 (0.35)
	Received IRS in last 12 months, n (%)		229 (86.4)	252 (87.2)
	Used insecticides or burned materials to repel mosquitoes or flies in last 24 hrs, n (%)		6 (2.2)	11 (3.7)
Indoor conditions				
	PM2.5 (μg/m^3) in bedrooms, mean (SD)		30.72 (26.16)	25.34 (30.22)
	Temperature (°C) in bedrooms, mean (SD)		23.51 (1.03)	23.38 (1.20)
	Relative humidity (%) in bedrooms, mean (SD)		72.79 (3.78)	73.01 (3.16)
Environmental characteristics:				
	LST (°C), current month, mean (SD)		27.06 (1.03)	26.94 (0.99)
	LST (°C), one-month lag, mean (SD)		30.39 (2.33)	30.13 (2.17)
	Rainfall (mm), current month, mean (SD)		130.67 (21.15)	130.42 (20.44)
	Rainfall (mm), one-month lag, mean (SD)		101.17 (30.60)	101.77 (30.18)

Cracks or openings in walls, windows, or doors were more commonly observed in control versus intervention houses, and primary cooking stoves in control houses were on average 2-3 meters closer to household toilets and rubbish piles compared to intervention houses at follow-up. Mean $PM_{2.5}$ concentrations were 30.7μg/m^3 (SD=26.1) in control bedrooms, which was slightly higher than 25.3μg/m^3 (SD=30.2) measured in intervention bedrooms. These values were below WHO interim targets of 35 μg/m^3 (WHO, 2021a). Rainfall and land surface temperatures in the month of and month prior to each visit were similar in each study arm.

4.3.2 Entomological outcomes

Anopheles mosquitoes

We collected 356 *Anopheles* mosquitoes over the course of 567 sampling nights via CDC light traps and Prokopacks. *An. gambiae* s.l. accounted for 82% of all *Anopheles* collected, whereas other species such as *An. ziemanni* were collected in lower numbers (Supplemental Table A-1). *Anopheles* density was 0.53 (SD=2.35) per sampling night in control houses and 0.72 (SD =2.95) in intervention houses, whereas median counts were 0 (range 0 – 31) in both arms (Table 4-3 & Figure 4-2).

Table 4-3: Effects of intervention on *Anopheles*, culicine, and filth fly density

	Control			Intervention			Adjusted RR	P value
Outcome	n visits	total collected	mean (sd)	n visits	total collected	mean (sd)		
Anopheles	271	143	0.53 (2.35)	296	213	0.72 (2.95)	1.58 (0.88, 2.85)	0.13
Culicines	271	894	3.3 (5.41)	296	1251	4.23 (8.7)	1.07 (0.78, 1.48)	0.67
Synanthropic flies	271	752	2.77 (4.84)	296	270	0.91 (2.69)	0.39 (0.27, 0.56)	<0.001

Rate ratios (RR) for *Anopheles* and culicine mosquitoes adjusted for maternal education, number of people that slept in house, cracks or openings in house, mud floors, bed net use, elevation, proximity to rice fields, and population density. RRs for flies adjusted for same variables except number of people that slept in house and rice field proximity

After adjusting for potential confounders, *Anopheles* rates were higher but not statistically significant in the intervention group compared to the control group (RR = 1.58, 95% CI: 0.88 – 2.85) (Table 4-3). The percent of occupants that slept under bed nets the night before and proximity to rice fields within 2km were positive predictors of *Anopheles* density, whereas elevation and population density were negative predictors. Full model results with unadjusted and adjusted effect estimates are presented in Supplemental Table A-2.

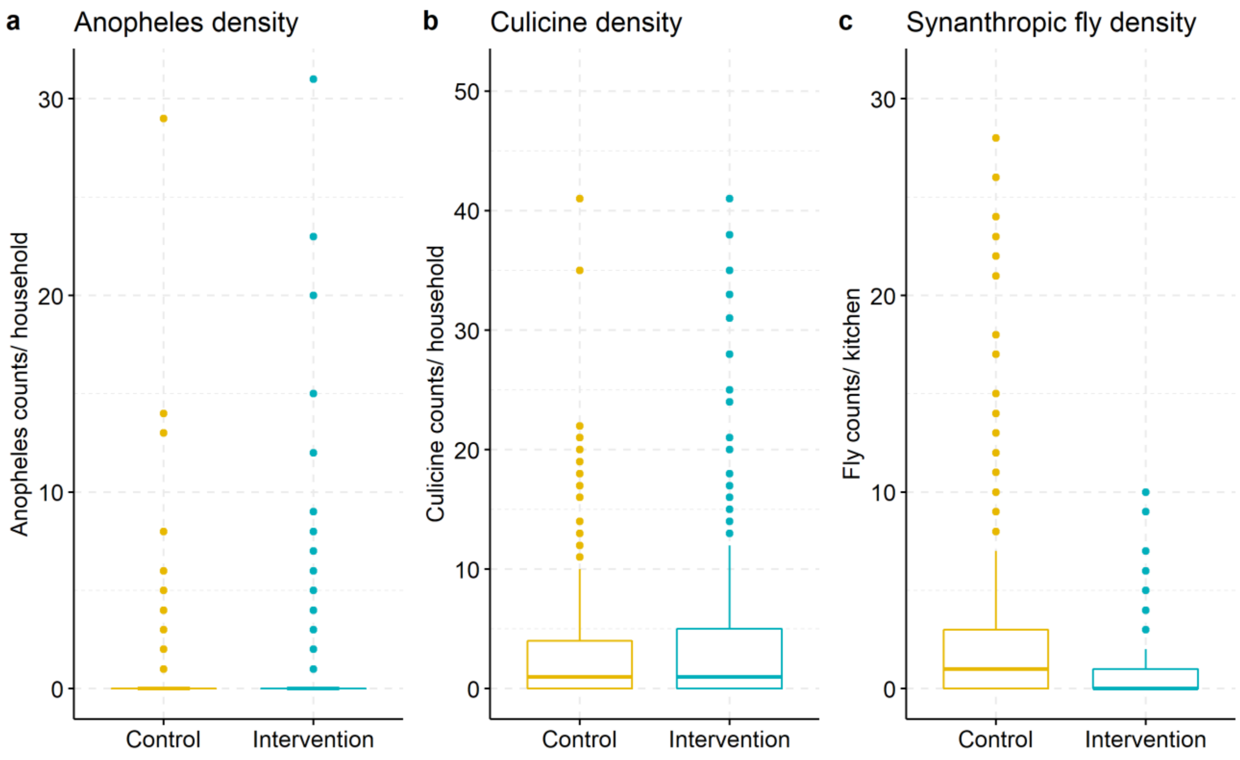

Figure 4-2: Densities of *Anopheles* mosquitoes (a), culicine mosquitoes (b) and synanthropic flies (c) by intervention status. Medians are shown with solid horizontal lines at the center of each box plot.

Few control houses in group 1 reported cooking indoors during the entomological sampling visits, so we included the group 2 houses to assess effect modification by cooking location. Including these houses, *Anopheles* density was 0.2 (SD=0.83) in control houses compared to 0.6 (SD=2.70) in intervention houses that cooked indoors at follow-up. After adjusting for potential confounders, this was associated with a substantial increase in *Anopheles* density, although confidence intervals included the null (RR = 3.66, 95% CI: 0.92 – 14.59). In contrast, the intervention did not appear to affect *Anopheles* density in houses that cooked outdoors (RR = 1.09, 95% CI: 0.31 – 3.83) (Supplemental Table A-3). We observed similar effects when we restricted the analysis to just the randomly selected group 1 houses, although the confidence intervals were wider (data not shown).

We then examined differences in $PM_{2.5}$ in the subset of 218 visits that had PATS+ measurements. Overall each standard deviation increase in $PM_{2.5}$ was negatively associated with *Anopheles* density, although the effect was not statistically significant (RR = 0.65, 95% CI: 0.15 – 2.82) (Supplemental Table A-4). Mean $PM_{2.5}$ concentrations were the highest in control houses which cooked indoors at follow-up. However, we did not statistically assess whether the effect of $PM_{2.5}$ was modified by cooking location because *Anopheles* counts were zero in all 17 control houses which had $PM_{2.5}$ measurements and cooked indoors at follow-up (Supplemental Table A-5).

A single *Anopheles* mosquito from a control household was *P. falciparum* sporozoite-positive, whereas no mosquitoes from intervention households were infected. Around 16% of *Anopheles* mosquitoes collected in control houses were bloodfed, compared to 5.2% from intervention houses. Bloodmeal composition was not different by intervention status (Supplemental Table A-6).

Culicine mosquitoes

We collected 2,145 culicine mosquitoes over the course of the study, 2,048 (96%) of which were *Cx. quinquefasciatus*. Mean culicine density was 3.30 (SD = 5.41) per sampling night in control houses, compared to 4.23 (SD = 8.70) in intervention houses (Table 4-3, Figure 4-2, & Supplemental Table A-1). After controlling for potential confounders, the intervention did not affect culicine density (RR = 1.07, 95% CI: 0.78 – 1.47). The number of people that slept in the house the night before, mud floors, and proximity to rice fields were positive predictors of culicine density, whereas elevation was negatively associated with culicine density (Supplemental Table A-2).

Cooking location did not modify the effect of the intervention, and neither $PM_{2.5}$ nor cooking location were predictors of culicine density (Supplemental Tables A-3 and A-4). Fewer than 1% of *Cx. quinquefasciatus* mosquitoes collected in control houses were bloodfed, compared to 1.2% from intervention houses. Bloodmeal composition was not different between intervention and control houses (Supplemental Table A-4).

Synanthropic flies

We collected 1022 synanthropic flies, 475 (46%) of which were Muscidae family 436 (43%) were Fanniidae. Mean synanthropic fly density was 2.77 (SD 4.84) in control houses compared to 0.91 (SD 2.69) in intervention houses (Table 4-3 & Supplemental Table A-1). Controlling for potential confounders, the intervention was associated with a 61% reduction in synanthropic fly density (RR = 0.39, 95% CI: 0.27 – 0.56).

This effect appeared to be primarily driven by changes in cooking location among households in the intervention arm. Close to 91% of intervention houses reported cooking indoors at follow-up whereas only 9% of these houses cooked indoors at baseline (Table 4-2). Intervention participants were encouraged to cook in a covered location in order protect LPG stoves (Clasen et al., 2020), and may have chosen to cook inside the main house out of convenience or to protect stoves and fuel from theft, as has been observed previously in Rwanda (Seguin et al., 2018). Cooking indoors at follow-up was associated with a 62% reduction in fly densities (RR = 0.38, 95% CI: 0.27 – 0.55), indicating that cooking location mediated the effect of the intervention (Supplemental Table A-4). Indoor kitchens were a mean of 2-3 meters further away from latrines and rubbish pits, which are common fly breeding sites. Animals were less commonly observed in or near indoor kitchens compared to outdoor kitchens, and house walls also could have inhibited fly entry into indoor kitchens. In contrast, $PM_{2.5}$ in bedrooms was not associated with fly density.

4.3.3 Secondary outcomes: Malaria and Diarrhea

A total of 69 malaria cases (39 control, 30 intervention) were reported among mothers. Mean longitudinal prevalence of malaria was 8.1% (SD = 13.0) among mothers in the control group and 5.8% (SD = 12.1) among mothers in the intervention group. After controlling for potential confounders, the intervention was not associated with malaria risk among mothers (LPR = 0.92, 95% CI: 0.56 – 1.47) (Table 4-4). The results were similar when we restricted the analysis to only confirmed malaria cases. $PM_{2.5}$ and cooking location were not predictors of reported malaria risk in mothers (Supplemental Table A-6).

Table 4-4. Effects of intervention on longitudinal prevalence of reported malaria in mothers and malaria and diarrhea in children

	Control			Intervention			Adjusted RR	P value
Outcome	obs. periods	n cases	mean longitudinal prevalence (sd)	obs. periods	n cases	mean longitudinal prevalence (sd)		
Malaria in mothers	471	39	8.09 (12.97)	512	30	5.78 (12.21)	0.92 (0.56, 1.47)	0.72
Malaria in mothers, confirmed	471	29	5.98 (12)	512	22	4.31 (9.87)	0.95 (0.53, 1.68)	0.86
Malaria in children	360	9	2.67 (9.83)	394	3	0.78 (4.65)	0.42 (0.09, 1.44)	0.20
Malaria in children, confirmed	360	6	1.83 (0.1)	394	3	0.78 (4.65)	0.56 (0.11, 2.36)	0.44
Diarrhea in children	359	32	8.83 (0.14)	397	30	7.4 (14.27)	0.96 (0.59, 1.56)	0.88

Obs. periods = periods of observation; RRs adjusted for maternal education, number of people that slept in house, cracks or openings in house, mud floors, bed net use, elevation, proximity to rice fields, and population density

Malaria in children was less common than in mothers, with 12 (9 control, 3 intervention) reported cases during 754 follow-up periods. Mean longitudinal prevalence of malaria was 2.7% (SD = 9.8) among children in the control group and 0.8% (SD = 4.6) among children in the intervention group. After controlling for potential confounders, malaria risk was lower children in the intervention arm compared to the control, although this effect was not statistically significant (LPR = 0.42, 95% CI: 0.09 – 1.44) (Table 4-4). The effect was slightly attenuated but similar when we restricted to only confirmed cases. We did not evaluate the effects of $PM_{2.5}$ on malaria risk in children because there were too few cases for which PATS+ measurements were available (n=5) (Supplemental Table A-6).

A total of 62 (32 control, 30 intervention) diarrhea episodes were reported among children during 756 follow-up periods. Mean longitudinal prevalence of diarrhea was 8.83% (SD = 0.14) among children in the control group and 7.4% (SD = 14.27) among children in the intervention group. After controlling for potential confounders, the intervention was not associated with diarrhea prevalence rates in children (LPR = 0.96, 95% CI: 0.59 – 1.56) (Table 4-4). Each standard-deviation increase in $PM_{2.5}$ was positively associated with diarrhea, although the effect was not statistically significant (PR = 1.59, 95% CI: 0.86 – 2.82) (Supplemental Table A-7).

4.4 Discussion

This was the first randomized controlled trial to measure the impact of a clean cooking intervention on entomological and epidemiological parameters of vector-borne disease. We found no effect of the intervention on *Anopheles* density, bloodmeal composition, or *P. falciparum* infection in *Anopheles* mosquitoes. These findings indicate that the replacement of biomass fuels with LPG as part of the HAPIN household air pollution intervention did not significantly change mosquito density or exposure to malaria-infected mosquitoes, despite clear indications of mosquito behavioral changes from our previous research in experimental huts (Hennessee et al., 2022). This study is important because cleaner cooking fuels are increasingly promoted and the effect of cooking fuels on vector behavior and exposure to vector-borne diseases remains poorly understood, especially under real-world conditions.

Although indoor cooking was uncommon among control households, *Anopheles* densities were higher in intervention houses that cooked indoors at follow-up compared to control houses, whereas no effect was apparent for houses that cooked outdoors. Similar findings were reported in two observational studies that found reduced *Anopheles* densities in houses that cooked with biomass indoors (Hiscox et al., 2013; McCann et al., 2017). This could be related to repellant effects of $PM_{2.5}$ or other pollutants from indoor biomass combustion, although low sample sizes limited our ability to assess this effect among houses which cooked indoors. Previously we observed that elevated indoor $PM_{2.5}$ concentrations were associated with decreased household entry and host-seeking by lab-reared *Anopheles* mosquitoes and increased mortality (Hennessee et al., 2022).

Nevertheless, other environmental determinants of *Anopheles* density such as altitude, proximity to breeding sites, and housing quality were more important for determining vector density across the overall study population in the context of high IRS coverage. The importance of environmental and housing characteristics has been observed in Rwanda and elsewhere (Diuk-Wasser et al., 2007; Tusting et al., 2017; Tuyishimire, 2016).

Although our study was not powered to detect this outcome and the effect estimates were imprecise, reported malaria longitudinal prevalence was lower among children in the intervention arm compared to the control arm (0.8% vs 2.7%). Reductions in household air pollution exposure have been shown to improve innate immune function, which could reduce susceptibility to infectious diseases such as malaria (Lee et al., 2015). Additional research could further investigate malaria risk and immune markers in the overall HAPIN population in order to shed light on this potential co-benefit of clean fuel adoption. Reported bed net use was also 17% higher among individuals in intervention households compared to control households, which could impact observed malaria outcomes.

We also found no effect of the intervention on culicine mosquito density, and the intervention was not associated with changes in bloodfed status or bloodmeal composition among *Cx. quinquefasciatus*. However, low counts of other less abundant species limited our ability to assess other species-specific effects of the intervention. This could be especially important for *Aedes aegypti,* which typically bites indoors or around houses during the day and therefore could be more influenced by changes in cooking fuels or cooking location (Captain-Esoah et al., 2020; Madewell et al., 2020; Reinhold et al., 2018). Further research in settings where *Ae. aegypti* and associated arboviruses such as Dengue Virus or Yellow Fever Virus are common is needed to further explore this potential effect.

A round of IRS was conducted approximately six months before the start of vector sampling for this study, which likely contributed to the low mosquito counts we observed. IRS coverage was not different by intervention status, and therefore should not have influenced empirically measured vector densities. However, reduced vector densities may have affected study power and our ability to detect differences between control and intervention households.

We observed a 64% reduction in the density of synanthropic flies in intervention houses, which appeared to be primarily driven by higher rates of indoor cooking among intervention households. However, we did not observe a strong association between the intervention and diarrhea prevalence in children. The study was not powered to detect relatively small changes in diarrhea prevalence as this was a secondary outcome. It is also possible that fly reductions were not adequate to affect diarrheal disease. Previous studies in the Gambia and Pakistan reported 22 to 23% reductions in reported diarrhea in children after insecticide spraying reduced synanthropic fly densities by 75% or more (Chavasse et al., 1999; Emerson et al., 1999). Fly reductions may also have pathogen-specific effects on diarrheal disease. For example, a fly control intervention in Israel led to major reductions in shigellosis among the study population, whereas no effect was seen for Norovirus gastroenteritis (Cohen et al., 1991; Cohen et al., 2002). Further investigations of diarrhea etiology could examine potential pathogen-specific effects of reduced fly densities in the HAPIN study.

