Host selection and feeding preferences of farm-associated mosquitoes (Diptera: Culicidae) in the United Kingdom

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Declaration

I, Victor Albert Brugman, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Livestock farms sit at the interface between humans, livestock species and wildlife. However, limited data exist on mosquito-vertebrate host interactions on farms in the United Kingdom. This thesis therefore aimed to understand mosquito-vertebrate host interactions on UK livestock farms using a combination of field collections, colony mosquito experiments and molecular techniques for species identification and blood meal analysis.

Field collections conducted between 2012 and 2014 yielded a total of 22 693 adult mosquitoes comprising 7 genera and 18 species. Fifteen species displayed human biting activity as assessed by human landing catch, with a maximum observed biting pressure at a single farm of up to 89 bites per 25 minutes at sunset. The avian biting rate, as assessed by the use of chicken-baited traps, was considerably lower than the human biting rate, but demonstrated the ornithophilic activity of three mosquito species, two of which had not previously been collected by such an approach in the UK.

Field-caught blood-fed mosquitoes were subjected to a three-stage, targeted analysis, demonstrating that a single DNA extract from an engorged mosquito abdomen provides sufficient DNA for species delineation of *Anopheles maculipennis* s.l., blood meal identification and detection of myxoma virus. This study implicated *Anopheles atroparvus*, for the first time, in the transmission of myxomatosis between wild rabbits. The blood meals of over 900 mosquitoes of nine species were identified, revealing feeding on 5 mammalian and 14 avian hosts. Importantly, this study identified key potential vector species *Culex pipiens f. pipiens* as feeding on both resident and migratory birds.

Collectively, these results demonstrate that UK livestock farms support ornithophagic, mammalophagic and anthropophagic mosquito populations which, at certain farms, can lead to a severe nuisance biting pressure on humans. The described feeding of potential vector species, such as newly-established *Culex modestus*, on farm-associated domestic and wild hosts, suggests that certain mosquito species could play a role in facilitating future pathogen transmission cycles on livestock farms in the case of a novel incursion.

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Finally, I am forever grateful for the on-going love and support of mum, dad and Merina.

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List of abbreviations

Abbreviation	Full description
Ace-2	acetylcholinesterase-2
APHA	Animal and Plant Health Agency
Arbovirus	Arthropod-borne virus
BATV	Batai virus
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CBT	Chicken-baited trap
CDC	Centers for Disease Control
cDNA	Copy DNA
CHIKV	Chikungunya virus
cm	Centimetre
CNS	Central nervous system
CO_2	Carbon dioxide
DB Barn owl	Dark-breasted Barn owl
DENV	Dengue fever virus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
GIMP	The GNU Image Manipulation Program
GLMM	Generalized linear mixed model
HLC	Human landing catch
INKV	Inkoo virus
ITS-2	Internal transcribed spacer gene 2
km	Kilometer
L.	Linnaeus 1758
LSHTM	London school of Hygiene and Tropical Medicine
m/m^2	Metre/metre squared
mm	Millimetre
MMP	Mosquito Magnet Pro
ng	Nanogram
NUTS3	Nomenclature of Territorial Units for Statistics, level 3
PCR	Polymerase chain reaction
PHE	Public Health England
RNA	Ribonucleic acid
SINDV	Sindbis/Sindbis-like viruses
sp./spp.	Unknown species/multiple species of a known genus
SSHV	Snowshoe hare virus
TAHV	Tahyna virus
TMAC	Tetramethylammonium chloride
TPI	The Pirbright Institute
UK	United Kingdom
USUTV	Usutu virus
WNV	West Nile virus

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Chapter 1 – Introduction

1.1 An introduction to the mosquitoes of the United Kingdom

1.1.1 Mosquito research in the United Kingdom

Sporadic interest in the biology of mosquitoes of the United Kingdom (UK) can be traced to the early 19th century (Snow et al. 1997), with accelerated interest accompanied by systematic studies taking place only at the start of the 20th century, shortly after the identification of the role of mosquitoes in malaria transmission (Ross 1897). Figure 1.1 illustrates the literature published concerning UK mosquitoes from 1825 to 2015, broadly separated into seven content categories according to subject (literature sources 1825-1997 in Snow et al., (1997), 1998-2015 in appendix A1). The circulation of indigenous *Plasmodium vivax* malaria, or "ague", into the early 20th century was an important driver of interest in UK mosquitoes. However, arguably the main motivation for early work into the distribution and life history of UK mosquitoes was the presence of nuisance biting populations (category 6), particularly on the south coast of England. The first handbook for the identification of 21 recognised UK mosquito species was published in 1920 (Lang 1920) (category 4). Efforts to control key species biting humans, including *Ochlerotatus detritus* Haliday 1833 and *Culiseta annulata* Schrank 1776 led to the formation of the British Mosquito Control Institute on Hayling Island in 1925 (see Snow & Snow, (2004) for a review). Additional studies over two subsequent decades revised the fauna to include 29 mosquito species (Marshall 1938) (category 2), with a peak in studies into the life history of UK species during this period (category 1).