It is also possible that other changes associated with clean fuel adoption could affect diarrheal disease. For example, reduced household air pollution exposure could improve immune function or gut health, which could reduce diarrhea risk (Gordon et al., 2014; Heft-Neal et al., 2018; Lee et al., 2015; Marynowski et al., 2015). Although we observed a positive relationship between $PM_{2.5}$ concentrations in bedrooms and diarrhea prevalence, the effect estimates were imprecise and included the null (Supplemental Table A-7). Bedroom $PM_{2.5}$ concentrations were not necessarily representative of overall particulate matter exposure among children, and other unmeasured pollutants such as carbon monoxide and black carbon could influence immune function, inflammation, and metabolism (Manisalidis et al., 2020; Sinharoy et al., 2020), which could in turn affect diarrhea risk. Follow-up studies could assess relationships between components of fuel combustion, immune and gut health biomarkers, and diarrhea risk across all HAPIN children to further assess potential relationships between household air pollution and diarrhea. Additional studies could also measure food contamination by flies in HAPIN kitchens in order to better assess the direct effects of fly reductions on exposure to enteric pathogens (Lindeberg et al., 2018).

Self-reported outcomes were limited by the potential for recall and interviewer bias. However, for reported malaria we observed similar effects when we restricted the analysis to only confirmed malaria cases. We were unable to confirm reported diarrhea cases. Further studies could include clinic-confirmed malaria and diarrhea cases in order to account for this possibility.

4.5 Conclusions

The adoption of cleaner-burning LPG stoves and fuel via a household air pollution intervention trial did not appear to affect mosquito exposure in this setting, whereas it resulted in a substantial reduction in synanthropic fly density in cooking areas. The intervention was not significantly associated with malaria or diarrhea risk among mothers or children.

These findings indicate that, at least in this setting, the adoption of cleaner cooking may have null or even beneficial effects on exposure to important vectors and the diseases they transmit. This is a reassuring finding for groups involved in the promotion of clean-cooking interventions. However, further research should be conducted in other eco-epidemiological settings to assess potentially context or species-dependent effects of clean fuel adoption, and whether vector control interventions should be promoted in tandem with cleaner fuels in certain contexts. For example, changes in cooking fuels may have a stronger effect in settings where indoor cooking is more common or where daytime-biting vectors such as *Aedes aegypti* are dominant.

These findings also point to potential benefits of a more holistic approach to household environmental health. HAP interventions, water, sanitation and hygiene (WASH) interventions, and vector control interventions are all targeted at the household level but are traditionally delivered by siloed disease control programs (Clasen & Smith, 2019). Efforts to bridge these fields could capitalize on potential synergies between interventions (for example, fly control as a co-benefit of clean cooking interventions or mosquito control as a benefit of piped water interventions) in order to maximize household environmental health benefits.

5 Research Aim 3. Impacts of insecticide resistance and environmental change on *Anopheles gambiae* reemergence and resurgent malaria transmission in eastern Rwanda, 2010 – 2020

5.1 Background

From 2000-2010, Rwanda achieved unprecedented gains in malaria control due to rapid scale-up of Long-Lasting Insecticide Treated Nets (LLINs) and malaria case management (Eckert et al., 2017). Yearly malaria hospital admissions declined from 35 per 1000 individuals in 2000 to less than 10 in 2009, and total malaria-related deaths declined from 4,275 per year in 2000 to 809 in 2009 (WHO, 2010). Outpatient incidence declined by 87% and test positivity declined 71% between 2005 and 2011 (PMI, 2019; RBC, 2017). Rwanda was also among the first countries to achieve universal insecticide treated net (ITN) coverage in 2011 (Karema et al., 2020). In light of these gains, malaria elimination was considered achievable and nation-wide pre-elimination was targeted for 2012 (PMI, 2009).

However, after 2011 the country experienced a dramatic malaria resurgence. Total reported cases increased 20-fold from less than 230,000 cases in 2011 to over 4,700,000 in 2016. Test positivity increased concurrently from under 15% to nearly 50% (PMI, 2019) (Figure 5-1). Nationally representative health surveys also demonstrated an increase in malaria prevalence: microscopy-confirmed prevalence among children under five increased from 1.4% in 2010 to 2.2% by 2014-2015, and then to 7.2% by 2017 (ICF, 2017; National Institute of Statistics of Rwanda et al., 2015; NISR & Macro, 2012). Although these trends were observed nation-wide, eastern Rwanda experienced the highest burden. Prevalence in children under five in Eastern Province rose from 3.4% in 2010 to 18% in 2017 (ICF, 2017; PMI, 2019) .

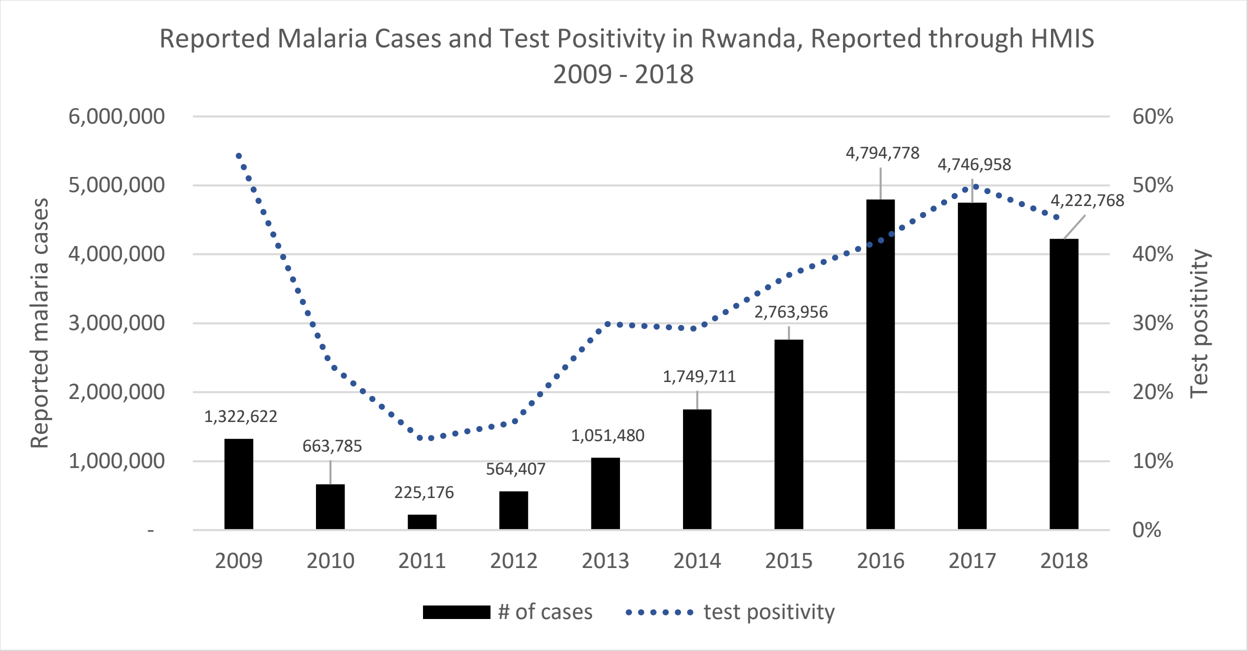

Figure 5-1. Reported malaria cases and tested positivity, 2009 – 2018. Data are from the Rwanda Health Management Information System (HMIS) and reported in Malaria Operational Plans from the President's Malaria Initiative

In response to the resurgence, the Rwandan National Malaria Control Program (NMCP) conducted a situational analysis and identified three groups of potential causes. These included 1) improved healthcare and surveillance (e.g., increased treatment seeking, diagnostic testing, and reporting), 2) environmental change (e.g., increased rice cultivation, increased rainfall and temperatures), and 3) vector control failures (e.g., insecticide resistance, inconsistent vector control campaigns, low ITN coverage, distribution of substandard ITNs, and ITN deterioration) (PMI, 2019; RBC, 2016, 2017).

Each of these explanations is plausible; for example, in DRC apparent malaria increases have been linked to scaled-up diagnostic testing and improved reporting (Lechthaler et al., 2019). Rice cultivation can increase *Anopheles* population density (Diuk-Wasser et al., 2007), and has been associated with dramatic outbreaks in Burundi and Kenya (Coosemans, 1985; Hunter et al., 1993). Research in southern Rwanda found spatial clustering of malaria cases near water-based agro-ecosystems such as rice and irrigated vegetable fields (Rulisa, Kateera, Bizimana, Agaba, Dukuzumuremyi, Baas, de Dieu Harelimana, Mens, Boer, et al., 2013). Furthermore, short-term increases in temperature and rainfall have historically been implicated in major malaria outbreaks in Rwanda (Hammerich et al., 2002; Loevinsohn, 1994). Highland areas in East Africa are considered especially vulnerable to climate change (Alonso et al., 2011; Paaijmans et al., 2009; Pascual et al., 2006; Ryan et al., 2020; Ryan et al., 2015; Siraj, Santos-Vega, Bouma, Yadeta, Ruiz Carrascal, et al., 2014), although the subject is debated (Chaves & Koenraadt, 2010; Hay et al., 2002). Insecticide resistance has been linked to the reemergence of *Anopheles funestus* and malaria resurgences in Kenya and South Africa (Hargreaves et al., 2000; McCann et al., 2014), whereas interrupted vector control programs led to massive outbreaks in northern Uganda (Raouf et al., 2017).

Although each of these hypotheses is credible, the increase in test positivity and malaria prevalence indicate that the observed resurgence was not solely an artifact of improved surveillance. Care seeking for fevers also remained relatively constant throughout the period (PMI, 2022b). NMCP policy required diagnostic confirmation of all suspect malaria cases starting in 2009 (Karema et al., 2020), and >99% of reported cases were diagnostically confirmed since 2011 (PMI, 2019, 2022b).

The other explanations (environmental changes and vector control failures) suggest that the resurgence was mediated by changes in vector ecology. However, no scientific studies were conducted to evaluate these hypotheses or identify the primary drivers of the resurgence. Furthermore, although the *Anopheles gambiae* s.l. species complex is the dominant vector group in Rwanda (Hakizimana et al., 2018), the relative importance of the two sibling species that are present in Rwanda, *An. gambiae* s.s. and *An. arabiensis,* has not been described in detail and the contribution of each species to the malaria resurgence is unknown. This information is essential for informing effective control measures and predicting and preventing future malaria resurgences in Rwanda and similar transmission settings in East Africa.

We therefore conducted a retrospective analysis of entomological, epidemiological, and meteorological data from four sentinel sites in Eastern Province, Rwanda in order to investigate the causes of resurgent malaria transmission in the area. Our objectives for this study were two-fold: 1) compare the bionomics of *An. gambiae* and *An. arabiensis* and identify the most important species for malaria transmission, and 2) investigate the impacts of environmental change and vector control failures on *Anopheles gambiae* s.l. species composition and malaria incidence from 2010 – 2020.

5.2 Methods

5.2.1 Study setting:

This study took place in four sites in Eastern Province, Rwanda. Eastern Province experienced the highest increase in prevalence between 2010 and 2020 (ICF, 2017), and has the highest malaria burden in the country (PMI, 2019). Universal ITN coverage was achieved in 2011, and large-scale IRS campaigns have been conducted in some high burden districts since 2008 (PMI, 2009). Malaria transmission is largely seasonal in the region, with spikes in May to June and November to December following the long and short rainy seasons (PMI, 2019).

The NMCP maintains a network of 12 entomology sentinel surveillance sites, four of which are in Eastern Province and were included in this study. Each entomology sentinel site is co-located with health centers which provide outpatient malaria diagnosis and treatment. Two sentinel sites, Bukora and Rukara, began collecting entomology data in 2010, whereas the other two sites, Mimuli and Mareba, began in 2012. The sites have similar elevations (Figure 5-2). Rice cultivation is common in wetland areas in Mimuli and Mareba (Hakizimana et al., 2016).

An. gambiae s.s. (hereafter *An. gambiae*) constituted 94 - 99% of the *An. gambiae* s.l. species complex in multiple sites in Rwanda in the mid-2000s (Howell, 2008; Konaté, 2007), but was largely replaced by *An. arabiensis* following the scale-up of ITNs and IRS from 2006-2011 (Hakizimana et al., 2018). This trend has been widely observed in sub-Saharan Africa, likely owing to the tendency of *An. arabiensis* to feed outdoors and on non-human hosts compared to more endophilic and anthropophagic vectors such as *An. gambiae* and *An. funestus* (Bayoh et al., 2010; Sinka et al., 2010). A 2013 study reported that the relative abundance of *An. arabiensis* compared to *An. gambiae* was 84%, 94%, and 96% in Bukora, Mareba, and Rukara. However, *An. gambiae* was more dominant in Mimuli and made up 62% of the species complex (Hakizimana et al., 2016). No studies have investigated changes in species composition or density since 2013.

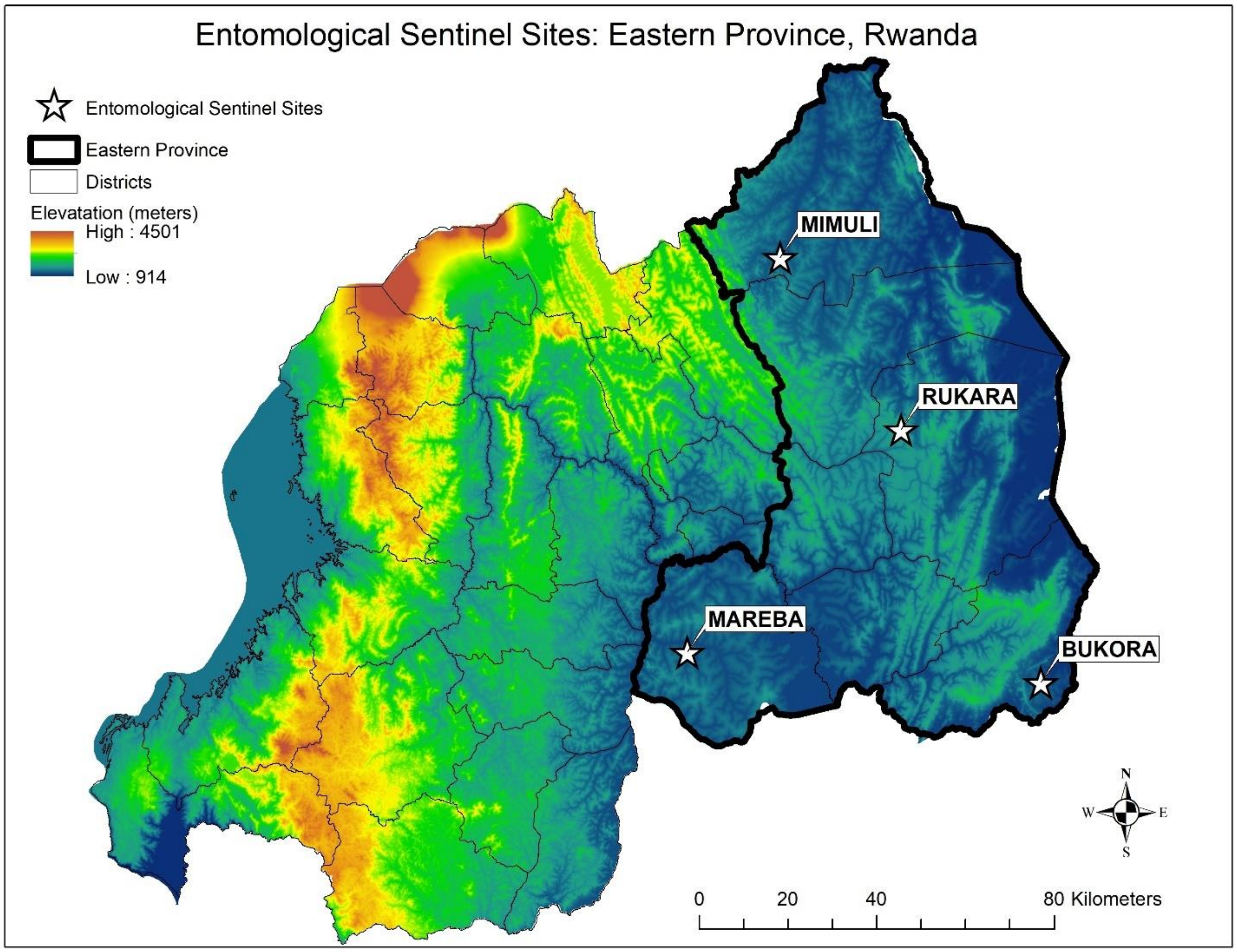

Figure 5-2: Locations of four entomology sentinel sites in Eastern Province, Rwanda.

5.2.2 Entomological data

Field entomology data

From 2010 to 2020 the NMCP conducted routine entomological surveillance on a monthly basis in three villages surrounding each sentinel site. Three houses were randomly selected from each village for human landing catch (HLC), such that each sentinel site included a network of 9 houses in which monthly HLC was conducted. HLC was performed in each house over two consecutive nights every month, as described previously (Hakizimana et al., 2018). Briefly, HLC was conducted from 6pm to 6am by two trained volunteers each night. One collector sat inside the house and the other sat outside approximately 5-8 meters from the entrance of the house. Host-seeking mosquitoes were collected with glass mosquito collection tubes and stored in envelopes by hourly intervals. Pyrethrum spray catch was also performed over two consecutive nights per month in five separate houses in each village to measure resting mosquito density.

After collection, mosquitoes were transported to field laboratories and were morphologically identified using standard morphological keys (Gillies & Coetzee, 1987). Because *An. gambiae* and *An. arabiensis* are morphologically identical, they were only identified to the species complex, *An. gambiae* s.l. Monthly densities for each species were calculated as the number of bites per person per night (bpn), defined as the total monthly species counts per village/ 2 sampling nights/ 3 houses/ 2 collectors. For this analysis, species counts were then pooled from the three villages within each sentinel site and species densities were averaged. This was done because laboratory analyses were conducted on pooled samples from each sentinel site (see below). HLC was conducted in Bukora and Rukara from January 2010 through December 2020, and in Mareba and Mimuli from March 2012 through December 2020.

Laboratory entomology data

Mosquito samples were pooled per sentinel site and sent to the central entomology laboratory in Kigali for laboratory analyses. Ten percent of *Anopheles* samples from each site were randomly selected for enzyme-linked immunosorbent assays (ELISA) to assess *P. falciparum* sporozoite infection (Benedict, 2007). Bloodfed mosquitoes were also tested with ELISA to determine bloodmeal composition. Finally, *An. gambiae* s.l. mosquitoes were selected for molecular characterization within the species complex using standard Scott polymerase chain reaction (PCR) assays (Scott et al., 1993). A random sample of 30% of *An. gambiae* s.l. mosquitoes was selected in sites where monthly *An. gambiae* s.l. HLC counts exceeded 100, whereas all *An. gambiae* s.l. mosquitoes were speciated by PCR in sites where counts were less than 100. ELISA and PCR were conducted on a monthly basis from July 2017 through December 2020.

5.2.3 Epidemiological data

Monthly records of diagnostically confirmed, outpatient malaria cases were collected for each of the four health facilities. NMCP policy required diagnostic confirmation of all suspect malaria cases throughout the time period. Populations in each health facility catchment area were estimated by the NMCP based on the 2012 national census and adjusted yearly to account for projected population increases. Malaria case data were not available by age, so overall monthly incidence was calculated as the total number of reported cases divided by the catchment population.

5.2.4 Outcomes

Objective 1 outcomes. The first objective of the study was to compare the bionomics of *An. gambiae* and *An. arabiensis* and identify the most important species for malaria transmission. This was assessed using the detailed 2017-2020 laboratory data. Primary outcomes included:

- *Human blood index*: the human blood index (HBI) was defined as the proportion of bloodmeals among each species which were from humans. This included mixed blood meals from human and non-human hosts. HBI is a key indicator for vector capacity because mosquitoes must feed on humans twice in their lifetime in order to be infected and then infect a subsequent host (Garrett-Jones, 1964).
- *Indoor biting*: we assessed indoor biting for each species as the proportion of overall HLC collections that were collected indoors. We excluded mosquitoes collected by PSC from this analysis because PSC is exclusively conducted indoors. Although outdoor biting is often speculated to be a potential source of residual malaria transmission following scale-up of indoor vector control interventions, numerous studies in East Africa have found that the majority of infective bites still occur indoors (Bayoh et al., 2014; Huho et al., 2013; Killeen et al., 2006; Seyoum et al., 2012).
- *Sporozoite rates:* Sporozoite rates were calculated as the proportion of each species which were positive for *Plasmodium falciparum* sporozoites out of the total that were tested via ELISA.

Sporozoite rates are a key indicator of mosquito infectivity and are widely used to implicate dominant malaria vectors.

- *Density*: We calculated and compared the density of each species by combining laboratory measurements of species composition with field-based density measurements for the species complex.
    - Species composition: We assessed species composition as the relative abundance of *An. gambiae* compared to *An. arabiensis*. Relative abundance of *An. gambiae* for each sentinel site was defined as the proportion of all *An. gambiae* s.l. tested by PCR each month that were identified as *An. gambiae*.
    - Density: We then applied our estimates of species composition to monthly HLC *An. gambiae* s.l. density estimates from each sentinel site from July 2017 – December 2020 in order to directly estimate and compare the density of *An. gambiae* and *An. arabiensis*. Density was defined as bites/person/night (bpn) for each species, as defined previously (Hakizimana et al., 2018; WHO, 2021b).
- *Entomological Inoculation Rate (EIR):* Finally, we multiplied the sporozoite rates by annualized density estimates (bites per person per year) to obtain the annual entomological inoculation rate (EIR). Annual EIR is the number of infective bites per person per year (Warrell & Gilles, 2017). EIR is widely used to measure malaria transmission intensity (Shaukat et al., 2010).

Two sites, Bukora and Mimuli, had received consistent indoor residual spraying (IRS) with a long-lasting organophosphate insecticide, pirimiphos-methyl, since at least September 2016. Mareba received pirimiphos-methyl IRS in February 2018, and Rukara was sprayed with Fludora®Fusion in April/May 2019. We therefore assessed each outcome overall and separately during pre- and post-IRS periods.

Objective 2a outcomes. The next objective was to investigate the effect of vector control failures and environmental change on *Anopheles gambiae* s.l. species composition and density during the study period. We defined species composition and density as above. We employed historical records of species composition from 2007 and 2013 (Hakizimana et al., 2016; Konaté, 2007) as baseline values before the period in which detailed lab data were available (2017-2020).

Objective 2b outcomes. The last objective was to assess the impacts of vector control failures and environmental change on malaria incidence in the area. We defined monthly malaria incidence per 1000 individuals as the number of diagnostically confirmed malaria cases at each health facility / catchment population * 1000.

5.2.5 Independent variables

We used a combination of programmatic data, ground-based observations, and remotely sensed data in order to assess trends in vector control and environmental parameters. These included:

Vector control variables:

- IRS: We collected detailed information about IRS campaigns in each site from 2010 to 2020 from NMCP records as well as programmatic reports from the President's Malaria Initiative and their implementing partner, Abt Associates (PMI, 2022a). This included the timing of each campaign, type and class of insecticide used, and administrative coverage. Administrative coverage was consistently >98% for targeted areas so was not considered further for this analysis. We estimated insecticide duration for each insecticide using World Health Organization (WHO) and other published estimates (Fongnikin et al., 2020; Haji et al., 2015; Rowland et al., 2013; WHO, 2015). IRS campaign timing in each site are plotted and expected residual duration is discussed in Supplemental Figure B-1.
- ITN coverage: Rwanda began distributing nets to high-risk groups in 2006, and the first universal ITN coverage campaign was started in May 2010. Universal coverage was achieved by February 2011 (Karema et al., 2020). Additional campaigns were conducted every two to three years, and nets were distributed through routine channels throughout the time period (PMI, 2022b). A detailed study by the Malaria Atlas Project used programmatic and Demographic and Health Survey (DHS)

and Malaria Indicator Survey (MIS) data to estimate ITN use at a 1km spatial resolution across Africa from 2000 to 2020 (Bertozzi-Villa et al., 2021). We used the *raster* package (Hijmans et al., 2015) in R version 4.0.2 to extract the Malaria Atlas Project ITN use estimates at the spatial coordinates of our study sites for each year of the study. We then used loess smoothing to produced continuous estimates of ITN use at each site on a monthly basis. ITN coverage estimates are plotted in Supplemental Figure B-2.

- Insecticide susceptibility: The NMCP conducted insecticide susceptibility tests on a yearly basis from 2011 to 2020. Susceptibility tests were conducted using WHO tube tests following previously described procedures (Hakizimana et al., 2016). Briefly, 25 unfed 2-3 day-old progeny of field collected *Anopheles gambiae* s.l. mosquitoes were exposed for 1 hour to diagnostic concentrations of permethrin, deltamethrin, bendiocarb, pirimiphos-methyl, and clothianidin. Mortality was assessed at 24 hours. Tests were repeated in four replicates, and susceptibility was defined as the average mortality across each test. All non-pyrethroid insecticides showed >90% susceptibility across the study period. We therefore considered them fully effective when employed in IRS. However, dramatic drops in permethrin and deltamethrin were apparent in each site. Trends in deltamethrin and permethrin susceptibility are shown in Supplemental Figure B-3.

Environmental variables:

- Rice cultivation: we assessed rice cultivation using published reports and high-resolution Google Earth Pro historical imagery from 2010 to 2020. Rice was consistently cultivated throughout the study period in Mareba and Mimuli (Hakizimana et al., 2016), whereas no evidence of rice cultivation was found within 2km of Bukora and Rukara. More than 95% of *An. gambiae* adults are typically found within this distance from larval sites (Gillies, 1961). We therefore determined that increased rice cultivation was not a viable explanation of resurgent malaria transmission across the four sites and excluded it from further analysis.

- Temperature: The NMCP maintains meteorological stations in Bukora and Rukara, which provided data on minimum and maximum monthly temperatures for each month from 2010 to 2020. Station measurements showed a dramatic spike in temperatures during the time period; average minimum temperatures increased approximately 1.5°C from 13.8°C in 2010 to 15.3°C in 2016. Maximum annual temperatures increased 2.3°C in the same period, from 26.3°C to 28.6°C. Maximum temperatures returned to normal after 2017, but minimum temperatures remained elevated relative to the earlier part of the decade (Supplemental Figure B-4). We then used generalized additive models to model monthly Tmin and Tmax as a function of remotely sensed environmental variables and predict temperatures at the two sites without meteorological stations. Additional details are provided in Supplemental materials, Appendix B.
- Rainfall: We retrieved monthly 5km-resolution gridded rainfall estimates from the Climate Hazards Infrared Precipitation with Stations (CHIRPS) project (Funk et al., 2015), and extracted values at each site to estimate rainfall. Rainfall trends were relatively constant throughout the study period (Supplemental Figure B-5).

5.2.6 Statistical analysis

All analyses were conducted in R version 4.0.2 (Team, 2013). We first conducted descriptive analyses of HBI, indoor biting, sporozoite rates, and density for the period when detailed laboratory data were available, 2017 to 2020. We conducted the analyses overall and then by IRS period as described above.

We then fit log-binomial models to assess the effects of IRS coverage, ITN use, insecticide susceptibility, and rainfall on species composition from 2010 to 2020. We dichotomized insecticide resistance by whether susceptibility was ≥ 80% (WHO, 2006). We evaluated both permethrin and deltamethrin resistance as potential predictors because both were used in ITNs during the period. We selected permethrin based on Akaike Information Criterion (AIC) values when both variables were added last to the full model. We fit interaction terms between ITN use and permethrin resistance to investigate whether resistance modified net effectiveness. We also hypothesized that there could have been interactions between deltamethrin resistance and deltamethrin-based IRS, but only non-pyrethroid based IRS was used from 2017 to 2020 when most of the species composition data were available. We therefore constructed an ‘effective IRS’ variable to represent any IRS campaign conducted with an insecticide for which susceptibility was > 80% (Brogdon & Chan, 2010; Dengela et al., 2018). We evaluated both variable specifications and selected the ‘effective IRS’ variable based on AIC values. We evaluated temperature but excluded it from the final model because it did not improve model fit. All models included random intercepts to account for repeated measurements by site. We also included sin and cosine functions with periodicities of one year and six months to model seasonal changes in species composition. Model fit was assessed via AIC and pseudo-R^2 values, and collinearity was assessed using variance inflation factor values. We used the final model to predict species composition, and then applied these estimates to monthly *An. gambiae* s.l. human landing catch counts from each sentinel site to estimate trends in *An. gambiae* density during the period.

Finally, we fit mixed effect Poisson models to evaluate the impacts of vector control and environmental variables on malaria incidence rates (IRR) in each site. We assessed the same vector control variables as described above and also assessed lagged temperature and rainfall. However, we classified permethrin susceptible by quartiles in order to more completely assess potential effects of increasing resistance levels. We compared Tmax and Tmin and selected Tmax based on improved AIC values. We modeled seasonality using sin and cosine functions with periodicities of one year and six months (Diggle & Giorgi, 2016), and included random intercepts for sites. We evaluated model fit based on AIC and pseudo-R^2 values.

5.2.7 Ethical considerations

This study was reviewed by the Rwanda National Ethics Committee and was determined to be exempt from IRB review because it relied on de-identified, routinely collected surveillance data. Data were provided under a data sharing agreement with the Rwanda Biomedical Center, which coordinated the collection of all data used in this study.

5.3 Results

5.3.1 Malaria trends and HLC results

Dramatic increases in malaria incidence were reported in each site during the study period. Annual reported incidence in Bukora increased 20-fold, from 113 cases per 1,000 in 2011 to 2,334 in 2015. Incidence in Mareba increased from 92 cases per 1,000 in 2011 to 1,008 in 2017. Incidence was lowest in Mimuli during 2010 at 212 cases per 1,000, and increased to 1,277 by 2016. Finally, incidence in Rukara increased 63-fold, from 21 cases per 1,000 in 2011 to 1,341 in 2018. Monthly incidence trends are shown in Figure 5-3.

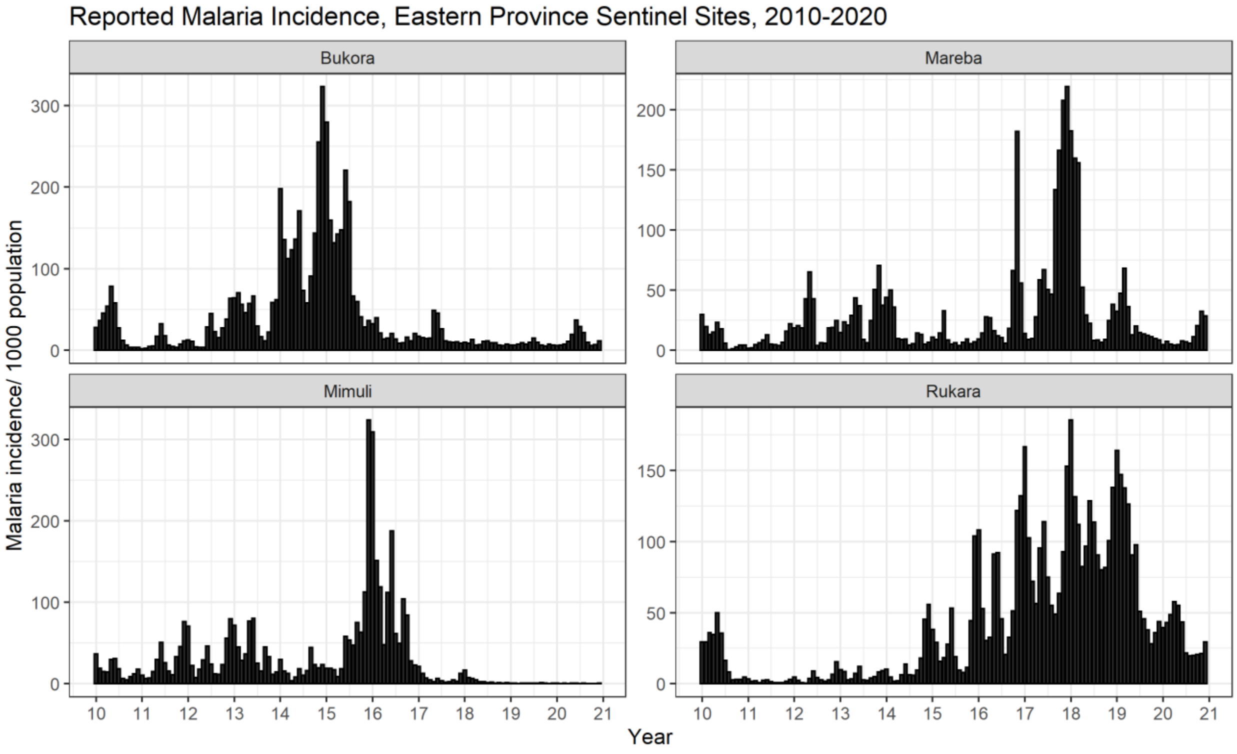

Figure 5-3: Monthly reported malaria incidence per 1000 population in four study sites

5.3.2 Objective 1: Comparing *An. gambiae* and *An. arabiensis*

We then compared the bionomics of *An. gambiae* and *An. arabiensis* from 2017 to 2020 in order to identify the most important species for malaria transmission in the region. Overall HBI was 80% in *An. gambiae*, which was four times higher than in *An. arabiensis* (20%). Because mosquitoes must bite at least twice in order to acquire malaria parasites and infect a new host, this means that *An. gambiae* vectorial capacity was 16 times higher than *An. arabiensis*, assuming other parameters such as longevity and gonotrophic cycle are similar (Garrett-Jones, 1964). *An. gambiae* was approximately 1.5 times more likely to bite indoors (66% vs. 40%).

Sporozoite rates were over 28 times higher in *An. gambiae* versus *An. arabiensis* (11.3% vs. 0.4%, respectively), whereas overall density was lower for *An. gambiae*, at 1.1 bpn compared to 3.0 bpn for *An. arabiensis* (Table 5-1). The overall annual entomological inoculation rate (EIR) for *An. gambiae* was 45 infective bites per person per year, compared to 4 for *An. arabiensis*.

Table 5-1. Human blood index, indoor biting, and sporozoite rates among An. gambiae complex, pre and post-IRS

		An. gambiae s.s.		*An. arabiensis*	
		Total tested	n (%)	Total tested	n (%)
Human Blood Index	Overall	15	12 (80%)	59	12 (20.3%)
	Pre-IRS	9	7 (77.8%)	9	1 (11.1%)
	IRS	6	5 (83.3%)	50	11 (22%)
Indoor biting*	Overall	125	82 (65.6%)	633	253 (39.97%)
	Pre-IRS	64	53 (82.8%)	49	33 (67.5%)
	IRS	61	29 (47.6%)	584	220 (37.7%)
Sporozoite Rate	Overall	151	17 (11.3%)	732	3 (0.4%)
	Pre-IRS	84	17 (20.2%)	65	2 (3.1%)
	IRS	67	0 (0%)	667	1 (0.2%)
		Total collected	Mean (SD)	Total collected	Mean (SD)
Density (bpn)	Overall	5622	1.1 (3.4)	15236	3.0 (5.88)
	Pre-IRS	3586	3.6 (6.1)	2963	3.0 (7.43)
	IRS	2036	0.5 (2.0)	12273	3.0 (5.5)

* Indoor biting compares proportions of mosquitoes caught during human landing catch indoors compared to outdoors. bpn = bites per person per night.

HBI was not strongly affected by IRS (Table 5-1, Figure 5-4). However, the proportion of mosquitoes that bit indoors was nearly 50% lower for both species after IRS compared to before IRS. Prior to IRS, sporozoite rates were 20.2% in *An. gambiae*, and were reduced to 0% after IRS. Sporozoite rates were also reduced for *An. arabiensis* from 3.1% to 0.2% after IRS, although only three *An. arabiensis* tested positive throughout the entire study period. Prior to IRS, *An. gambiae* exceeded *An. arabiensis* densities at 3.6 bpn compared to 3.0 bpn. IRS reduced *An. gambiae* densities 86% to 0.5 bpn, whereas they remained unchanged among *An. arabiensis* pre and post IRS. Prior to IRS, EIR for *An. gambiae* was 265 infective bites/person/year, whereas it was 34 for An. arabiensis. Following IRS, EIR was 0 for *An. gambiae* and 2 for *An. arabiensis*.