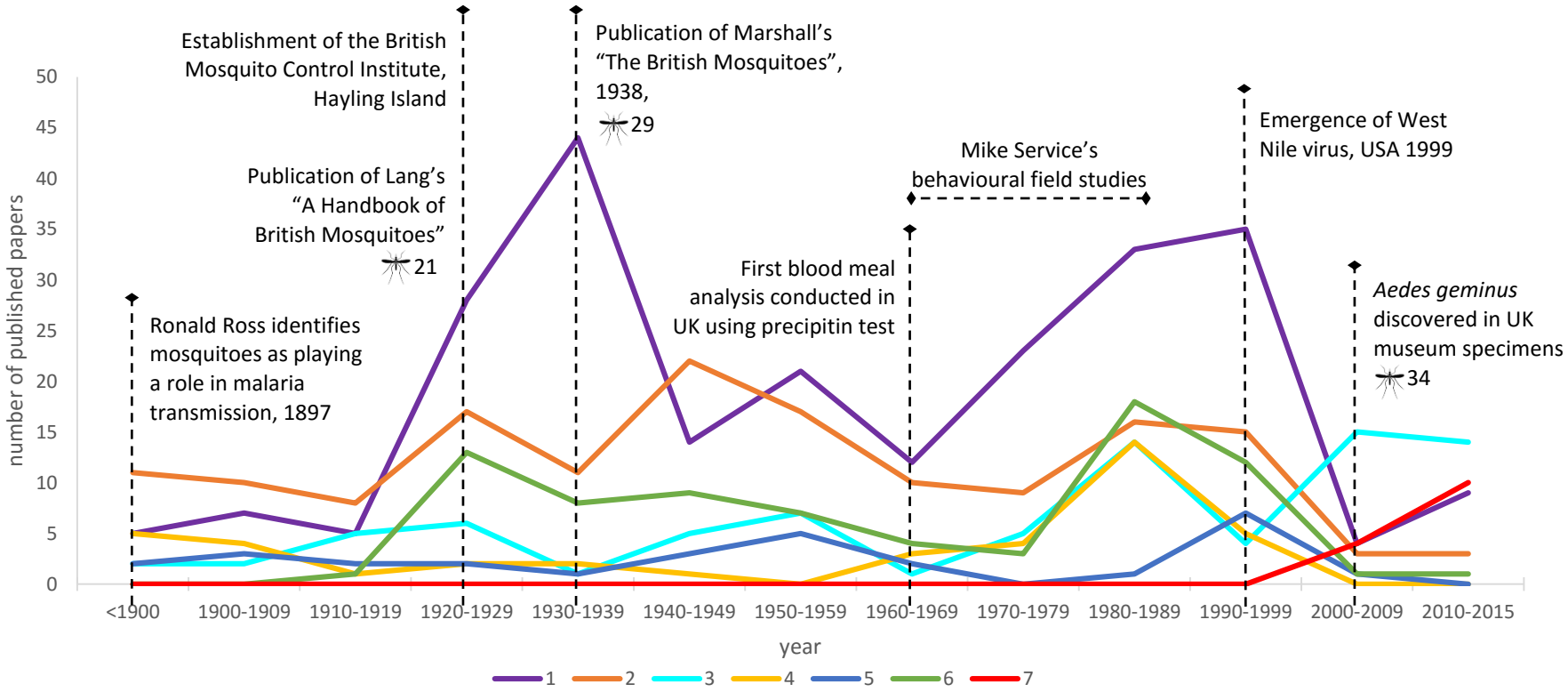


Figure 1.1: Published literature (634 references) concerning mosquitoes in the UK, according to the titles of published literature up until 1997, after Snow et al. (1997) and the title and/or abstract of papers published 1998-2015 (see reference list in appendix A1). Annotations are provided for noteworthy events. indicates the number of species recognised at each time point.

Categories for literature: (1) Biological field/lab studies, (2) Distribution studies/species checklists, (3) Papers referring to specific mosquito-borne pathogens, (4) Identification keys/technical papers, (5) Miscellaneous papers e.g. entomologists' obituaries, (6) Control/nuisance biting papers, (7) Surveillance/risk assessments for mosquito-borne pathogens.

Through the 1930s, 1940s and 1950s, research into the distribution and occurrence of mosquitoes in the UK continued to be accompanied by interest in understanding the declining transmission and occasional outbreaks of indigenous *P. vivax* (see (Dobson 1989) for a review). The work of Percy George Shute played a major role during this period, which notably included his demonstrating that *Anopheles atroparvus* van Thiel 1927 was refractory to infection with tropical strains of *Plasmodium falciparum* (Shute 1940). A further important contribution was his work investigating the severe nuisance biting of humans caused by *Culex pipiens f. molestus* Forskål 1775, which plagued Londoners sheltering at Liverpool Street underground station during the Blitz of World War II (Shute 1951). The *Culex pipiens* (L.) complex continued to be studied in detail during this period, particularly in the work of Peter Frederick Mattingly. Morphological, behavioural and genetic crossing observations led to the conclusion that the *Cx. pipiens* complex was a single, polytypic species characterised by two major forms in the UK: *Cx. pipiens f. pipiens* (L.) and *Cx. pipiens f. molestus* (see Mattingly (1967) for a review). These forms differed in phenotype, and hybridisation between the forms was prevalent where promoted by environmental conditions (Mattingly 1967). At this time *Culex torrentium* Martini 1925 was also recognised for the first time in UK, occupying sympatric habitats, particularly containers, with *Culex pipiens* (L.) (Mattingly 1951). This brought the number of recognised UK mosquito species to 31.

Systematic behavioural studies of UK mosquitoes were not widely conducted until the 1960s and 1970s. These field studies were focused primarily on Brownsea Island in the Poole harbour area of Dorset and in Monks Wood, Cambridgeshire, and were among the first to use host-baited collection methods for trapping mosquitoes in the UK. Traps baited with rabbits (*Oryctolagus cuniculus* Linnaeus 1758)) (Service 1971a; Service 1969c) and chickens (*Gallus gallus* L. 1758) (Service 1969c) received only limited use, however, the human landing catch technique was used extensively. This methodology involved the collection of mosquitoes into test tubes from a stationary human, generally the author of the study (almost invariably Dr Mike Service) but on occasion involving up to twelve additional collectors (Service 1969a). The

simplicity, flexibility and capacity for detailed observation of anthropophagic mosquito behaviour using this technique facilitated the collection of much of the fundamental behavioural data on UK mosquitoes that exists to date (covered in detail later on in this chapter). These data include the identification of spatial (Service 1971d), seasonal (Service 1969a) and diel patterns of mosquito activity (Service 1971c). Host-baited collections were occasionally supplemented with light or suction trap catches to provide unbiased (although more limited in number) collections of mosquitoes at different heights from the ground (Service 1969b; Service 1971c). Particular focus was given to the ecology of the woodland species *Ochlerotatus cantans* Meigen 1818 (Lakhani & Service 1974; Service 1977; Service 1971c) due to its abundance close to favoured field sites. It was during this same period that the precipitin test, first implemented to analyse the origin of mosquito blood meals during the 1920s in the USA (Bull & King 1923), was integrated into ecological studies to elucidate host feeding patterns of UK mosquitoes (Service 1969a) and the range of arthropod predators which fed on them (Service 1973).

Some behavioural field studies continued into the 1980s and 1990s, with focus shifting to the biology of larval (Renshaw et al. 1995) and adult (Renshaw et al. 1994) *Oc. cantans* in woodland populations to the south of Liverpool, reflecting the move of Mike Service to the Liverpool School of Tropical Medicine (Service 2010). Here, the enzyme-linked immunosorbent assay (ELISA) technique was first used for investigating blood-feeding patterns of *Oc. cantans* (Renshaw et al. 1994). This technique had been optimised not long before using blood-fed *Oc. cantans*, *Ochlerotatus punctor* Kirby 1837 and *Culicoides obsoletus* Meigen 1818 group collected from the UK (Service et al. 1986). This period also saw an increased focus on consolidating the taxonomy, distribution (including the production of distribution maps (e.g. Snow (1998)) and nomenclature of UK mosquitoes, in large part driven by the work of Keith Snow. Of arguably greatest importance was the assembly of two taxonomic keys to the mosquitoes of the UK (Cranston et al. 1987; Snow 1990). These collated and presented morphological identification details for the eggs, larvae, pupae and adults of the 32 mosquito

species recognised at the time and remain the main reference point for separation of the UK mosquitoes.