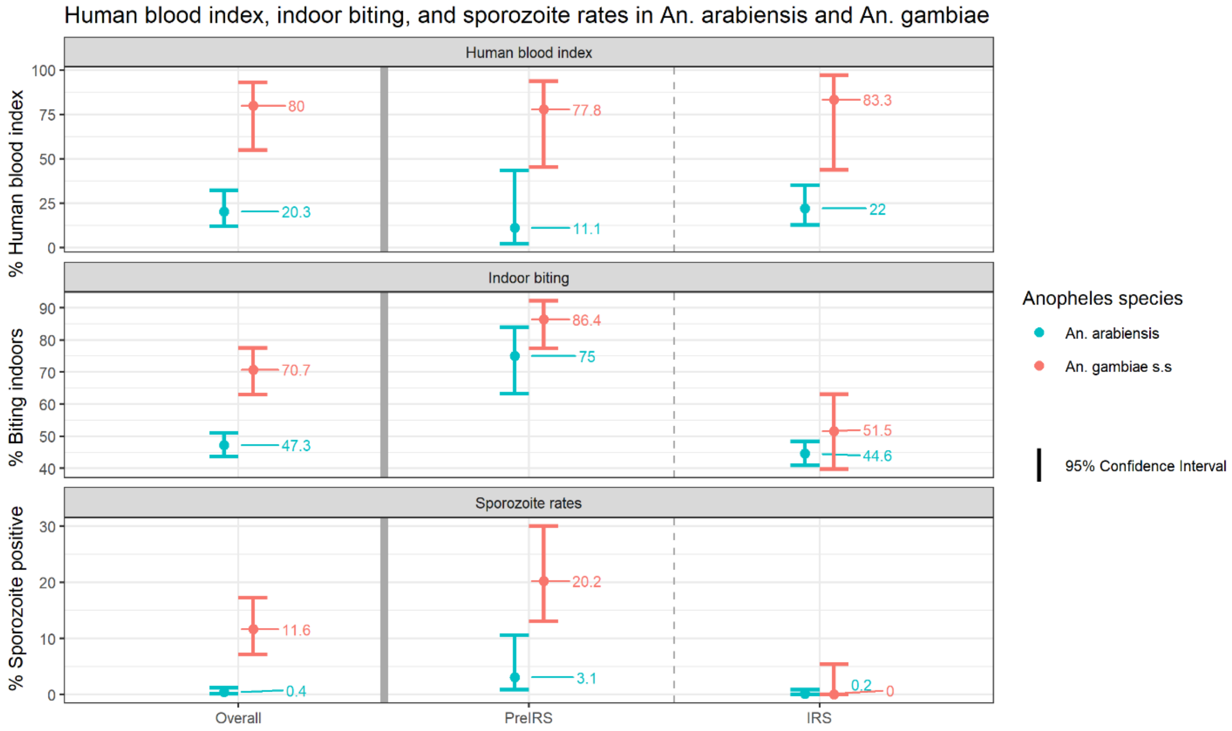

Figure 5-4: Human blood index, indoor biting rates, and sporozoite rates in *An. arabiensis* and *An. gambiae* from 2017. Overall estimates are shown on the left and estimates prior to and post IRS implementation are shown on the center and right, respectively.

An. gambiae was virtually absent in Bukora and Mimuli from 2017 – 2020 (Figure 5-5), which corresponded to a period of low malaria incidence in both sites (see Figure 5-3). Both of these sites had received pirimiphos-methyl IRS since 2016. On the contrary, *An. gambiae* was the dominant species in Mareba and Rukara in 2017, both of which were experiencing their highest peaks in malaria incidence. *An. gambiae* populations dropped almost immediately following the first pirimiphos-methyl IRS campaign in Mareba in February 2018, but persisted in Rukara until 2019 when Fludora®Fusion IRS was implemented. In contrast, *An. arabiensis* densities remained the same or increased after IRS, but no concurrent resurgence in malaria was observed.

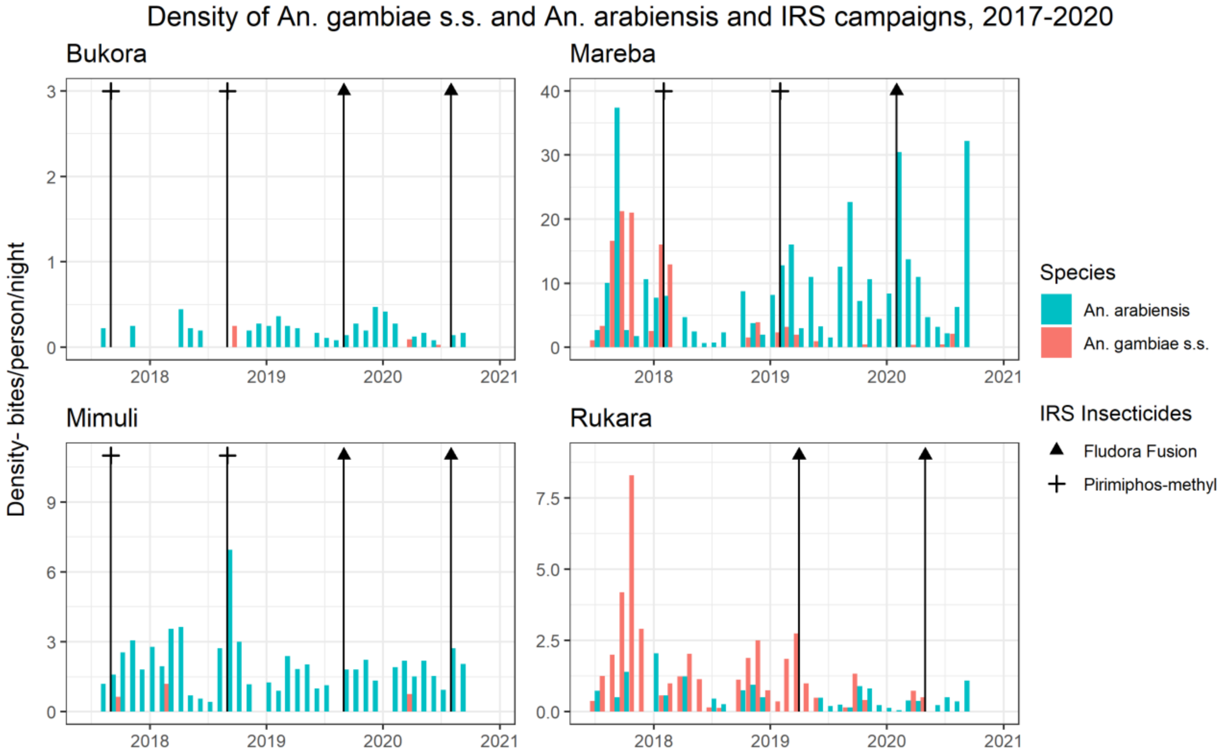

Figure 5-5: Density (bites per person per night) of *An. gambiae* and *An. arabiensis* from 2017 to 2018. Densities are plotted in relation to implementation of IRS campaigns in each site. Both insecticides used during this period were expected to have 11-month residual action.

5.3.3 Objective 2a: Changes in species composition from 2010 to 2020

We then modeled changes in species composition as a function of IRS coverage, ITN coverage, and permethrin resistance. In adjusted models, IRS coverage with effective insecticides was associated with an 86% lower prevalence rate of *An. gambiae* compared to *An. arabiensis* (PR = 0.14, 95% CI: 0.05 – 0.36). Each standard deviation increase in ITN coverage was also associated with dramatically lower *An. gambiae* prevalence rates. However, increased permethrin resistance dramatically increased *An. gambiae* relative abundance and modified the effect of ITNs, indicating a declining protective effect of bed nets in the presence of high resistance levels. Rainfall also was positively associated with *An. gambiae* relative abundance, which suggests the species may exploit rainfed larval habitats more readily than *An. arabiensis* (Table 5-2).

Table 5-2: Effects of vector control, insecticide resistance, and rainfall on relative abundance of *An. gambiae* s.s. compared to *An. arabiensis*

	PR (95% CL)	P-value
IRS*	0.14 (0.05, 0.36)	<0.001
ITN use[ǂ]	0.13 (0.04, 0.44)	<0.001
Permethrin resistance	9.08 (4.76, 17.34)	<0.001
Lagged monthly rainfall[ǂ], mm	1.58 (1.24, 2.01)	<0.001
Interaction: ITN * Permethrin resistance	5.11 (1.6, 16.29)	0.0058

PR = Prevalence ratio*IRS defined as coverage of IRS where insecticide susceptibility was >80%. [ǂ] continuous variables were scaled to standard deviations. Permethrin resistance defined dichotomously as susceptibility <80%

Model predictions of *An. gambiae* relative abundance are shown in Figure 5-6. ITNs and pyrethroid IRS appeared highly effective in suppressing *An. gambiae* abundance in all sites in 2010 and 2011 when universal ITN coverage was first implemented in Rwanda. However, *An. gambiae* quickly reemerged as a dominant species in Mimuli, which was the first site to develop high rates of pyrethroid resistance. Relative abundance then increased steadily in Mareba and Bukora, but was suppressed in 2014 and 2015 following bendiocarb IRS campaigns in both sites. It spiked again during brief gaps in IRS coverage in Mimuli and Mareba in 2016 and 2017, respectively, which corresponded with peaks in incidence in both sites (see Figure 5-3). It reemerged the latest in Rukara where pyrethroid susceptibility persisted the longest, but then remained dominant until the 2019 IRS campaigns.

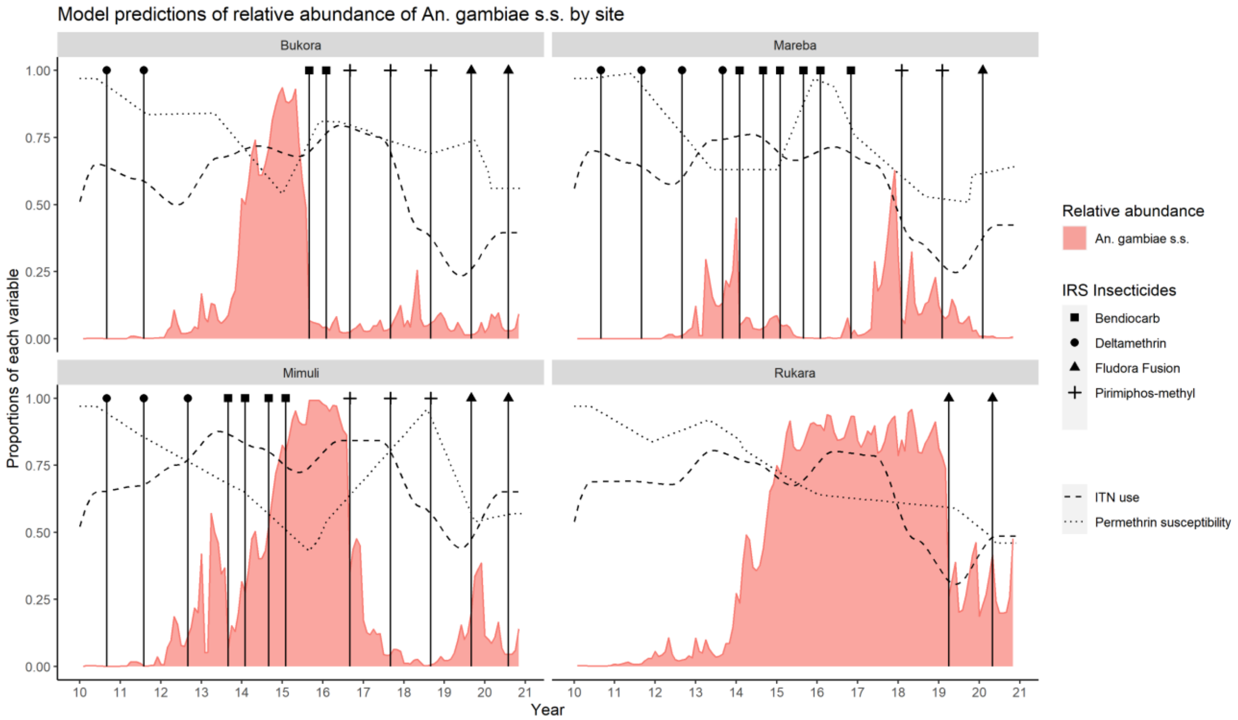

Figure 5-6: Model predictions of *An. gambiae* abundance relative to *An. arabiensis* during study period. Relative abundance is shown as a proportion of the overall *An.gambiae* s.l. species complex. IRS campaigns are plotted with vertical lines. The bold dashed line shows ITN use, whereas the fined dotted line shows permethrin susceptibility.

We applied the species composition predictions to the field-measured *An. gambiae* s.l. human landing catch counts in order to characterize trends in *An. gambiae* density over time (Figure 5-7). *An. gambiae* densities showed pronounced spikes which corresponded almost identically with the periods of high malaria transmission in each site. Model predictions also showed high densities in Mimuli early in the period. Although reported incidence was lower at that point than during the most pronounced peak in 2016, HMIS records indicate that the district where Mimuli is located accounted for 42% of all cases in the country in 2011 (Hakizimana et al., 2016). It is possible that early reemergence of *An. gambiae* was responsible for high transmission throughout the district during that period.

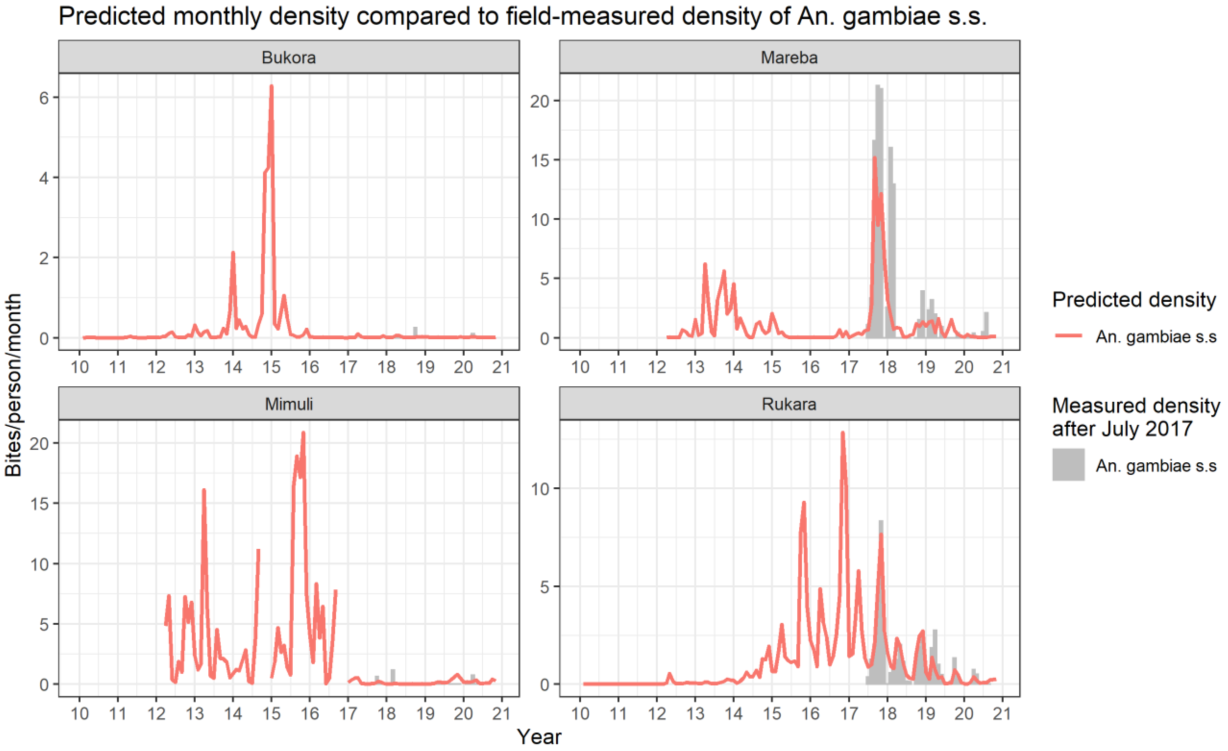

Figure 5-7: Model predictions of *An. gambiae* density during study period. Model predictions are shown with the red line, whereas empirical measurements from July 2017 are shown in grey bars. Gaps in predicted density in Mareba and Mimuli are due to missing data.

5.3.4 Objective 2b: Causes of increased malaria incidence

Finally, we examined the impacts of the previous vector control variables as well as temperature and rainfall on reported malaria incidence over the period. After controlling for other covariates, IRS was associated with a 75% reduction in malaria incidence rates. ITNs were also highly protective. However, each quartile increase in pyrethroid resistance was associated with substantial increases in incidence, and resistance significantly moderated the protective effect of ITNs. Each 1 degree increase in one-month lagged maximum temperatures was associated with an 23% increase in malaria incidence rates (Table 5-3). Annual maximum temperatures increased 2.3°C between 2011 and 2016 (Supplemental Figure B-4), which would have contributed to a 61% increase in malaria incidence, controlling for other variables.

Table 5-3: Adjusted effects of vector control, insecticide resistance, temperature and rainfall on malaria incidence in study sites

	IRR (95% CL)	P-value
IRS*	0.25 (0.25, 0.25)	<0.001
ITN use[ǂ]	0.47 (0.46, 0.49)	<0.001
Permethrin resistance, Q2	1.4 (1.38, 1.42)	<0.001
Permethrin resistance, Q3	2.74 (2.7, 2.78)	<0.001
Permethrin resistance, Q4	5.94 (5.86, 6.02)	<0.001
Interaction: ITN use * Resistance Q2	2.87 (2.78, 2.96)	<0.001
Interaction: ITN use * Resistance Q3	2.55 (2.47, 2.63)	<0.001
Interaction: ITN use * Resistance Q4	1.94 (1.88, 2)	<0.001
Lagged monthly maximum temperature[ǂ], °C	1.23 (1.23, 1.24)	<0.001
Lagged monthly rainfall[ǂ], mm	1.15 (1.14, 1.15)	<0.001

IRR = incidence rate ratio; *IRS defined as coverage of IRS where insecticide susceptibility was >90%; ǂ continuous variables were scaled to standard deviations with the exception of temperature. Permethrin resistance levels defined according to quartiles of susceptibility: Q1 = 85.1 - 100%, Q2 = 72.1 - 85%, Q3 = 62.1 - 72%, Q4 = 43 - 62%. Q1 (low resistance) was considered the reference group

Finally, we compared model predictions of malaria incidence with actual reported incidence (Figure 5-8). The trends were nearly identical, indicating these factors explained the vast majority of the observed malaria trends (marginal $R^2 = 0.94$).

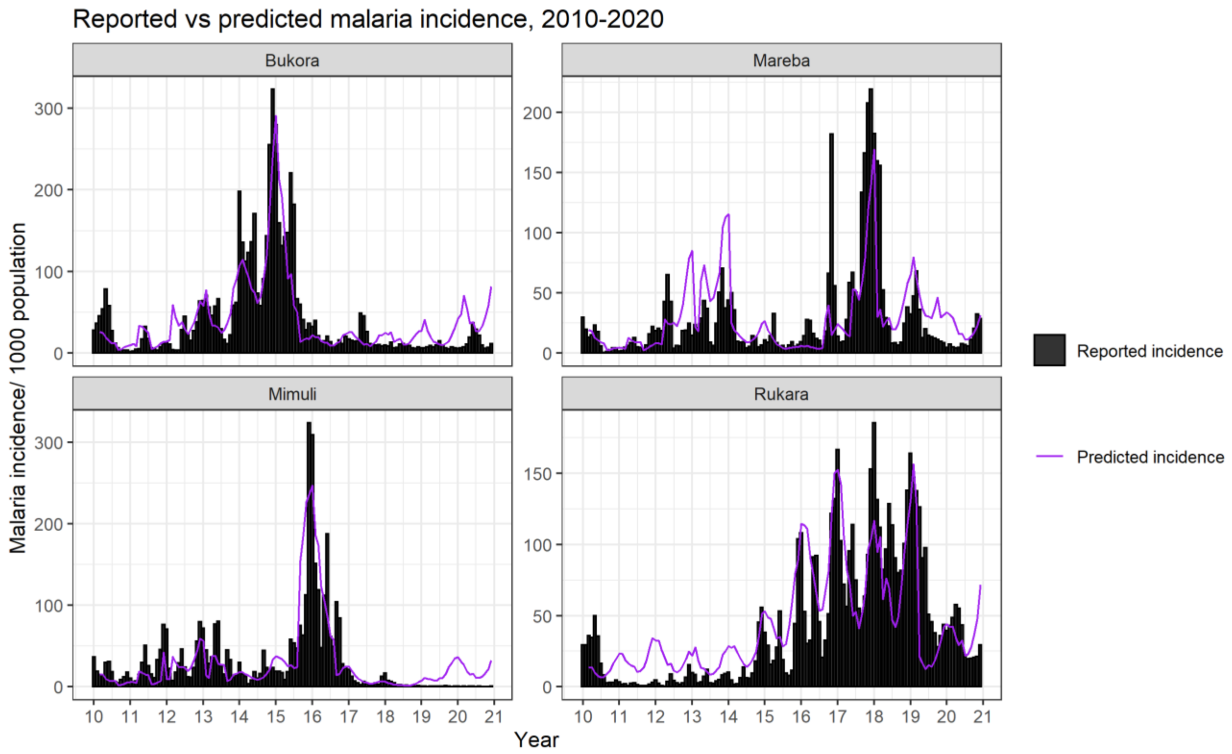

Figure 5-8: Reported malaria incidence compared to model predictions from 2010 to 2020. Model predictions are shown with the purple line, whereas health-facility reported incidence is shown with black bars.