The emergence and rapid spread of West Nile virus (WNV) in the USA in 1999 (Lanciotti 1999) led to a change in the focus of mosquito research in the UK, towards assessing the risk of exotic mosquito-borne pathogen emergence (see Gould et al. (2006); Higgs et al. (2004); Medlock, Snow, et al. (2007) for reviews). Since the turn of the century, this has resulted in a considerable proportion of research papers concerned with UK mosquito biology focussing on the potential for pathogen emergence (category 3). Passive surveillance of wild birds for antibodies towards WNV (and more recently viral RNA) was initiated in 2001 (Phipps et al. 2008; Brugman et al. 2013) and for Usutu virus (USUTV) in 2005 (Horton et al. 2013). A single trial that collected and tested mosquitoes for WNV was conducted in 2003 (Department of Health 2004) followed by more systematic collections and arbovirus testing of mosquitoes which remain ongoing (Vaux et al. 2015). To date, surveillance activities have not provided evidence of WNV or USUTV presence in the UK. However, two separate studies indicated through detection of antibodies that wild birds (Buckley et al. 2003) and sentinel chickens (Buckley et al. 2006) may have been exposed to arboviruses including WNV, USUTV and Sindbis virus (SINDV). The status of these studies remains uncertain; while it is not impossible that these arboviruses are circulating endemically in the UK among bird populations with limited manifestation of clinical disease, the lack of confirmation in more recent studies means that their conclusions should be treated with caution.

1.1.2 Bionomics of UK mosquitoes

Over 103 mosquito species of nine genera have been reported in Europe (Snow & Ramsdale 2003; Linton et al. 2005; Schaffner et al. 2009; Snow 2010). The UK fauna is classified under eight of these genera: *Aedes* (3 species), *Anopheles* (6), *Coquillettidia* (1), *Culex* (4), *Culiseta* (7), *Dahliana* (1), *Ochlerotatus* (11) and *Orthopodomyia* (1) constituting a current total of 34 species (Medlock & Vaux 2009; Medlock & Snow 2008a; Snow 2010). Figure 1.2 shows the distribution (10km squares) of these genera across the UK. The two most recent additions to the UK fauna are *Anopheles daciae* Linton, Nicolescu & Harbach 2004 and *Aedes geminus* Peus 1971. *Anopheles daciae* was identified as a cryptic member of the *An. maculipennis* complex (alongside *An. atroparvus* and *Anopheles messeae* Falleroni 1926)) in the UK in the early 2000s (Linton et al. 2005), shortly after its identification based on ribosomal *ITS-2* gene sequences in Romania (Nicolescu et al. 2004). *Aedes geminus* was discovered as a result of re-inspection of museum specimens previously identified as *Aedes cinereus* Meigen 1818 (Medlock & Vaux 2009), with separation only possible by comparison of the male genitalia. Populations of a third species, *Culex modestus* Ficalbi 1889, were re-discovered in 2010 as larvae (Golding et al. 2012) and subsequently as adults (Medlock & Vaux 2012; Vaux et al. 2015) after more than 70 years of apparent absence since initial reports from the coastal region of Hampshire in the 1940s (Marshall 1945).

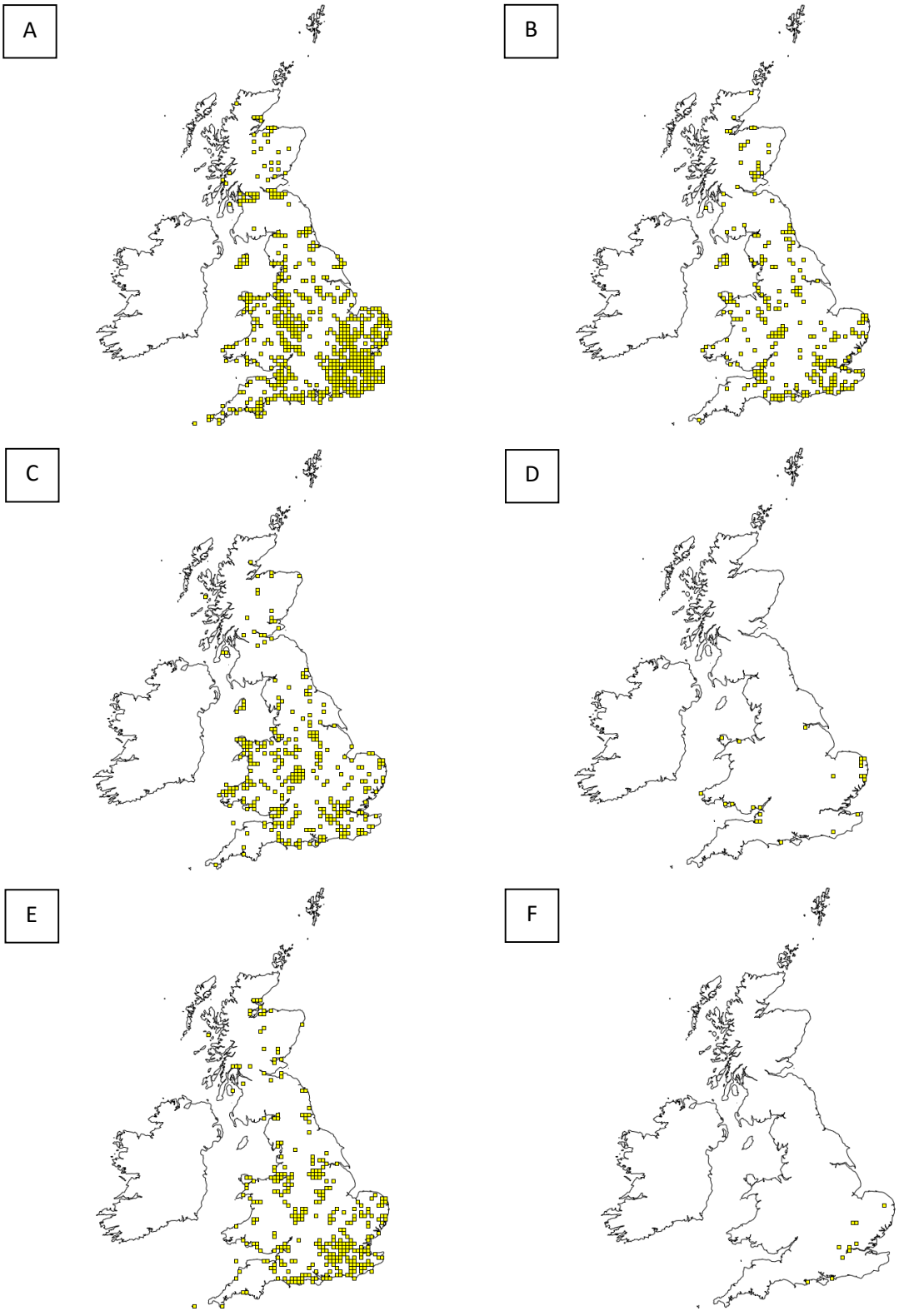


Figure 1.2: Distribution (10 km squares) of UK mosquito genera according to publically-accessible records on the National Biodiversity Network (NBN) Gateway 1900-2015 (correct as of September 2015). Records for *Aedes*, *Dahliana* and *Ochlerotatus* are combined as recent changes in nomenclature (see Snow (2010)) are not reflected in the database. A: *Anopheles*; B: *Culiseta*; C: *Culex*; D: *Coquillettidia*; E: *Aedes/Ochlerotatus/Dahliana*; F: *Orthopodomyia*.