5.4 Discussion

We retrospectively assessed trends in species composition and causes for major outbreaks that occurred at each site from 2010 to 2020. While pyrethroid IRS and ITNs were associated with reductions in *An. gambiae* relative abundance early in the decade, pyrethroid resistance and gaps in IRS coverage led to rapid reemergence of *An. gambiae*. Similarly, the protective effects of ITNs on malaria incidence were progressively reduced with increasing levels of resistance. Reported incidence followed almost identical patterns to *An. gambiae* densities in each site, indicating that the malaria resurgence was primarily driven by the reemergence of *An. gambiae* after prior control with ITNs and IRS. A dramatic increase in maximum temperatures from 2015 to 2017 magnified transmission in sites where *An. gambiae* had reemerged. However, implementation of non-pyrethroid IRS appeared extraordinarily effective in reducing *An. gambiae* abundance and controlling malaria transmission.

These findings indicate that *An. gambiae* was clearly the dominant vector in this area. Sporozoite rates among *An. gambiae* were 28 times higher than *An. arabiensis*, and it was four times more likely to have fed on humans. Prior to IRS, entomologic inoculation rates were 234 in *An. gambiae*, which is similar to levels reported in holoendemic areas in East Africa prior to the era of universal ITN coverage (Beach et al., 1993; Beier et al., 1990; Hay et al., 2000) and to one site in southwest Rwanda in the early 2010s (Hakizimana et al., 2018). Numerous studies in East Africa have described *An. gambiae* and/or *An. funestus* as dominant vectors compared to *An. arabiensis* (Beier et al., 1990; McCann et al., 2014; Okello et al., 2006; Taylor et al., 1990). However, this was the first study to assess the relative importance of the two *An. gambiae* s.l. sister species to malaria transmission in Rwanda. As has been observed elsewhere (Abong'o et al., 2020), the relative proportion of *An. arabiensis* rose following IRS, but malaria incidence and sporozoite rates declined.

Although outdoor biting increased in both species following IRS, cases declined precipitously as IRS reduced *An. gambiae* densities, indoor biting, and sporozoite rates. This indicates that indoor IRS with non-pyrethroid insecticides remains highly effective for malaria control in this setting, primarily by controlling indoor-biting *An. gambiae*. Previous studies have found that indoor biting is likely the main driver of residual malaria transmission despite high ITN coverage (Bayoh et al., 2014; Huho et al., 2013).

We observed a strong effect of pyrethroid resistance on malaria incidence, which supports previous NMCP technical reports that noted correlations between the increased resistance levels and increased malaria incidence from 2011 to 2016 (RBC, 2016). Although resistance has been implicated as a primary driver of malaria resurgence elsewhere, other multi-site cohort studies found no effect of resistance on malaria incidence (Kleinschmidt et al., 2018; Lindblade et al., 2015; Ochomo et al., 2017). A key issue with these studies is that standard bioassays do not directly assess the functional consequences of resistance or what resistance thresholds that could actually lead to control failure (Grossman et al., 2020). Unmeasured functional resistance characteristics could determine whether highly efficient vectors reemerge or remain controlled after developing resistance. For example, *An. funestus* and *An. gambiae* s.l. densities remained low in Malawi despite high levels of resistance (Lindblade et al., 2015; Wondji et al., 2012). In contrast, *An. funestus* was fully eliminated in northern South Africa in the 1950s but reemerged in the 1990s due to high insecticide resistance and likely contributed to a major malaria resurgence in the area (Hargreaves et al., 2000).

Differences in resistance mechanisms may also be related to divergent conclusions about the importance of resistance. Numerous studies have demonstrated that piperonyl butoxide (PBO) treated ITNs restore the effectiveness of pyrethroid ITNs and reduce malaria incidence compared to standard ITNs in settings with highly resistance vector populations (Gleave et al., 2021). This provides indirect evidence that metabolic resistance is leading to control failures. Metabolic resistance has been confirmed in *Anopheles gambiae* s.l. populations in our study area (Hakizimana et al., 2016). In contrast, other studies in the region have found no associations with target site resistance and control failure (Mathias et al., 2011).

A similar trend to that reported in this study appears to have taken place in western Kenya over the last twenty years. Prior to widespread ITN coverage, *An. gambiae* and *An. funestus* were the dominant vectors, and *An. gambiae* constituted >85% of the *An. gambiae* complex. An ITN trial in the 1990s followed by universal ITN campaigns in the mid-2000s effectively controlled both species, leading to major decreases in malaria burden (Bayoh et al., 2010; Gimnig et al., 2003; Lindblade et al., 2006). By 2009 *An. gambiae* accounted for only 1.2% of the species complex and both *An. gambiae* and *An. funestus* were rare relative to *An. arabiensis* (Bayoh et al., 2010; McCann et al., 2014).

However, malaria incidence and mortality in western Kenya began to resurge in the late 2000s (Hamel et al., 2011). A study of two sites which experienced strong increases in malaria incidence found that *An. gambiae* and *An. funestus* densities increased 5 to 10-fold in the same period (Zhou et al., 2011). A follow-up study in the same sites found high pyrethroid resistance among *An. gambiae* s.l. in the site which had the largest malaria resurgence (Kapesa et al., 2017). The authors also reported a 2°C increase in mean air temperatures from 2011-2015, which is nearly equivalent to what we observed in our study setting. A later study linked pyrethroid resistance to the reemergence of *An. funestus* and increased malaria incidence in the region. Additionally, sporozoite rates were nearly twice as high in *An. gambiae* compared to *An. funestus* and both species fed almost exclusively on humans, although *An. funestus* densities were higher (McCann et al., 2014). It is therefore plausible that the reemergence of both species may have driven increased malaria transmission in the area.

Another study in Uganda reported the reemergence of *An. gambiae* in two sites shortly after LLIN distributions, whereas it was fully controlled in a third site following bendiocarb IRS campaigns (Mawejje et al., 2021). Reported malaria also increased at the two sites which saw increased *An. gambiae* densities, and declined precipitously in the site which received IRS (Katureebe et al., 2016). In northern Uganda, the discontinuation of bendiocarb IRS in 2014 led to a 9-fold increase in malaria prevalence (Nuwa et al., 2022; Raouf et al., 2017). However, the increase was almost completely reversed following the reimplementation of IRS with pirimiphos-methyl in 2017 (Namuganga et al., 2021). In western Kenya where *An. funestus* had reemerged, pirimiphos-methyl IRS campaigns in 2017 dramatically reduced *An. funestus* and led to 44 - 65% reductions in health facility-reported malaria cases (Abong'o et al., 2020). These trends are strikingly similar to our study site, where *An. gambiae* density and malaria incidence surged in the mid-2010s but were controlled following pirimiphos-methyl and clothianidin IRS campaigns.

We observed a sustained increase in ambient air temperatures during the study period, which was associated with a >60% increase in malaria incidence rates. Similar effects have been observed previously in the 1980s in Rwanda when a pronounced period of warming led to a 3 to 5-fold increase in incidence (Loevinsohn, 1994). Incidence increased the most in higher altitude areas, which has also been observed elsewhere in the East African highlands (Pascual et al., 2006; Siraj, Santos-Vega, Bouma, Yadeta, Ruiz Carrascal, et al., 2014; Zhou et al., 2004b). These findings have implications for the effects of climate change in East Africa, which is considered especially vulnerable to climate-driven increases in transmission suitability (Alonso et al., 2011; Caminade et al., 2014; Githeko & Ndegwa, 2001; Lindsay & Martens, 1998; Ryan et al., 2020; Ryan et al., 2015). Temperatures in East Africa in the last thirty years have increased faster than the global average (Gebrechorkos et al., 2019; Ngarukiyimana et al., 2021). In addition to long-term trends, dramatic spikes in minimum and maximum temperatures were observed between 2014 and 2017 (Harris et al., 2020). WHO malaria incidence estimates showed substantial increases in Kenya, Uganda, Tanzania, and Burundi in addition to Rwanda during the same time period (WHO, 2017b, 2022). However, *An. gambiae* density and malaria incidence in our study area remained low in sites with non-pyrethroid IRS coverage, despite elevated temperatures. This indicates a potentially important role of vector control in mitigating vulnerability to climate-related malaria resurgences.

One limitation of this study was our inability to quantify other components of net integrity besides ITN use. A previous study by Hakizimana and colleagues showed that approximately 50% of ITNs in this setting deteriorate to the point of being ineffective in two years (Hakizimana et al., 2014). Furthermore, there were reports that substandard nets with reduced insecticidal content were distributed in 2013 and 2016 (Karema et al., 2020). Substandard nets could have had lower bioefficacy against host-seeking mosquitoes (Vinit et al., 2020), or could have accelerated the development of resistance due to delivery of sub-lethal pyrethroid doses. These trends likely occurred colinearly with increased resistance and would require more detailed data to investigate. It is also possible that increased insecticide resistance exacerbated the effect of net deterioration. For example, in western Kenya, Ochomo and colleagues found that deteriorated nets remained effective against susceptible mosquitoes, but did not prevent entry or feeding by pyrethroid resistant mosquitoes (Ochomo et al., 2013).

There is relatively little historical data on *An. gambiae* s.l. species composition in Rwanda prior to the scale-up of ITNs and IRS. We relied on two reports which demonstrated that *An. gambiae* was the dominant species (94 - 99% of all *An. gambiae* s.l. samples) in 2007 - 2008. Although we cannot confirm this was the case for our sites, other work from East Africa showed that *An. gambiae* was the dominant species in the *gambiae* complex prior to widespread vector control (Bayoh et al., 2010). Species replacement of *An. gambiae* by *An. arabiensis* in Rwanda has been documented following ITN distributions (Hakizimana et al., 2018), and more recently in a technical report following IRS campaigns (Niyituma et al., 2019). However, our study was the first to describe the reemergence of *An. gambiae* in Rwanda after prior control.

Another limitation of our study was our reliance on reported case data, which may not fully reflect malaria transmission trends. However, the tight relationship between *An. gambiae* density and observed incidence suggests that reported cases at least captured overall trends if not the full magnitude of transmission. Finally, we pooled the field HLC samples by sentinel site because all laboratory analyses were pooled by site. Villages where HLC was conducted were all within 5km of their corresponding sentinel site, and vector control interventions were conducted at a district level. It is possible though that *An. gambiae* s.l. species composition is spatially heterogenous at a smaller scale than what we were able to observe. Finer scale entomological studies are needed to investigate this possibility.

These findings indicate the need for new sustainable interventions in addition to existing vector control measures, which are challenged by insecticide resistance and short gaps in coverage. The Rwanda NMCP has invested in a number of measures such larval control with *Bacillus thuringiensis var israelensis* (Bti), stocking larvivorous fish in permanent breeding sites, and community engagement for environmental management and larval control (Hakizimana et al., 2018; Ingabire et al., 2017; Murindahabi et al., 2018; RBC, 2020b). Further characterization of the larval ecology of *An. gambiae* could enable more precise targeting of larval source reduction measures. The NMCP has also implemented an insecticide resistance management policy in which IRS insecticides are rotated every two years (RBC, 2020a). This strategy may have contributed to the success of non-pyrethroid IRS campaigns by mitigating carbamate and organophosphate resistance. With support from the Global Fund and the President's Malaria Initiative, the NMCP distributed PBO-treated ITNs in five districts in 2020 (PMI, 2022b). This could restore insecticidal activity against pyrethroid-resistant vectors (S. G. Staedke et al., 2020). Evaluation efforts should explore species-specific effects of the intervention and resistance profiles. The potential agonistic effects of pirimiphos-methyl and PBO should also be considered in sites where IRS and next generation ITNs are delivered together (Syme et al., 2021).

5.5 Conclusions:

Malaria control in this region of Rwanda is fragile. Insecticide resistance increased dramatically over just two to three years, and malaria transmission spiked rapidly with declining ITN effectiveness and gaps in IRS effectiveness. The region is also vulnerable to climate-driven warming, especially in instances where control measures are less effective or interrupted. However, the success of non-pyrethroid IRS suggests that existing indoor control measures remain highly effective, and can mitigate vector reemergence and climate-driven increases in transmission. Surveillance efforts should focus on *An. gambiae* as a key vector, and further innovative interventions should be targeted and evaluated specifically for their effects on this species.

6 Summary, reflections, and directions for future research

6.1 Summary of findings

Smoke has been used as an insect repellent for centuries (Torr et al., 2011), and numerous experimental and observational studies have shown that smoke from biomass combustion repels mosquitoes and flies. This has led to a concern that the adoption of cleaner cooking fuels such as liquid petroleum gas could create an environment that is more favorable for mosquitoes and flies and potentially increase exposure to vector-borne diseases (Biran et al., 2007; Yamamoto et al., 2011). However, no prior studies have directly compared the effects of traditional and cleaner fuels on *Anopheles* mosquito behavior. This information is critical for assessing the potential impacts of clean cooking interventions and promoting the delivery of additional vector control interventions in tandem with clean fuels if necessary.

We used a semi-field experimental design to assess the effects of three common cooking fuels on multiple parameters of *Anopheles* mosquito behavior, including household entry, host-seeking, household entry, and mortality. Cooking with LPG fuels was associated with increased household entry and host-seeking compared to cooking with wood or charcoal, and to baseline conditions where no fuels were used. Mosquito mortality was reduced in LPG huts compared to those that cooked with wood or charcoal. $PM_{2.5}$ and temperature were elevated in charcoal and wood-burning huts compared to LPG, and showed inverse associations with mosquito household entry and host-seeking.

These findings demonstrate that, at least in highly controlled conditions, the adoption of cleaner fuels could reduce or reverse repellent and deterrent effects from biomass fuels, potentially altering human exposure to *Anopheles* mosquitoes and malaria parasites or other *Anopheles*-transmitted pathogens. Although prior studies have suggested that reductions in indoor mosquito collection in wood-burning houses were due to increased exophily rather than actual reductions in host-seeking (Bockarie et al., 1994), we found that biomass fuels deter both entrance and host-seeking. Furthermore, the differences were pronounced during the entire collection period (6pm – 6am), rather than just during cooking, meaning the repellent effects of biomass combustion endure after the fuels themselves are extinguished. Household entry and host-seeking were also elevated in LPG huts compared to baseline conditions where no fuels were used, indicating a potential attractant effect of LPG fuels. Although more research is needed to investigate this finding, it is potentially linked to high CO_2 emissions from LPG stoves and higher relative humidity levels, both of which attract host-seeking mosquitoes.

These findings demonstrate the biological plausibility that changes in human exposure to important disease vectors could occur following adoption of cleaner cooking fuels. However, the study was conducted with one species of lab-reared mosquitoes, and it is unknown whether field populations or different species would react differently. Furthermore, $PM_{2.5}$ levels may have been higher in the small, poorly ventilated experimental huts compared to typical field conditions. Kitchens are often located in a separate structure or outdoors, and it is possible that biomass emissions in outdoor kitchens would be irrelevant to indoor-biting vectors in these scenarios. Finally, incidence of malaria is typically not linearly related to vector density. Other host-factors such as immune function could be improved by household air pollution interventions and reduce infectious disease burden independently of changes in vector density. We therefore concluded that additional entomological and epidemiological studies were needed to evaluate the real-world impact of clean fuel adoption on insect vectors and the disease they transmit.

Our second aim consisted of a randomized controlled trial to assess the impacts of cleaner fuel adoption on mosquitoes and flies in houses in Eastern Rwanda which traditionally rely on biomass fuels for cooking. This was the first study explicitly designed to investigate the effects of LPG adoption on the bionomics of important disease vectors in field conditions. We also assessed longitudinal prevalence of reported malaria and diarrhea as secondary outcomes. This information remains essential for understanding and, if necessary, mitigating potential risks associated with cleaner fuel adoption.

We found that *Anopheles* mosquito densities were slightly elevated in intervention houses compared to control houses, although the differences were not statistically significant. We also found no differences in *Plasmodium falciparum* sporozoite rates in mosquitoes and no change in bloodmeal composition between intervention and control houses. Furthermore, reported malaria incidence was lower among children in the intervention arm than the control arm, although the effect estimates were imprecise and not statistically significant. It is possible that low overall vector densities reduced our study power to detect true differences. Nevertheless, the overall coherence of these findings suggests that this intervention did not significantly increase exposure to *Anopheles* mosquitoes or malaria incidence in this context. Housing quality, environmental characteristics, and vector control efforts were more important determinants of vector density and malaria risk in this real-world setting. We also found no differences in culicine densities or bloodmeal composition between study groups, which lends support to this conclusion.

Cooking inside the main house was rare among biomass users in this study, and it is possible that indoor-biting mosquitoes were largely unaffected by $PM_{2.5}$ or other repellent components of fuel emissions in houses which cooked outside. Indeed, we observed some evidence for effect modification by cooking location; mosquito densities were more than three times higher in intervention houses compared to control houses which cooked indoors, although the effect estimates were imprecise and not statistically significant. We were unable to directly assess the effects of $PM_{2.5}$ on vector density in these houses due to limited observations in the control arm. Further studies in areas with higher rates of biomass cooking inside the main home could help shed light on potential effects of clean fuel adoption in these settings. This effect may also be important for other vector species. For example, *Aedes aegypti* typically lives in houses and bites during the day, including the mornings and early evenings (Captain-Esoah et al., 2020; Madewell et al., 2020; Reinhold et al., 2018). Cooking often occurs at the same time, and changes in fuel emissions could therefore be more relevant for this species.