Mosquitoes exploit a variety of temporary and more permanent freshwater and saline habitats, subterranean water sources as well as container habitats and dendrolimnic (tree hole) habitats (Medlock & Snow 2008a). Table 1.1 provides an overview of the preferred habitats (categories 1-6), voltinism, seasonal activity patterns and reported general feeding preferences of 19 mosquito species commonly reported in the literature. The distribution of UK mosquitoes is largely determined by the availability of larval development sites, although factors such as climate that may modulate occurrence have not been investigated at a national level. Within the current literature, coastal, marshy saline areas (category 2) exploited by species such as *Oc. detritus* and *An. atroparvus* have received particular attention (Snow 1987). This is largely due to the proximity of human habitation to biting populations and in the case of the latter species, studies of the transmission of *P. vivax* malaria (see discussions in Marshall, (1938); Ramsdale & Snow, (1995)). Of those species colonising container habitats, the *Cx. pipiens* complex has received a substantial amount of attention, not least due to uncertainty surrounding the influence of genotype and hybridisation on field phenotype (Malcolm 2009; Cranston et al. 1987; Curtotti 2009). The prevalence and abundance of *Cx. torrentium* remains less well understood and has not been satisfactorily separated from that of the *Cx. pipiens* complex due to morphological similarity (a difficulty that persists in Europe as a whole (Hesson et al. 2011)). There is also some limited evidence pointing to the increasing use of container habitats by the normally dendrolimnic species *Anopheles plumbeus* Stephens 1828 in the UK, particularly in urban areas (Townroe & Callaghan 2014). The trend of increasing exploitation of container habitats by this species in and around pig stables in Belgium has been responsible for significant local nuisance biting of humans in recent years (Dekoninck et al. 2011). However, in the absence of longer-term UK studies (Townroe and Callaghan (2014) conducted studies over two years) the existence of this trend in the UK cannot be confirmed.

Mosquito species	Habitat	Voltinism	UK seasonal activity adults larvae J F M A M J J A S O N D	Reported feeding behaviour (mammal, bird, reptile)
Aedes cinereus (Meigen 1818)	1	M		mammals, birds
Anopheles atroparvus (van Thiel 1927)	2	M		mammals, birds
Anopheles claviger (Meigen 1804)	5	M		mammals
Anopheles messeae (Falleroni 1926)	5	M		mammals, birds
Anopheles plumbeus (Stephens 1828)	6	B		mammals, birds
Coquillettidia richiardii (Ficalbi 1889)	5	U		mammals, birds
Culex pipiens f. pipiens (Linnaeus 1758)	3	M		birds
Culex pipiens f. molestus (Forskål 1775)	4	M		mammals, birds
Culex torrentium (Martini 1925)	3	M		birds
Culiseta annulata (Schrank 1776)	3	M		mammals, birds
Culiseta litorea (Shute 1928)	5	U		mammals, birds, reptiles
Culiseta morsitans (Theobald 1901)	5	U		mammals, birds, reptiles
Ochlerotatus annulipes (Meigen 1830)	1	U		mammals
Ochlerotatus cantans (Meigen 1818)	1	U		mammals, birds
Ochlerotatus caspius (Pallas 1771)	2	M		mammals
Ochlerotatus detritus (Haliday 1833)	2	M		mammals, birds
Ochlerotatus flavescens (Müller 1764)	1	U		mammals
Ochlerotatus punctor (Kirby 1837)	1	U		mammals, birds
Ochlerotatus rusticus (Rossi 1790)	1	U		mammals

Table 1.1: The ecology of commonly-reported UK mosquitoes after Marshall (1938); Medlock (2015); Medlock et al. (2005); Service (1969a); Service (1971b); Medlock & Snow (2008); Cranston et al. (1987); Snow (1990); Medlock & Vaux (2015b). Habitat types after Medlock & Snow (2008): (1) temporary freshwater pools, (2) temporary saline pools, (3) artificial water collections, (4) underground water, (5) permanent ground water, (6) tree holes. Voltinism: (U) univoltine, (B) bivoltine, (M) multivoltine.

Mosquitoes in the UK exhibit species-specific seasonal activity patterns (Marshall 1938; Cranston et al. 1987; Snow 1990; Medlock & Vaux 2015b; Service 1969a) (Table 1.1). Mosquitoes may either be univoltine (producing only one generation per year), bivoltine (producing two generations per year) or multivoltine (producing multiple generations per year). Univoltine species such as *Coquillettidia richiardii* Ficalbi 1889 emerge as adults during the spring, after which abundance declines throughout the summer. Bivoltine species such as *An. plumbeus* exhibit two peak periods of adult emergence, the first in May-June and the second in August-September. Multivoltine species include *Cs. annulata* and *Cx. pipiens f. pipiens* and may display overlapping peaks in adult abundance with the emergence of adults of multiple generations between spring and late autumn. Recent seasonal data collected using artificial traps from Woodwalton fen in Cambridgeshire have provided evidence for *Ae. cinereus* Meigen 1818 being multivoltine, producing peaks in abundance at the end of June and a second, larger peak, towards the end of September (Medlock & Vaux 2015b). This contrasts with an earlier study which showed a single peak in human biting activity in July followed by continuous adult population decline, leading to the conclusion that this species was univoltine (Service 1969a). Whether these differences are a result of population-level changes over time, field site location or result from a delayed emergence of a single egg batch (i.e. a univoltine population with staggered emergence due to incomplete submergence in early habitat re-wetting) is unclear.