In contrast, the intervention was associated with a 61% reduction in densities of synanthropic flies. This finding was unexpected as woodsmoke is often used to repel flies (Torr et al., 2011), but it appeared to be primarily driven by increased rates of indoor cooking in the intervention houses. Indoor kitchens were further from latrines, rubbish piles, and animal feces in the compound, all of which are important sources of flies (S. W. Lindsay et al., 2012; T. Lindsay et al., 2012; Sharma & Jain, 2019; Wolfe et al., 2017).

Exterior household walls may also physically prevent fly entry. Reductions in flies could convey important health benefits. Synanthropic flies mechanically vector numerous enteric pathogens including *Shigella, E. coli,* and *Salmonella* (Greenberg, 1973). Previous studies have reported 22 – 26% reductions in child diarrhea following successful fly control interventions (Chavasse et al., 1999; Emerson et al., 1999).

We did not observe a significant effect on reported diarrhea in our study population. We are hesitant to draw conclusions about this finding, as our study was not powered to detect small changes in diarrhea prevalence. However, it is possible that reductions in flies were not adequate to reduce food contamination by flies, or that reductions in flies may cause pathogen-specific reductions in diarrheal etiology which are not observable when considering all-cause reported diarrhea as an outcome. It is also possible that fly densities in cooking locations do not adequately reflect overall contamination by flies throughout the household and community. Other benefits of reduced household air pollution such as improved immunity or gut health could reduce diarrhea independently of its effect on flies (Gordon et al., 2014; Heft-Neal et al., 2018; Lee et al., 2015; Marynowski et al., 2015). Additional studies in this population could illuminate pathogen-specific effects of the intervention and potential changes in upstream risk factors for diarrhea which may be unrelated to flies.

Finally, we conducted a retrospective entomological and epidemiological study to investigate the causes of a major malaria resurgence in eastern Rwanda in the last decade. We first assessed the importance of the two most common malaria vectors, *An. gambiae* and *An. arabiensis*, using detailed entomological data from four sentinel sites. We then assessed the impacts of vector control failures and environmental changes on species composition and reported malaria incidence in the same sites from 2010 to 2020. Although numerous hypotheses have been posited to explain the major malaria resurgence, this was the first scientific study to evaluate the role of each of these factors. It was also the first study to compare the importance of the two dominant malaria vectors in malaria transmission in the study area. Such information is essential for informing effective malaria control strategies and predicting and preventing future malaria outbreaks and epidemics.

Although *An. arabiensis* is common in the study area, *An. gambiae* was clearly the dominant vector. More than 80% of bloodfed *An. gambiae* had fed on humans, compared to 20% of *An. arabiensis*. Because a mosquito must feed at least twice in order to acquire and transmit malaria parasites, this means that *An. gambiae* is 16 times more likely to transmit malaria, controlling for other parameters of vectorial capacity such as longevity and gonotrophic cycle. Overall sporozoite rates were 28 times higher in *An. gambiae* compared to *An. arabiensis. An. gambiae* densities were also slightly higher prior to IRS. Although many other studies in East Africa have also reported that *An. gambiae* is a more important vector than *An. arabiensis* (Beier et al., 1990; McCann et al., 2014; Okello et al., 2006; Taylor et al., 1990), this is the first study of its kind to do so in Rwanda. Additional studies should evaluate the role of *An. gambiae* in other parts of the country. If these findings are confirmed, control efforts should be targeted and evaluated specifically for their effects on this species.

We then retrospectively assessed trends in species composition and malaria transmission since 2010. We found that the scale-up of universal ITN coverage was associated with major declines in *An. gambiae* and concurrent reductions in malaria early in the decade. However, pyrethroid resistance increased quickly from 2011 to 2013, leading to the reemergence of *An. gambiae* as a dominant vector. IRS control with bendiocarbs mitigated its reemergence in some sites, but small gaps in coverage led to major spikes. Malaria outbreaks followed almost identical patterns, highlighting the important role of *An. gambiae* reemergence during this period. Finally, a >2°C increase in ambient air temperatures appeared to increase incidence during this period, likely by magnifying the vectorial capacity of *An. gambiae* and malaria parasite development rates (Mordecai et al., 2019). However, the initiation of IRS campaigns with long-lasting insecticides effectively controlled *An. gambiae,* and malaria incidence declined concurrently in the latter part of the study period.

It is very likely that similar trends took place in other countries in the region during the time period. For example, long-term studies in western Kenya implicated pyrethroid resistance in the reemergence of *An. funestus* and resurgent malaria transmission following universal ITN scale-up (McCann et al., 2014).

Additionally, dramatic increases in ambient air temperature were recorded throughout the region from 2014 to 2017, and WHO reported increased incidence in Kenya, Uganda, Tanzania, and Burundi in addition to Rwanda during the same time period (WHO, 2017b, 2018b). Long-term warming trends in the region have also exceeded the global average in the last thirty years (Gebrechorkos et al., 2019; Ngarukiyimana et al., 2021). However, few other studies have assessed the simultaneous impact of insecticide resistance, gaps in vector control, and short and long-term warming in the reemergence of a previously controlled vector species and resurgent malaria transmission.

The findings in our study and those from others in East Africa suggest that: 1) malaria control in the region is highly vulnerable to insecticide resistance and to the reemergence of previously controlled vector species, and 2) short and long-term climate warming may magnify these trends in the absence of additional control measures. However, these trends have not been adequately investigated in the region, and many control programs remain reactive rather than proactive in dealing with resurgences. Multi-site studies are needed to compare entomological and epidemiological dynamics of malaria transmission across the region following the advent of universal ITN campaigns. Such efforts should pay special attention to factors involved in the reemergence of the two most important vectors, *An. gambiae* and *An. funestus*, including the role of insecticide resistance and declining ITN effectiveness. Similarly, larger scale retrospective studies are needed to assess the effects of regional warming on malaria transmission in the region. Although many models project that East Africa is highly vulnerable to climate-driven increase in transmission suitability, surprisingly few studies have assessed the impacts of warming in the last decade, which was the hottest in recorded history (Lindsey & Dahlman, 2021). Furthermore, studies rarely consider the combined effects of changes in vector control and environmental changes, meaning most models do not provide realistic estimates of climate-induced changes in malaria suitability (Rocklöv & Dubrow, 2020). Empirical evidence of the effects of short-term warming on malaria transmission in the context of high ITN coverage could enable more accurate predictions of malaria transmission dynamics under future climate scenarios.

A third conclusion from this research and other recent studies in the region (Abong'o et al., 2020; Namuganga et al., 2021) is that non-pyrethroid IRS remains highly effective. This is a reassuring finding for malaria control programs that largely rely on just two main interventions, IRS and ITNs, for the vast majority of vector control activities. Further multi-site studies could compare the factors that enable the success of these interventions, with particular focus on restoring control of reemergent vectors such as *An. funestus* and *An. gambiae*. On the other hand, the success of non-pyrethroid IRS campaigns provides indirect evidence that pyrethroid resistance is contributing to net failures more than has previously realized. Further research should critically assess the factors contributing to the decline of net effectiveness. Key questions include whether ITNs still provide individual protection even if they are not effectively killing mosquitoes, and whether insecticide resistance magnifies the effects of declining net integrity as has been observed elsewhere (Ochomo et al., 2017). The impact of substandard nets on accelerating resistance and declining net effectiveness should also be further assessed (Vinit et al., 2020).

Numerous studies have observed increased exophilic behavior of malaria vectors following ITN and IRS scale-up (Cooke et al., 2015; Kreppel et al., 2020; Sherrard-Smith et al., 2019). Such studies often recommend strong investments in outdoor mosquito control, insecticide-treated netting, spatial repellents, and traps. However, the success of non-pyrethroid IRS in our study and elsewhere in East Africa indicates that indoor interventions remain an important tool for malaria control in the region. Further studies should critically assess the importance of outdoor transmission while accounting for human behavioral patterns. For example, nightly outdoor human landing catch counts likely overestimate outdoor exposure in settings where the majority of the population sleeps indoors (Bayoh et al., 2014). Carefully designed intervention trials could assess the benefits and cost-effectiveness of outdoor interventions compared to traditional control measures in order to make more rigorous recommendations about the situations in which outdoor interventions are needed.

6.2 Reflections and directions for future research

Aim 1 was the product of the combined efforts of a wonderful team of colleagues and friends. These included my advisors, Dr. Tom Clasen and Dr. Uriel Kitron from Emory, Dr. Josh Rosenthal from the Fogarty Center of the National Institutes of Health, Dr. Miles Kirby from Harvard (previously at Emory), and Dr. Emmanuel Hakizimana, Xavier Misago and Jackie Mupfasoni from the Malaria and Other Parasitic Diseases Division (MOPDD) of the Rwanda Biomedical Center. I am grateful to this team, whose technical advice, day-to-day support, and friendship improved the study in too many ways to count. I am especially grateful to Xavier and Jackie, whose entomological expertise and dedication helped translate the study from an idea to reality. Long nights and early mornings with them at the experimental huts in Ruhuha were some of my favorite times during my PhD journey.

My contributions to the study included conceptualizing the study design, designing the mosquito traps, developing all data collection tools, training the study team, conducting the analyses, and drafting the manuscript. Xavier and Jackie trained the collectors, managed to coordinate an large team of tailors and welders to make 18 mosquito traps, and supervised the day-to-day implementation of the study and all data collection after I left Rwanda. Miles Kirby trained our team on the use of UCBs and PATS+, helped us procure equipment, and provided technical support for numerous other details of the study implementation. All study team members provided invaluable feedback on the study protocol and manuscript drafts.

There are a number of things I would do differently if I could repeat the study. We focused on indoor cooking because we hypothesized that mosquitoes would be most affected by indoor emissions from cooking fuels. However, we later learned that indoor cooking was relatively uncommon among biomass users in Eastern Province compared to other parts of Rwanda. In the future I would add another round of the study to assess the effects of cooking outdoors or near the house on household entry and indoor host-seeking. This study also reinforced the importance of piloting study methods prior to implementation. We assumed that the experimental huts would be representative of indoor cooking conditions, but the experimental huts were very small, and $PM_{2.5}$ levels were elevated compared to levels reported by many field-based studies. Additional pilot work prior to the study start could have enabled us to better calibrate the conditions to better mimic real-world cooking conditions. Finally, I hope to conduct a follow-up study with other species, particularly *Aedes aegypti*, which may be more affected by emissions from cooking fuels based on the timing and location of its biting behavior.

I am grateful to have had the support of the same study team for my second aim. Aim 2 also benefited enormously from the support of the HAPIN study staff, particularly Florien Ndagijimana, Ephrem Dusabimana, Jean de Dieu Ntivuguruzwa, Jean Uwizeyimana, and Dr. Ghislaine Rosa. They helped secure IRB approval, translated the research tools, and provided support with on-the-ground logistics. I am also grateful to Dr. Seth Irish from CDC who very generously gave me a crash course in synanthropic fly identification in return for a six-pack of IPAs. This study could not have taken place without the technical expertise of an incredibly talented team of MOPDD entomology officers and technicians, and I gratefully acknowledge their hard work. I am also grateful to the Clean Cooking Alliance for financially supporting this study and Aim 1.

My contributions to Aim 2 were to design all the data collection tools, develop the sampling procedures, train the staff, generate maps and schedules of all the study visits, procure study materials, and design and code the data collection tools. I conducted all data analysis and drafted the manuscript for publication. I am grateful Xavier Misago and Jackie Mupfasoni for supervising day-to-day activities, and to the entomology team for conducting all the entomological sampling, species identification, and lab work. I am grateful to the entire HAPIN team for their incredible work on the broader HAPIN trial, particularly during times when COVID prevented me from returning to the field, for providing access to their resources and study population, and for sharing baseline data as well as the reported malaria and diarrhea outcomes. All members of the study team provided invaluable feedback and advice on the study protocol, day-to-day implementation challenges, and manuscript drafts.

This study highlighted the challenges in establishing causation, even in the context of a randomized controlled trial. For example, were differences in fly densities due to changes in cooking location or changes in fuels? Similarly, were the lack of differences we observed in diarrhea outcomes due to low study power, a lack of effect of flies on diarrhea, pathogen-specificity in the role of flies as vectors, or an insufficient reduction in flies in order to observe a true effect? These questions strengthened my interest in causal reasoning in epidemiology and pushed me to seek additional training in causal mediation analysis. It also underscored the importance of triangulating multiple studies with different study designs and disciplines in order to improve inference. I find Dr. Raina Plowright's discussion of triangulation for causal inference in disease ecology particularly insightful in this respect (Plowright et al., 2008).

This study also highlighted the limitations of vector counts as a proxy for exposure to vector-borne diseases. For example, we observed slightly higher densities of *Anopheles* mosquitoes in study houses, but malaria rates were not significantly different and actually appeared lower in the intervention participants. This is in agreement with the well-known phenomenon where higher mosquito levels are not necessarily correlated with increased malaria incidence, a phenomenon originally termed, "Anophelism without malaria" (Inhorn & Brown, 1990). Although we also evaluated more direct parameters of vectorial capacity in mosquitoes such as human blood index and sporozoite rates, we did not originally power the study to detect differences in these outcomes, especially as sporozoite rates were extraordinarily low after IRS. Future research could be designed specifically to investigate differences in sporozoite rates or entomological inoculation rates across study arms, which could more directly characterize differences in malaria transmission risk.

We started this study after all houses had been randomized and intervention houses had received LPG stoves and fuel. However, causal inference in this study would have been improved if we could have started sampling prior to the delivery of the intervention. We could have then used a difference in differences approach to more directly assess the impacts of LPG fuel adoption in the intervention houses themselves. Additional baseline data could have helped ensure balance between study arms, particularly as it relates to determinants of vector abundance such as population density, housing quality, and proximity to breeding sites. Finally, I would ideally like to conduct two follow-up studies 1) in a malaria endemic setting where indoor cooking is common, and 2) in a setting where *Aedes aegypti*-transmitted pathogens (e.g., DENV) are common. This would help shed light on the two major lingering questions in our study, namely whether the effects of clean fuel adoption are more pronounced in houses which use biomass fuels indoors and among vector species that generally bite during peak cooking hours.

The differences we observed in flies also presents some important avenues for future research. A first step would be to characterize whether this effect is generalizable to other settings. Additional studies could be repeated at other HAPIN sites such as India or Guatemala. It is likely that the differences would be most pronounced in settings where traditional cooking locations are frequently outdoors or in separate cooking structures. A second critical research question is whether observed reductions in flies actually reduce food contamination and exposure to enteric pathogens. One study in Bangladesh assessed the effects of flies on food contamination by placing containers of cooked white rice in study kitchens. The authors then quantified rice contamination by flies using fecal indicator bacteria (Lindeberg et al., 2018). A similar design could be employed in the context of our research to better characterize the importance of flies as potential mechanical vectors of enteric pathogens. However, previous work has shown the importance of flies as mechanical vectors of enteric pathogens may be pathogen-specific (Cohen et al., 1991; Cohen et al., 2002). Characterization of specific pathogens in contaminated food would therefore be preferable compared to just using fecal indicator bacteria as an indicator for contamination. An ancillary HAPIN study collected stool samples from children in order to assess the effects of the intervention on enteric infection, the gut microbiome, and enteric dysfunction. Further analyses of these samples could shed important light on the effects of fly reductions.

The third aim relied on secondary surveillance data from the MOPDD. I am grateful to the entomology technicians, officers, and collectors that conducted entomological surveillance over the last ten years, as well as the health facility staff and surveillance personnel that collected the malaria case data. I gratefully acknowledge Dr. Hakizimana and his team at MOPDD for leading the surveillance efforts over the years and generously sharing the data. My own contribution to this research was developing the analysis plan, conducting the analyses, and drafting the manuscript. All co-authors provided invaluable feedback on the original study proposal as well as the manuscript drafts.

This study underscored the value of combined entomological and epidemiological analyses. For example, it would have been difficult to evaluate the actual importance of changes in *Anopheles* species composition during the period without health facility-based malaria case data. Likewise, we may not have been able to understand the true drivers of resurgent malaria transmission without detailed entomological data. Many studies also do not account for concurrent environmental changes when assessing the effects of malaria control interventions. For example, recent studies in northern Uganda have linked the discontinuation of indoor residual spraying to a dramatic malaria resurgence (Namuganga et al., 2021; Nuwa et al., 2022), and another study in Burundi concluded that major malaria epidemics during the last decade were due to improved testing and reporting, despite increases in test positivity and malaria deaths during the time period (Sinzinkayo et al., 2021). Neither of these studies assessed temperature trends during the period, and it is possible that increased regional temperatures helped drive the observed case increases. On the other hand, recent research in Ethiopia found that a 'slowdown' in global warming in the early 2000s happened concurrently with the scale-up of vector control interventions and may have contributed to decreased transmission during the period (Rodó et al., 2021).

Although the field entomology surveillance was conducted at the village level, these data were pooled by sentinel site prior to laboratory analyses. Malaria case data were also available at the sentinel site level rather than by each patient's village of residence. In future studies I hope to investigate variations in vector bionomics and malaria transmission at the village level. This would enable us to better characterize environmental drivers of *An. gambiae* ecology and malaria outcomes. For example, additional spatial entomological analyses could assess the relationship between proximity to larval sites (e.g., rice fields, dams, irrigation ditches, etc.) and household density of *An. gambiae* and *An. arabiensis*. This could help target larval control interventions that MOPDD is currently piloting (Kapesa et al., 2017; Rulisa et al., 2021) towards larval sites which are particularly favorable for *An. gambiae*. Ideally these efforts would be coupled with additional field investigations of *Anopheles* larval ecology in order to validate observed spatial associations. We could then assess whether these factors led to spatial clustering or heterogeneity in malaria risk at the village level.

On the other hand, this study also highlighted the need for a larger-scale analysis of these trends in other sites across Rwanda and ideally elsewhere in East Africa. This would enable us to assess the generalizability of our current findings and generate broader recommendations for vector control across the region. Although it appears likely that vector reemergence drove malaria increases across the region over the past decade, this trend has only been investigated in isolated studies (Katureebe et al., 2016; Mawejje et al., 2021; McCann et al., 2014). The impact of large-scale warming during the period has also only been investigated in a few small studies (Kapesa et al., 2017; Kreppel et al., 2019), and not at a regional scale. Other reports have noted striking increases in cases and test positivity during the same time period, but did not investigate the role of vector bionomics or meteorological trends in resurgent transmission (Sinzinkayo et al., 2021; WHO, 2017b, 2018b). A multi-country combined entomological and epidemiological study could investigate whether these trends indeed occurred throughout the region and the primary drivers of those trends. It could also illuminate the most effective interventions for mitigating malaria resurgences as a result of vector reemergence and climate warming.