Overwintering mechanisms are also species-specific and can occur at the egg stage (all *Aedes* spp., the majority of *Ochlerotatus* spp.), as larvae (e.g. *An. claviger*, *Cq. richiardii*) or as adults (e.g. *Cx. pipiens f. pipiens*, *Cs. annulata*) (Snow 1990; Cranston et al. 1987). Some species will overwinter at different stages depending on environmental conditions. For example, *Dahliana geniculata* Olivier, 1791 will overwinter either as eggs or larvae, although only the eggs can withstand freezing, whilst the egg rafts, all larval instars, pupae and adults of *Cs. annulata* have been observed throughout the year (Cranston et al. 1987). Larvae of certain species such as *An. plumbeus* can survive using surface respiration alone when the water in their tree hole habitats ices over (Snow 1990). Fertilised females of *Cx. pipiens f. pipiens* and *Cs. annulata*

overwinter inside artificial structures such as stables or barns, the former species entering into hibernation from as early as August (Service 1969a; Onyeka & Boreham 1987). Males of *Cs. annulata* have also been observed between October and February in the UK (Service 1969a). During hibernation, mosquitoes undergo considerable mortality caused by fungi (e.g. those of the genera *Cephalosporium* and *Entomophthera*), depletion of fat reserves and predation by spiders such as *Meta* spp. (Service 1969a; Onyeka & Boreham 1987). *Culex pipiens f. pipiens* and *An. messeae* can undergo complete hibernation, relying on nutrient stores in their fat bodies to last them until the breaking of hibernation in the spring. Fat reserves may decrease by up to 80% over hibernation for *Cx. pipiens f. pipiens* (Onyeka & Boreham 1987). *Anopheles atroparvus* and *Cs. annulata* undergo incomplete hibernation (although *An. atroparvus* is also capable of complete hibernation), in which females periodically become active and blood-feed in order to replenish their fat reserves (Ramsdale & Wilkes 1985). Increases in water temperature and prolonged periods of light exposure are key factors in stimulating larval development and the termination of adult hibernation respectively, upon arrival of spring (Cranston et al. 1987). *Culex pipiens f. molestus* alone does not undergo winter hibernation, with adult activity occurring year-round in permissive subterranean habitats (Snow 1990).

1.1.3 Host-seeking, biting and feeding behaviour

The host seeking behaviour of UK mosquitoes is poorly understood, despite being a major focus of investigations of behaviour worldwide (see Gibson & Torr (1999); Pickett et al. (2010); Takken & Verhulst (2013) for reviews). Studies have investigated host-seeking, biting and blood feeding behaviour primarily using human landing catch collections, with available information in large part derived from studies focused on *Oc. cantans* only, as mentioned previously (see review by Service, (1972)). One advantage of direct collection from baits is that it allows recording of subtle variations in behaviour. *Coquillettidia richiardii*, for example, was observed to arrive at human bait in 'waves' of several individuals interspersed with periods of no arrivals (Service 1969a). This species also rested on nearby vegetation for up to five minutes if disturbed before attempting to feed again and took approximately 3.5 minutes to feed, slightly longer than the two-three minutes taken by *An. plumbeus* and *Aedes* species also observed taking blood meals (Service 1971b).

The use of other potential vertebrate hosts of UK mosquitoes in trapping studies is far more limited, with rabbits (Service 1969c; Service 1971a), mice and a chicken (Service 1969c) the only other species to be utilised. In many cases the studies trialled different trap types with different hosts over different numbers of trap nights, therefore precluding direct comparison of trap efficiency or host attractiveness. For example, Trinidad number 10 traps, a baffle-based trap design, were used with mice as bait, with collections over 20 trap days and 36 trap nights yielding eight *Oc. detritus* and 40 *Cq. richiardii* (Service 1969c). In the same study, rabbits and a chicken were placed, separately, in cylindrical-type traps. Rabbit-baited traps collected 833 mosquitoes of nine species whilst the chicken-baited trap collected only 12 *Culex pipiens* s.l. and 8 *Culiseta morsitans* Theobald 1901; rabbits, however, were used over 32 trapping sessions whilst the chicken was used only in 13, and each was run on different days (Service 1969c). The same study did directly subsequently compare rabbit and chicken baited traps to avoid the latter issue by placing the baited traps 5m apart on the same trap night. In this case, 96 mosquitoes of seven species were collected in the rabbit-baited traps whilst none were collected from the

chicken-baited trap. Similarly, direct collections from a tethered rabbit yielded 206 mosquitoes of ten species whilst yielding none from a tethered chicken (Service 1969c).

There are two further important limitations of this and a further host-baited trap study (Service 1971a). Firstly, baited collections were conducted either from ground level or within about 1 metre from it and thus did not account for potential vertical differences in mosquito flight and host-seeking behaviour. In separate collections from suction traps placed at increasing height from the ground, the majority of the total *Cx. pipiens* s.l. and *Cs. morsitans* were found in the highest trap (550 cm) whilst the majority of *Oc. cantans* were collected at 23 cm from the ground (Service 1971c). Secondly, only a single bait animal was used in the collections. Variation in attraction of host-seeking mosquitoes to individual humans has been demonstrated worldwide in several species, including representatives of the genera *Aedes* (Khan et al. 1970; Logan et al. 2008), *Anopheles* (Knols et al. 1995; Lindsay et al. 1993) and *Culex* (Knols et al. 1995). These variations are in part considered to be a result of differences in the volatile odour profiles produced by each host (see Takken & Verhulst, (2013); Zwiebel & Takken, (2004) for reviews). Although Khan et al., (1970) did not find significant individual differences between animals of the same species, infection of birds with malarial parasites has been shown to influence the attractiveness of chronically infected birds to uninfected mosquito biting, indicating an ability of mosquitoes to distinguish between individual hosts of varying infection status (Cornet et al. 2013a; Cornet et al. 2013b). Taken together, these data suggest that mosquitoes are able to detect differences in the attractiveness of individual hosts across species groups and therefore highlight the importance of using multiple bait hosts where possible in trapping studies. The twelve human landing collectors used by Service, (1969a) remains the only use of multiple hosts to date in the UK.

The diel flight and biting periodicity of mosquitoes in the UK has been investigated in a limited series of studies primarily using human landing catches (Service 1969a; Service 1971c) with some use of suction traps (Service 1969b; Service 1971c). Available evidence indicates that

flight and biting behaviour is overwhelmingly crepuscular (Service 1969a). Occasional opportunistic daytime biting is generally associated with active disturbance of resting habitat by vertebrate hosts (Service 1971d). Service (1969a) demonstrated that human-biting mosquito species assemblages, including members of the genera *Anopheles*, *Culiseta* and *Ochlerotatus* displayed the greatest peak in biting activity around sunset with a smaller peak in biting occurring at sunrise. In a separate study, collections from six 24-hour human landing catches collected 82.1% of *Oc. cantans* in the period between 17:00 and 24:00 (Service 1977b). Some contrasting results have however been observed for UK populations, with distinct crepuscular biting peaks of *Cq. richiardii* observed by human landing catches (Service 1969a) whilst mean collections from rabbit-baited traps run during the day yielded almost twice the number of those run overnight, the latter incorporating sunset and sunrise (means of 12 and 6.8 per trap period, respectively) (Service 1969c). Detailed biting periodicity data exist only for eight UK mosquito species: *An. plumbeus*, *Cq. richiardii*, *Cx. pipiens* s.l., *Cs. annulata*, *Da. geniculata*, *Oc. cantans* and *Oc. punctor*. This is in part a reflection of the species availability at favoured field sites, but means that considerable gaps exist in the current understanding of flight and biting periodicity of the other UK species.