A. Appendix A: Research Aim 2 Supplemental Materials

Independent variables

Intervention: The primary exposure variable was intervention status. Intervention households had received LPG stoves and a consistent supply of LPG fuel as part of the HAPIN trial, whereas control households had not and were encouraged to continue cooking with traditional biomass fuels (Clasen et al., 2020). Control households were considered the reference group.

We also measured numerous other potential determinants of vector density. These included:

Cooking practices:

- Fuel used in primary stove: at each sampling round, participants were asked whether they had cooked in the prior 24 hours and what type of fuel they had used for their primary stove (LPG, biomass, or other/unknown).
- Cooking location: participants were also asked where their primary cooking location was (outdoors or in a separate cooking structure, indoors in main house, or unknown).

Housing characteristics:

- Number of people that slept in house night before: at each sampling round, participants were asked how many people slept in the house the night before.
- Presence of openings in windows, doors, and/or walls: study staff also visually observed the presence of any cracks or openings in windows, doors, or walls wider than 1cm, which could facilitate entry by mosquitoes or flies.
- Presence of open, water-holding containers: open, water-holding containers were counted and inspected for the presence of mosquito larvae and pupae.
- Toilet/ latrine covered: Staff also observed whether houses had a latrine and if yes, whether it was covered.
- Distance from latrine to kitchen (m): study staff measured distance in meters from the latrine to the center of the primary cooking location.
- Distance from rubbish pile to kitchen (m): study staff asked participants where their primary rubbish or garbage disposal area was and measured distance in meters to the center of the primary cooking location.
- Domestic animals in compound: study staff visually observed the presence of any animals in the compound
- Feces in compound: study staff visually observed the presence of any animal or human feces in the compound
- Uncovered, cooked food in kitchen: study staff visually observed the presence of any cooked food that was either uncovered or not covered with a tight-fitting lid in the cooking area.

Vector control:

- % of occupants that slept under net: at each sampling round, participants were asked how many individuals which slept in the house the night before slept under an insecticide treated bed net.

- Received IRS in last 12 months: Participants were asked whether they had received IRS in the last 12 months prior to the survey, which was confirmed by observing government-issued IRS cards.
- Used insecticides or burned materials to repel mosquitoes or flies in last 24 hrs: participants were also asked if they had used insecticides or burned any materials to repel mosquitoes or flies in the 24 hours prior to the study team visit.

Indoor conditions:

- $PM_{2.5}$ concentrations in µg/m^3: $PM_{2.5}$ concentrations were measured in a subset of 144 participants' bedrooms during each sampling round as described above. $PM_{2.5}$ levels for each round were averaged as the mean concentrations from 4pm on the day of the initial visit until 10am on the day of the return visit the next morning.
- Temperature (°C): temperature was measured along with $PM_{2.5}$ and averaged as described above.
- Percent relative humidity (RH): relative humidity was measured along with $PM_{2.5}$ and temperature and averaged as described above.

Environmental characteristics:

- Elevation (m): the elevation of each household was derived using a 30-meter digital elevation model (DEM) from the Shuttle Radar Topography Mission (RCMRD, 2015).
- Distance (m) to closest rice fields and dams: a cloud-free 30-meter Landsat 8 L1 retrieval of the study area from March 04, 2020 was downloaded from the U.S. Geological Survey (Roy et al., 2014; USGS, 2021). We used supervised image classification to classify rice fields in the study area, following previously published methods (Diuk-Wasser et al., 2007). Briefly, we first classified potential wetlands where rice is typically grown using elevation and slope models (Mahdavi et al., 2018). We then used principal components analysis (PCA) to remove correlation among bands 1 through 11 (Lei et al., 2008). The first three components of the PCA analysis accounted for 99% of variation in the image. We used high resolution google earth imagery as well as in situ observations to manually assign training samples for six land-use classifications (rice, natural wetlands, dams/lakes, row-crop agriculture, scrubland, and settlements). We then employed supervised image classification in ArcMap version 10.8.1 (ESRI, Redlands, CA) and assessed model results via the kappa coefficient. We then calculated Euclidean distance from each study household to the nearest rice field using the *rgeos* package in R version 4.0.2 (Bivand et al., 2017). We used the same method to calculate distance from each house to the closest dam.
- LST (°C): we downloaded 6km resolution monthly daytime land surface temperature (LST) averages from MODIS Terra MOD11B3 products in the NASA Earthdata portal (NASA, 2021). We calculated current and one-month lagged LST for each household at each study visit as the mean LST within a 2500m buffer area around each household using the *SP* and *Raster* packages in R (Hijmans et al., 2015; Pebesma & Bivand, 2005).
- Rainfall (mm): we downloaded monthly 6km resolution gridded rainfall estimates from The Climate Hazards group Infrared Precipitation with Stations (CHIRPS) (Funk et al., 2015). We then estimated current and one-month lagged rainfall values for each household at each study visit as the mean rainfall within a 2500m buffer area around each household using the *SP* and *Raster* packages in R
- Population density/ km^2: we downloaded high resolution (100m) gridded population density estimates from world pop for the study area in 2019 (Lloyd et al., 2019). We then estimated local

population density for each study household as the mean population density within a 250m buffer area around each household using the *SP* and *Raster* packages in R.

Follow-up time: We calculated follow-up time for mothers as the number of days between the date of randomization into the trial to the last completed assessment. Follow-up time for children was calculated as the number of days from birth until the last completed assessment (e.g. B4). If one or more planned assessments were not completed, we subtracted the average follow-up time for that assessment from the overall follow-up time for each individual.

Supplemental tables

Table 6-1: Vector density by intervention status

			Total		Control		Intervention		P-value
			n	Density (SD)	n	Density (SD)	n	Density (SD)	
Mosquito density									
	Anopheles spp.		356	0.63 (2.68)	143	0.53 (2.35)	213	0.72 (2.95)	0.87
		An. gambiae s.l.	291	0.51 (2.56)	107	0.39 (2.21)	184	0.62 (2.85)	0.94
		An. ziemanni	18	0.03 (0.24)	8	0.03 (0.26)	10	0.03 (0.23)	0.86
	Culicines		2145	3.78 (7.32)	894	3.30 (5.41)	1251	4.23 (8.70)	0.37
		Culex quinquefasciatus	2048	3.61 (7.02)	846	3.12 (5.10)	1202	4.06 (8.39)	0.41
		Aedes spp.	11	0.02 (0.21)	3	0.01 (0.10)	8	0.03 (0.27)	0.88
		Mansonia spp.	11	0.02 (0.18)	4	0.01 (0.12)	7	0.02 (0.22)	1.00
Fly density									
	Synanthropic flies		1022	1.80 (3.98)	752	2.77 (4.84)	270	0.91 (2.69)	<0.001
		Muscidae	475	0.84 (2.87)	369	1.37 (3.22)	106	0.36 (2.40)	<0.001
		Calliphoridae	72	0.13 (1.23)	62	0.23 (1.75)	10	0.03 (0.29)	0.22
		Fanniidae	436	0.78 (2.19)	297	1.10 (2.87)	139	0.48 (1.21)	<0.001
		Sarcophagidae	39	0.07 (0.65)	24	0.09 (0.86)	15	0.05 (0.38)	0.99
	Domestic flies		8737	15.41 (27.73)	4982	18.38 (35.27)	3755	12.69 (17.90)	0.03
		Drosophila	6604	11.65 (25.25)	3769	13.91 (32.13)	2835	9.58 (16.41)	0.03
		Psychodidae	1031	1.82 (10.28)	577	2.13 (13.91)	454	1.53 (5.06)	0.99

Table 6-2. Unadjusted and adjusted effects of intervention and other variables on *Anopheles*, culicine, and synanthropic fly densities

	***Anopheles* density**				**Culicine density**				**Synanthropic fly density**			
	Unadjusted		*Adjusted*		*Unadjusted*		*Adjusted*		*Unadjusted*		*Adjusted*	
	RR (95% CI)	*P value*	*RR (95% CI)*	*P value*	*RR (95% CI)*	*P value*	*RR (95% CI)*	*P value*	*RR (95% CI)*	*P value*	*RR (95% CI)*	*P value*
Intervention *(ref = control)*	0.92 (0.33, 2.55)	0.87	1.58 (0.88, 2.85)	0.13	1.17 (0.83, 1.63)	0.37	1.07 (0.78, 1.48)	0.67	0.31 (0.22, 0.45)	<0.001	0.39 (0.27, 0.56)	<0.001
Maternal education *(ref = primary or less)*	0.24 (0.09, 0.66)	0.01	0.47 (0.23, 0.98)	0.04	0.7 (0.49, 0.98)	0.04	0.79 (0.56, 1.12)	0.19	0.49 (0.33, 0.74)	<0.001	0.68 (0.46, 1.02)	0.06
# People slept in house	0.98 (0.74, 1.29)	0.87	1.05 (0.84, 1.31)	0.68	1.1 (0.99, 1.24)	0.09	1.14 (1.02, 1.28)	0.03	-	-	-	-
Openings in house *(ref = no)*	7.96 (2.4, 26.39)	<0.001	1.54 (0.62, 3.82)	0.35	0.83 (0.57, 1.19)	0.31	0.88 (0.58, 1.34)	0.56	1.72 (1.11, 2.67)	0.01	1.31 (0.8, 2.15)	0.284
Mud floors *(ref = no)*	6.09 (2.09, 17.78)	<0.001	1.22 (0.53, 2.84)	0.64	1.61 (1.13, 2.29)	0.01	1.73 (1.18, 2.54)	0.01	2.51 (1.69, 3.73)	<0.001	1.58 (1.01, 2.45)	0.04
% of occupants slept under net	2.39 (0.87, 6.57)	0.09	2.36 (1.02, 5.46)	0.0441	1.27 (0.86, 1.88)	0.23	1.35 (0.89, 2.04)	0.15	-	-	-	-
Elevation (m)ǂ	0.23 (0.16, 0.34)	<0.001	0.38 (0.27, 0.52)	<0.001	1.15 (0.98, 1.36)	0.09	1.41 (1.18, 1.68)	<0.001	0.77 (0.63, 0.93)	0.01	0.89 (0.75, 1.07)	0.219
Rice field within 2km *(ref = no)*	20.38 (7.53, 55.12)	<0.001	4.85 (2.5, 9.43)	<0.001	2.16 (1.44, 3.23)	<0.001	2.51 (1.63, 3.86)	<0.001	-	-	-	-
Population density / sq. kmǂ	0.05 (0.01, 0.2)	<0.001	0.13 (0.03, 0.52)	0.001	1.09 (0.91, 1.29)	0.35	1.13 (0.9, 1.41)	0.31	0.7 (0.56, 0.86)	<0.001	1.06 (0.8, 1.4)	0.69

RR= Rate Ratio; ǂ = continuous variables scaled to standard deviations;

Table 6-3. Effects of the intervention on vector density by cooking location

		Control		Intervention			
	Primary cooking location*	n obs	Mean (SD)	n obs	Mean (SD)	Adjusted RR	P value
Anopheles	Inside main house	56	0.16 (0.83)	309	0.6 (2.70)	3.66 (0.92, 14.59)	0.07
	Outdoors/ separate	262	0.72 (2.99)	34	0.97 (3.25)	1.09 (0.31, 3.83)	0.89
Culicines	Inside main house	56	4.2 (6.38)	309	4.71 (10.46)	0.75 (0.43, 1.3)	0.31
	Outdoors/ separate	262	3.16 (5.32)	34	3.82 (5.73)	1.08 (0.57, 2.07)	0.81
Synanthropic flies	Inside main house	56	1.11 (2.56)	309	0.82 (2.44)	1.09 (0.53, 2.25)	0.81
	Outdoors/ separate	262	2.79 (4.86)	34	2 (6.82)	0.64 (0.29, 1.41)	0.27

* location of primary cooking stove used in 24 hrs before vector sampling visits; n obs = number of observations; Rate ratios (RR) for malaria adjusted for maternal education, number of people that slept in house, cracks or openings in house, mud floors, bed net use, elevation, proximity to rice fields, and population density. RRs for diarrhea adjusted for same variables except number of people that slept in house and rice field proximity

Table 6-4. Effects of potential mediators (PM2.5, and cooking location) on vector density

		Anopheles mosquitoes		Culicine mosquitoes		Synanthropic flies	
		RR (95% CI)	*P value*	*RR (95% CI)*	*P value*	*RR (95% CI)*	*P value*
PM2.5 (µg/m$^{3)}$		0.65 (0.15, 2.82)	0.57	0.98 (0.77, 1.26)	0.90	0.96 (0.72, 1.28)	0.78
Cooking location at follow-up							
	Indoor	NA*		1.25 (0.92, 1.69)	0.15	0.38 (0.27, 0.55)	<0.001
	Outdoor/separate (ref)						

*Cooking location was evaluated as a modifier of the effect of the intervention on *Anopheles* density. Rate ratios (RR) for *Anopheles* and culicine mosquitoes adjusted for maternal education, number of people that slept in house, cracks or openings in house, mud floors, bed net use, elevation, proximity to rice fields, and population density. RRs for flies adjusted for same variables except number of people that slept in house and rice field proximity

Table 6-5. $PM_{2.5}$ and *Anopheles* density by cooking location

	Control			
		PM2.5 (μg/m^3)		*Anopheles*
	n obs	Mean (SD)	Median (IQR)	Mean (SD)
Inside main house	17	61.17 (120.2)	27.18 (17.96, 48.02)	0 (0)
Outdoors/ separate	74	36.94 (61.32)	20.92 (14.26, 29.28)	0.45 (1.64)
	Intervention			
		PM2.5 (μg/m^3)		*Anopheles*
	n obs	Mean (SD)	Median (IQR)	Mean (SD)
Inside main house	120	24.43 (28.63)	17.41 (11.81, 25.52)	0.16 (0.88)
Outdoors/ separate	7	15.75 (8.27)	13.94 (10.45, 15.71)	2.14 (5.67)

Table 6-6. Bloodfeeding and sporozoite infections among *Anopheles* and *Culex* mosquitoes, by intervention status

			Control	Intervention	P-value
			n (%)	n (%)	
Anopheles					
	Bloodfed		33/204 (16.2)	11/213 (5.2)	0.344
		Human	22/33 (66.7)	6/11 (54.5)	0.631
		Bovine	2/33 (6.1)	0/11 (0.0)	
		Mixed	7/33 (21.2)	4/11 (36.4)	
		Other	2/33 (6.1)	1/11 (9.1)	
	Pf +		1/33 (0.5)	0/11 (0.0)	0.983
Culex					
	Bloodfed		9/1071 (0.8)	20/1602 (1.2%)	0.321
		Human	1/9 (11.1)	1/20 (5.0)	0.459
		Bovine	0/9 (0.0)	1/20 (5.0)	
		Mixed	7/9 (77.8)	11/20 (55.0)	
		Other	1/9 (11.1)	7/20 (35.0)	

Table 6-7. Adjusted effects of $PM_{2.5}$ and cooking location on reported health outcomes

	Malaria in mothers		Malaria in children		Diarrhea in Children	
	LPR (95% CI)	*P value*	*LPR (95% CI)*	*P value*	*LPR (95% CI)*	*P value*
$PM_{2.5}$ (μg/m$^{3)}$	0.95 (0.45, 1.32)	0.83	NA*		1.59 (0.86, 2.82)	0.12
Cooking location at follow-up						
Indoor	1.11 (0.7, 1.74)	0.66	0.38 (0.08, 1.28)	0.15	0.95 (0.59, 1.51)	0.81
Outdoor/separate (ref)						

*Effect of $PM_{2.5}$ on malaria in children not evaluated because too few cases were observed among children with PM2.5 measurements (n=5). Longitudinal prevalence ratios (LPR) for malaria adjusted for maternal education, number of people that slept in house, cracks or openings in house, mud floors, bed net use, elevation, proximity to rice fields, and population density. LPRs for diarrhea adjusted for same variables except number of people that slept in house and rice field proximity.

B. Appendix B: Research Aim 3 Supplemental Materials

Supplemental figures

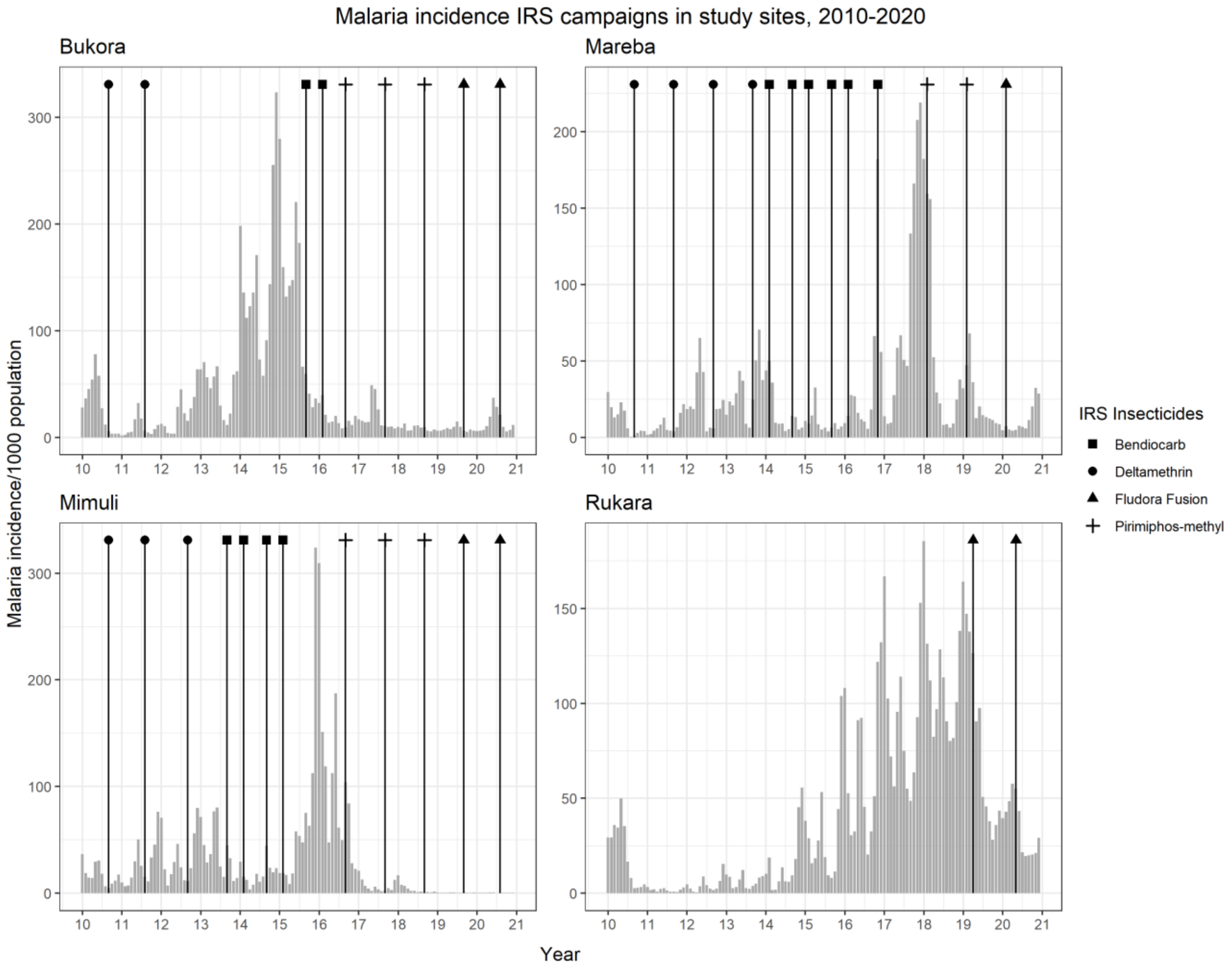

Figure B-1. IRS campaigns in relation to malaria incidence in study sites. Bendiocarb and Deltamethrin were expected to have a residual efficacy of 3-6 months, whereas pirimiphos-methyl and Fludora®Fusion were expected to last 9-12 months (Fongnikin et al., 2020; Mashauri et al., 2017; Rowland et al., 2013; WHO, 2015). A duration of 5 months and 11 months was chosen for *An. gambiae* relative abundance and malaria incidence models based on AIC values.