The majority of information on the feeding preferences of UK mosquitoes derives from analysing the blood meals of engorged individuals collected in the field, with the blood meal origin of twenty species investigated to date (Table 1.2). Seventeen UK species of six genera have been investigated using the precipitin test: *Anopheles* (2), *Aedes* (1), *Ochlerotatus* (8), *Culex* (2), *Culiseta* (3) and *Coquillettidia* (1) (Service 1969a; Service 1971b; Service 1971a; Onyeka & Boreham 1987; Curtotti 2009) or ELISA: *Oc. cantans* and *Oc. punctor* (Service et al. 1986; Renshaw et al. 1994). One of these studies (Renshaw et al. 1994), is the only one to date to combine blood meal data with host abundance and host size approximation as part of feeding index calculations (Kay et al. 1979) to compare preferential feeding of mosquitoes on different hosts. Local host abundance and defensive behaviour were found to influence feeding rates, with the largely opportunistic blood feeding species *Oc. cantans* preferentially feeding on horse

blood when one of two horses present in the area was sick, but shifting to feeding on cattle and sheep the following year when only the healthy horse remained. Both the precipitin test and ELISA require the preparation of specific anti-sera for potential blood-feeding host species, introducing a selection bias towards expected hosts (mostly humans, livestock and the most abundant wildlife species). Identification was frequently possible only to the level of broad groupings such as 'unidentified bovid' (i.e. the family *Bovidae*, including cattle, sheep and goats) or 'mammal' (Table 1.2). This is illustrated by the study conducted by Service (1971b) which could only identify 698/1416 (49%) of tested samples to broad categories (Table 1.2). Although feeding on at least nine mammalian species has been identified, the lack of resolution provided for bird feeding is of particular note and determination of mosquito feeding on specific bird species to date in the UK is limited to the feeding of both ecoforms of *Culex pipiens* s.l. on rock pigeon *Columba livia* Gmelin 1789 (Curtotti 2009).

Although farm-associated mammalian species including cattle, sheep, goats and horses have been identified as blood-feeding hosts for UK mosquitoes, UK mosquito populations closely associated with livestock farm environments have not been well characterised. Thus no studies have identified mosquito larval habitats found on livestock farms and the biting and feeding activity of these populations on humans, livestock and wild animals all found in close association on these sites. Elsewhere in Europe, this trend is continued; the inclusion of farms as field sites for mosquito studies has mainly been driven by the circulation of arthropod-borne viruses (arboviruses) (e.g. WNV on Italian horse farms (Sebastian et al. 2008)) with only limited inclusion of farms in studies targeted at understanding mosquito feeding behaviour (e.g. studies investigating the feeding behaviour of *Cx. pipiens* s.l. in Portugal (Osório et al. 2014)). Further afield, arbovirus outbreaks have also been the drivers for entomological studies on farm sites, for example Japanese Encephalitis virus (JEV) outbreaks on pig farms in Malaysia (Vythilingam et al. 1993).

Mosquito species	Human	Cow	Sheep	Goat	Sheep/goat	Horse	Pig	Deer	Dog	Rabbit	Bovid	Mammal	Reptile	Bird	Pigeon	Specific bird species
*Ae. cinereus/geminus**	✓[1,2]	✓[2]								✓[2,5]	✓[2]	✓[2]		✓[2]		
An. atroparvus	✓[6]	✓[7]		✓[6]				✓[6]		✓[7]						
An. claviger		✓[2]								✓[2,5]						
An. daciae	✓[6]	✓[6]		✓[6]		✓[6]		✓[6]						✓[6]		[6]*R. pennata#, L. meridonalis¥*
An. messeae	✓[6]			✓[6]				✓[6]						✓[6]		[6]*L. meridonalis ¥*
An. plumbeus	✓[1,2]	✓[2]								✓[1,2]	✓[2]			✓[1,2]		
Cq. richiardii	✓[1,2]	✓[2]								✓[1,2]	✓[2]	✓[2]		✓[1,2]		
Cs. annulata	✓[1,2]	✓[2]					✓[1,2]			✓[1,2]	✓[2]	✓[1]		✓[1,2]		
Cs. litorea	✓[1,2]									✓[2]		✓[1]	✓[1,2]	✓[1,2]		
Cs. morsitans	✓[1,2]	✓[1,2]								✓[2,5]	✓[2]	✓[1]	✓[1,2]	✓[1,2]		
*Cx. pipiens s.l.***	✓[1,2]									✓[2,5]				✓[1,2,8]		[8]identified feeding on a 'passerine bird'
Cx. pipiens f. pipiens															✓[7]	
Cx. pipiens f. molestus	✓[7]								✓[7]						✓[7]	
Cx. torrentium										✓[2]				✓[1,2]		
Da. geniculata	✓[2]	✓[2]									✓[2]					
Oc. annulipes		✓[2]														
Oc. cantans	✓[1,2,4]	✓[2-4]	✓[3,4]		✓[2]	✓[4]	✓[2]			✓[2-5]	✓[2]	✓[2]		✓[2,4]		
Oc. caspius		✓[2]			✓[2]						✓[2]					
Oc. detritus	✓[1,2]	✓[2]					✓[2]				✓[2]	✓[1,2]		✓[1,2]		
Oc. dorsalis	✓[2]	✓[2]				✓[2]	✓[2]			✓[2,5]	✓[2]					
Oc. flavescens		✓[2]			✓[2]						✓[2]					
Oc. punctor	✓[1,2]	✓[1-3]	✓[3]		✓[2]	✓[3]				✓[3,5]	✓[2]	✓[2]		✓[2]		

Table 1.2: Mosquito host preferences as identified by blood meal analysis studies in the United Kingdom, collated from the following references (in superscript): (1) Service (1969a), (2) Service (1971b), (3) Service et al. (1986), (4) Renshaw et al. (1994), (5) Service (1971a) (6) Danabalan et al. (2014) (7) Curtotti (2009) (8) Onyeka & Boreham (1987). **Aedes cinereus/geminus* not separated. **Studies did not separate the ecoforms of *Culex pipiens* s.l. ¥ Only 95% similarity in BLAST searches. # Non-native species, captive.