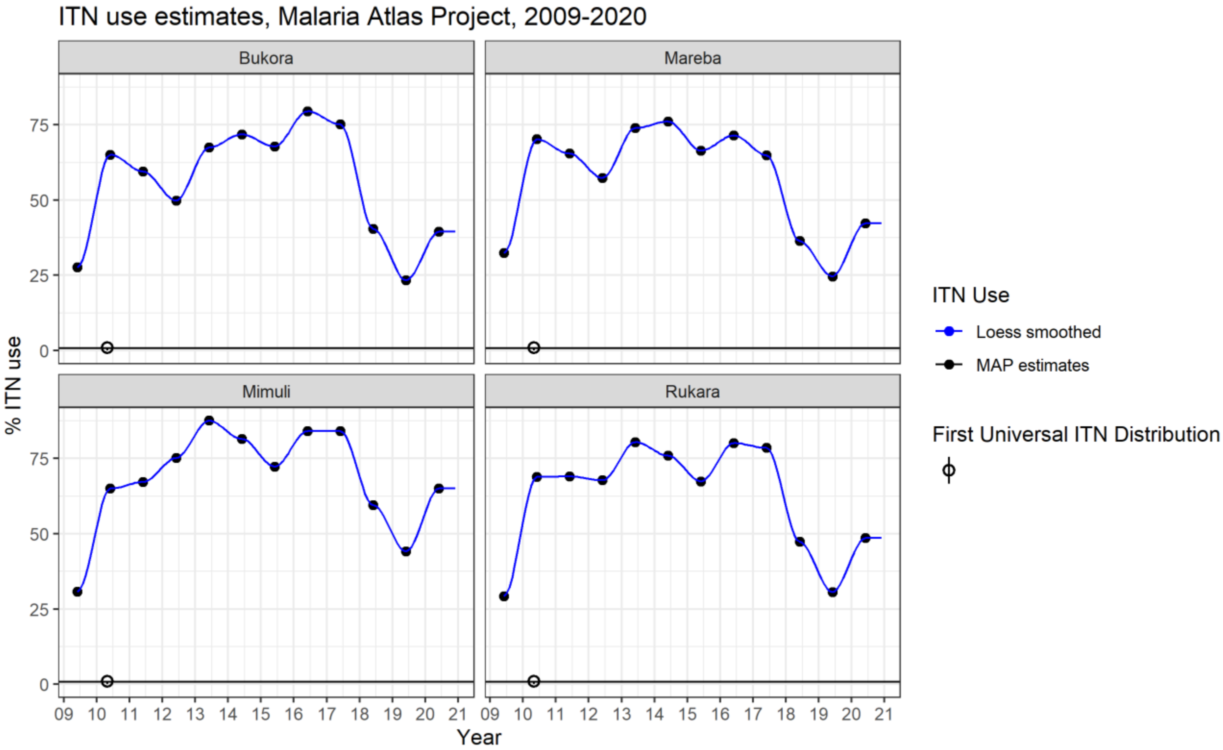

Figure B-2: ITN use estimates in each site. Yearly ITN use estimates (black dots) were derived from the Malaria Atlas Project (Bertozzi-Villa et al., 2021). Loess smoothing (blue line) was used to provide continuous trend estimates. A universal coverage campaign began in May 2010, which rapidly increased net use.

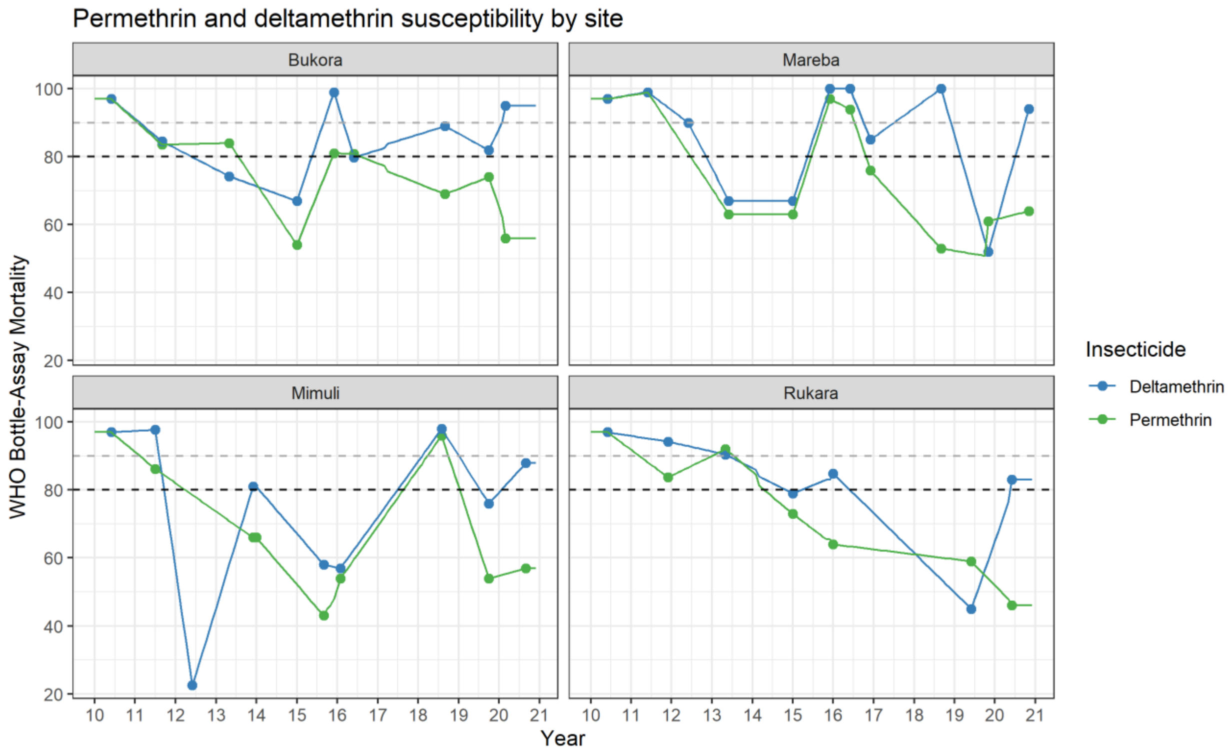

Figure B-3: Deltamethrin and permethrin susceptibility by site. WHO bottle-assays were conducted on a semi-regular basis with field collected An. gambiae s.l. as described previously (Hakizimana et al., 2016).

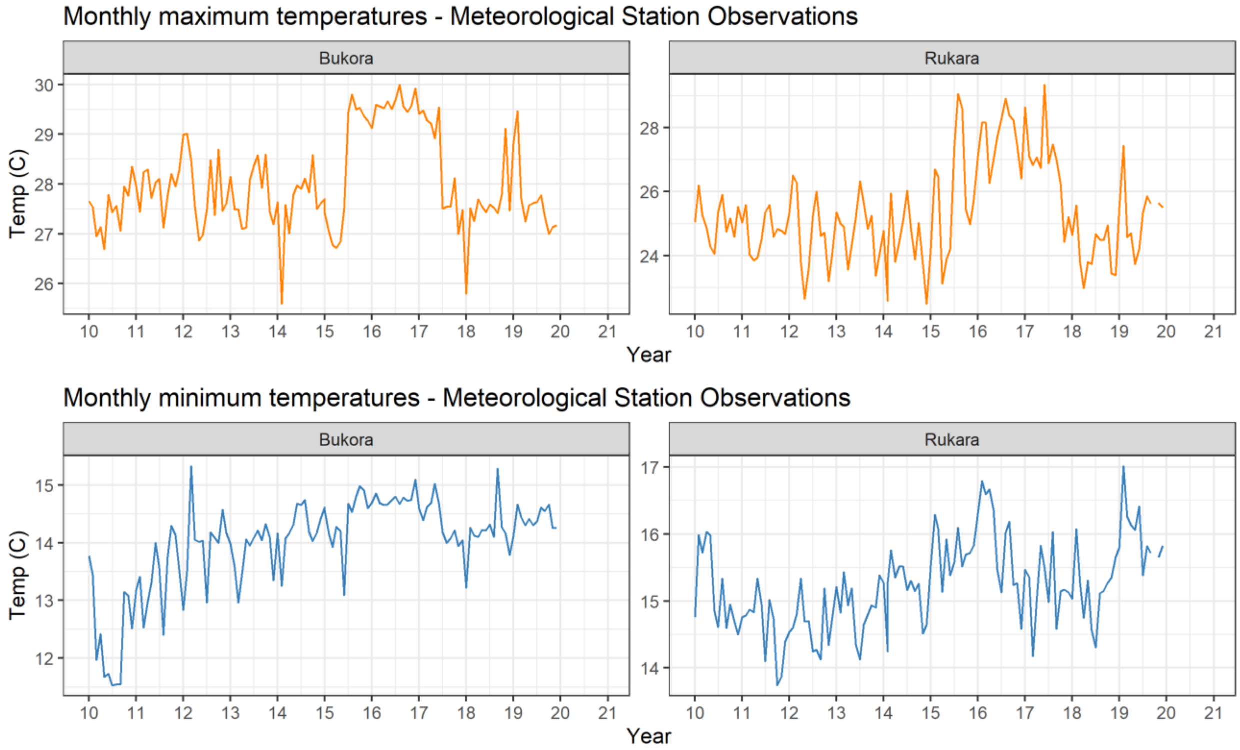

Figure B-4: Minimum and maximum monthly temperature trends in study site. Meteorological sites were located in Bukora and Rukara.

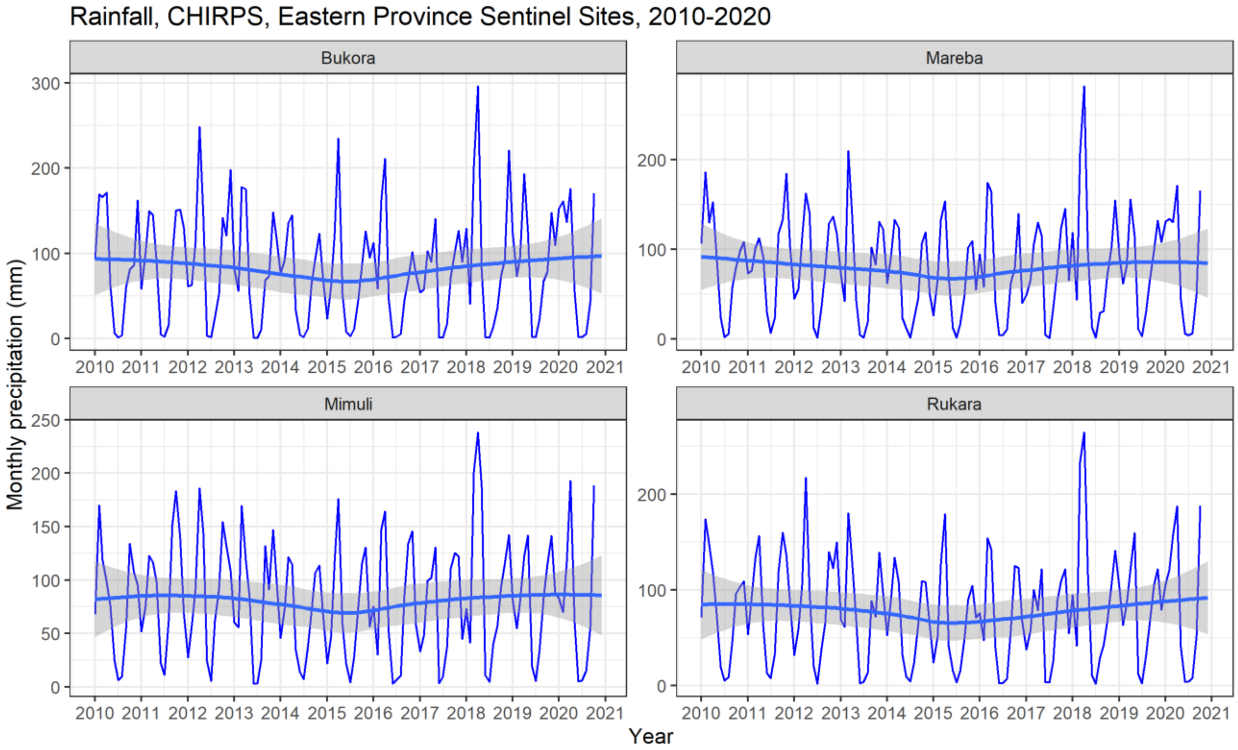

Figure B-5: Monthly rainfall in mm at each site. Rainfall estimates are from the Climate Hazards Infrared Precipitation with Stations (CHIRPS) project (Funk et al., 2015). The trend line is a loess smoothed trend, which shows essentially no trend in rainfall during the study period.

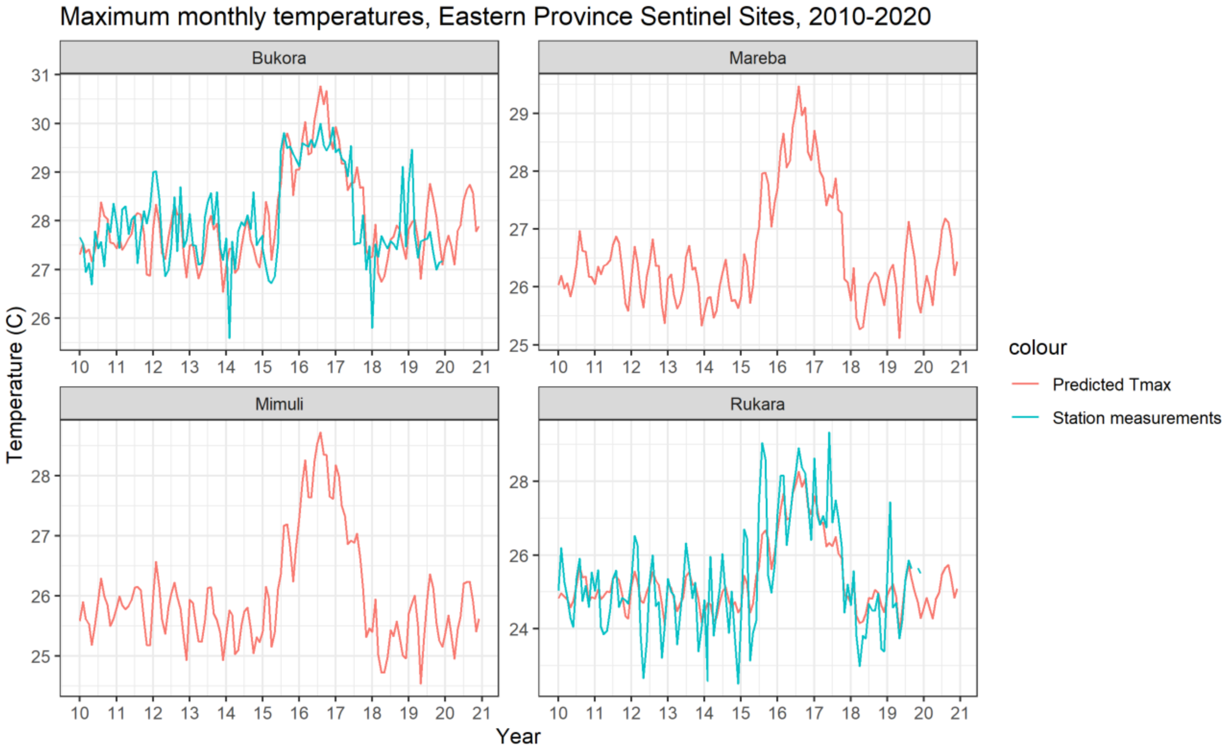

Figure B-6: Measured and predicted maximum temperatures at each site. Meteorological station measurements are shown in blue, whereas model predictions are shown in red.

Supplemental methods

Modeled temperature estimates: station-based minimum and maximum monthly temperature records were available for Bukora and Rukara, but not for the other two sites. We therefore used generalized additive models (GAMs) to model Tmin and Tmax as a function of remotely sensed environmental variables, following previously described methods (Lin et al., 2012; Weiss et al., 2014). Temporal correlation was modeled via a smoothed term for each month. Remotely sensed variables were first downloaded and monthly values extracted at each sentinel site using the *raster* package in R (Hijmans et al., 2015). Covariates included:

- Monthly nighttime and daytime land-surface temperature (LST) measurements from MODIS MOD11C3 Version 6 L3 satellite retrievals at ~5km spatial resolution (Wan et al., 2015)
- Monthly gridded surface Tmin and Tmax estimates from the university of East Anglia Climate Research Unit (CRU) Version 4 time series product at ~50km spatial resolution (Harris et al., 2020)
- Monthly normalized difference vegetation index (NDVI) from MODIS MOD13A3 products at 1x1km resolution (Beck et al., 2006)
- Monthly rainfall estimates from the Climate Hazards Infrared Precipitation with Stations (CHIRPS) project at a 5km spatial resolution (Funk et al., 2015)

We fit separate models for Tmin and Tmax. After fitting the models, we conducted repeated k-fold cross validation to assess predictive performance. Both models had high accuracy (Tmin $R^2 = 0.67$ and Tmax $R^2 = 0.82$) and low RMSE values (Supplemental Table B-1). We then used the models to predict Tmin and Tmax for each site. Supplemental Figure B-6 shows Tmax predictions compared to meteorological station measurements.

Table B-1: Predictive performance of GAM temperature models

	RMSE	R^2	MAE
Tmin	0.56	0.67	0.41
Tmax	0.78	0.82	0.60

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