1.2 The economic and social importance of mosquitoes in the United Kingdom

1.2.1 Nuisance biting

Nuisance biting of humans by mosquitoes is a significant and often overlooked problem in some parts of the UK, with biting reported from both rural and urban environments as far north as Scotland (Malcolm 2009; Medlock et al. 2012). Tidally flooded coastal areas associated with *Oc. detritus* populations are frequently associated with nuisance biting as are sewage works, associated with biting by *Cx. pipiens f. molestus* (Malcolm 2009; Medlock & Vaux 2015a). Changes in land use which introduce new areas for colonisation may also contribute to nuisance biting. Methods to prevent biting nuisance include the wearing of topical repellent products (e.g. N,N-Diethyl-meta-toluamide: DEET), treatment of larval development sites with insecticides or biological agents by pest control workers and limited bed net usage in certain areas of England, such as the Isle of Sheppey, where known biting nuisance mosquitoes are abundant (Hutchinson & Lindsay 2006b). Six local council authorities in the UK maintain specific budgets for mosquito control, at a cost of between £50 and £50 000 per year (Medlock et al. 2012).

Four surveys have been carried out in order to assess the extent of biting nuisance in the UK: from 1969-1970 (Service 1970), 1985-1986 (Snow 1986), 1996 (Snow 1996) and in 2009 (Medlock et al. 2012). The most recent of these surveys identified five species as responsible for the majority of reports: *An. maculipennis* s.l., *Cs. annulata*, *Oc. detritus*, *Cx. pipiens* s.l. (attributed to the *molestus* ecoform) and *Oc. cantans*, although seven additional species, *An. claviger*, *Cq. richiardii*, *Da. geniculata*, *Oc. annulipes*, *Oc. caspius*, *Oc. punctor* and *Oc. rusticus* were also reported. The results of the most recent survey largely reflected those of the previous surveys although the authors noted a two-fold increase in the number of nuisance biting reports since the 1996 survey (Medlock et al. 2012). This may reflect an increased mosquito biting nuisance, but could also be due to increased public awareness of mosquitoes in

the UK generated by passive reporting schemes such as the Mosquito Recording Scheme which has operated since 2005[1].

1.2.2 Historical circulation of mosquito-borne pathogens in the UK

Historically, there is evidence for the circulation of three mosquito-borne pathogens of human importance in the UK: Tahyna virus (TAHV), Yellow fever virus (YFV) and *P. vivax*. Only one record exists for TAHV, where antibodies were detected in a wood mouse (*Apodemus sylvaticus* L.) and bank vole (*Clethrionomys glareolus* Schreber 1780) in Devon (Chastel et al. 1985). This study was not followed up and the status of the virus remains unknown in the UK. During the 19th century, prior to the link between mosquitoes and transmission of YFV being established, yellow fever was a frequent cause of illness and death of sailors on ships returning from tropical destinations (Ramsdale & Snow 1995). The water barrels used to hold drinking water in the hold at the time are hypothesised to have supported populations of the mosquito *Aedes aegypti* Linnaeus 1762 which perpetuated virus transmission among the sailors on board. In 1865, the only confirmed case of local YFV transmission in the UK occurred, resulting in the infection of up 30 residents of the port area of Swansea, Wales. These infections occurred within a week of the arrival of the ship *Hecla* from Cuba, on which many deaths from the virus were reported (Buchanan 1866). It has been suggested that *Ae. aegypti* from this ship were responsible for on-shore transmission to the indigenous population and that the death of the mosquitoes as winter approached led to the cessation of the outbreak (Ramsdale & Snow 1995). Since these reports, *Ae. aegypti* has only been reported once in the UK, collected together with *Orthopodomyia pulcripalpis* Rondani 1872 from a beech tree hole in Epping Forest, Essex (MacGregor 1919).

Until the early 20th century, malaria attributable to *P. vivax* infection was a significant cause of morbidity in the UK, particularly in low-lying marshland areas of Kent and Essex (Dobson

[1] Internet link currently archived, see http://webarchive.nationalarchives.gov.uk/20140714084352/http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/Mosquitoes/MosquitoRecordingScheme/

1989). Transmission was primarily attributed to the *Anopheles maculipennis* complex, chiefly *An. atroparvus* (see Dobson (1989) and Ramsdale and Snow (1995) for historical reviews and Hutchinson and Lindsay (2006) for discussion of historical mortality attributable to malaria). Several interrelated factors contributed to the eventual disappearance of malaria in the UK including drainage of marshland which reduced mosquito breeding habitats and improvements to housing, including drier, windowed rooms containing fewer occupants which reduced mosquito resting sites and accordingly indoor biting and human transmission rates (Hutchinson 2004; Ramsdale & Snow 1995). Since the early 20th century, only sporadic malarial cases have been reported, the largest attributable to *P. vivax* imported by servicemen convalescing in the Thames/Medway marsh area after World War I. This region supports large populations of *An. maculipennis* s.l., including *An. atroparvus*, and the result was a prolonged localised epidemic with 481 confirmed human cases between 1917 and 1921 (Ramsdale & Snow 1995; Shute & Maryon 1969).

1.2.3 Current and potential mosquito-borne pathogen threats

The agricultural industry, comprising arable and livestock farming, is of significant importance to the food security of the UK. In 2014, agriculture contributed an estimated £9.9 billion to the UK economy and provided 476 000 jobs (Defra 2015). Livestock holdings across the UK consist of an estimated 9.8 million cattle and calves, 33.7 million sheep and lambs, 406 000 pigs and 169.7 million poultry (Defra 2015). Almost one million horses are also kept in the UK as part of the equine industry which is valued at £3.8 billion per year (British Equestrian Trade Association 2011), a figure further boosted by the £3.45 billion contributed by the British horse racing industry (British Horseracing Authority 2013). A farm-associated outbreak of a pathogen could therefore have the potential to cause significant economic disruption in addition to medical and veterinary health issues.

At present there is no evidence of the transmission of mosquito-borne pathogens to humans, livestock animals or horses in the UK (Medlock, Snow, et al. 2007; Malcolm 2009;

Medlock & Leach 2015). With the exclusion of TAHV for which only limited data evidencing its circulation exist (Chastel et al. 1985), two wildlife pathogens transmissible by mosquitoes are known to circulate in the UK. The first of these is the myxoma virus, causative agent of the widespread and usually fatal disease myxomatosis in wild and domestic rabbit (*O. cuniculus*) populations. The virus was first introduced into the UK in 1953 for control purposes and within two years had killed 99% of the wild UK rabbit population (Armour & Thompson 1955; Hudson et al. 1955). Mainly transmitted by the rabbit flea *Spilopsyllus cuniculi* Dale 1878 in the UK, the mouthparts of fleas and other biting insects including mosquitoes may become contaminated with the virus upon feeding through a characteristic skin lesion (Fenner & Woodroofe 1953). Mechanical transmission to another rabbit may then occur upon a subsequent blood-feed.

Avian malaria is the second pathogen transmitted by mosquitoes currently circulating in the UK. Distributed worldwide, this group of at least 25 protozoan parasites of the genera *Plasmodium* and *Haemoproteus* can pose a risk to the health of wild and domestic birds, but does not pose a zoonotic threat (Lapointe et al. 2012). Occasional high-profile cases of mortality attributable to avian malaria have drawn some attention to the disease in the UK, such as the deaths of African black-footed penguins at London Zoo (Quintavalle Pastorino et al. 2015). However, the transmission and ecological impact of UK endemic species of this genus which include *P. relictum* and *P. circumflexum* remains poorly understood (Lachish et al. 2011b; Lachish et al. 2011a). Studies into the clinical responses to infection in various bird species are also limited; for example, *P. gallinaceum* causes severe, age-dependent clinical disease in chickens (*G. gallus*) (Williams 2005), whilst *P. relictum* caused variable disease outcomes in experimentally infected Passerines (Palinauskas et al. 2008). Complete sporogony of *P. relictum* has been experimentally shown in *Cx. pipiens f. molestus* suggesting that this mosquito, which is present in the UK, may play a role in natural transmission cycles (Valkiūnas et al. 2015), although the generally restricted distribution of this mosquito (Medlock 2015; Malcolm 2009) may preclude its widespread involvement.

The re-establishment of *P. vivax* malaria in the UK is considered to be a present but low risk (Lindsay et al. 2010) and currently it is thought that arboviruses pose the greatest threat of emergence (Gould et al. 2006; Medlock, Snow, et al. 2007; Medlock & Leach 2015). At least ten mosquito-borne arboviruses currently circulate in Europe (Table 1.3). These include those that are proven or suspected aetiological agents of disease in humans (WNV, USUTV, Sindbis virus (SBV), TAHV, Batai virus (BATV), Inkoo virus (INKV), Chikungunya virus (CHIKV) and Dengue fever virus (DENV)); horses (WNV, TAHV, Snowshoe Hare virus (SSHV)), livestock species (TAHV, BATV, INKV) and wildlife (SSHV, Lednice virus) (Lundström 1999; Medlock, Snow, et al. 2007; Hubálek 2008; Tomasello & Schlagenhauf 2013; Becker et al. 2012). Aside from one vector competence study testing the competence of *Oc. detritus* for JEV (Mackenzie-Impoinvil et al. 2015), the potential role of UK species as enzootic or bridge vectors of arboviruses is primarily based on their host preferences.

The basic reproduction number, R_0, is a fundamental model for infectious disease spread and is defined as the number of secondary cases a single infectious case produces in a completely susceptible population (Dietz 1993). This model was refined for malarial transmission and subsequently its entomological parameters consolidated into a related model of vectorial capacity (Garrett-Jones & Shidrawi 1969; Reisen 1989): $C = \frac{ma^2P^n}{-lnP}$, where C = vectorial capacity, m = number of female mosquitoes per person, a = mosquito biting rate in bites/host/day, P = daily survival rate of mosquito, n = the extrinsic incubation period, in days. The figure for biting rate is a squared factor in the model as two bites are required for transmission, one from the infective host and one to the new host; this shows that the model is particularly sensitive to changes in this parameter (Dye 1992). Therefore, in order to facilitate modelling of disease risk, it is essential to have accurate data for biting rates on epidemiologically relevant hosts and on the factors influencing these. Factors include environmental variables and mosquito host preference, the latter of which is a measure of the

range of and preferences towards feeding on different vertebrate hosts (Dye & Hasibeder 1986; Kingsolver 1987).

Virus	Classification	Primary vertebrate hosts	Medical/veterinary importance
West Nile virus	*Flaviviridae Flavivirus*	Wild birds. Mammals including horses and humans incidental hosts.	Limited avian mortality in Europe, equine febrile illness with ~25% mortality. Severe neurological disease in <1% human infections.
Sindbis/Sindbis-like viruses; includes Ocklebo virus, Pogosta virus, Karelia virus	*Togaviridae Alphavirus*	Birds (Passeriformes), occasionally rodents and amphibians.	Sporadic illness in birds, including mortality in chickens. Fever, malaise and potentially chronic arthritis in humans, no mortality.
Tahyna virus	*Bunyaviridae Orthobunyavirus*	Brown hares, hedgehogs, rodents.	Influenza-like illness in humans with occasional CNS involvement.
Batai virus	*Bunyaviridae Orthobunyavirus*	Pig, horse, ruminants, and isolations from wild birds.	Mild illness sheep/goats. Influenza-like illness in humans.
Inkoo virus	*Bunyaviridae Orthobunyavirus*	Mountain hares.	Influenza-like illness in humans.
Lednice virus	*Bunyaviridae Bunyavirus*	Birds, primarily of the order Anseriformes.	Unknown, avian fatalities not recorded
Usutu virus	*Flaviviridae Flavivirus*	Birds, particularly the Passeriformes.	Avian mortality recorded in several species. Limited neuroinvasive cases reported from Italy.
Chikungunya virus	*Togaviridae Alphavirus*	Humans as primary reservoirs during epidemics. Non-human reservoirs include monkeys, rodents and birds.	Fever, joint pain (also chronic), occasional neurological involvement with some deaths reported.
Dengue fever virus	*Flaviviridae Flavivirus*	Humans.	Serotype 1 recorded from Europe. Cases range from asymptomatic to severe haemorrhagic fever.
Snowshoe hare virus	*Bunyaviridae Orthobunyavirus*	Snowshoe hare, voles, lemmings.	Non-fatal encephalitis in horses. Fever and occasional CNS involvement in humans.

Table 1.3: Mosquito-borne arboviruses of medical and veterinary importance currently circulating in Europe after Becker et al., (2012); Hubálek, (2008); Lundström, (1999); Medlock, Snow, et al., (2007); Pecorari et al., (2009); Tomasello & Schlagenhauf, (2013). CNS = central nervous system.

1.3 The changing environment and potential impact on mosquito populations The global climate is changing; current predictions indicate that the planet will experience average temperature increases of at least 1.5°C by 2100, accompanied by more frequent extreme weather events (IPCC 2013). The issue of anthropogenic climate change has garnered considerable controversy worldwide and its impacts are difficult to predict (see Karl & Trenberth, (2003); Rohr et al., (2011) for reviews). In the UK the potential effects of climate change on the UK mosquito fauna and mosquito-borne pathogen risk to the country are similarly complex, and have been the subject of several recent reviews (Snow & Medlock 2006; Gould et al. 2006; Gale et al. 2009; Medlock & Leach 2015). Detailed predictions are difficult to make owing to the complex and interrelated ecological, entomological and host parameters which may lead to both increases and reductions in the suitability of conditions for mosquito populations. This uncertainty is illustrated by the potential effects of drought on mosquitoes and mosquito-borne pathogens (see Stanke et al. (2013) for a detailed review). Short term periods of drought may lead to the aggregation of birds at a limited number of nutrient-rich aquatic habitats capable of supporting mosquito development. High mosquito abundance in these habitats may be facilitated by a lack of competitor species and predators if these are unable to survive the increasingly inhospitable local conditions. These factors may increase local mosquito biting rates and the risk of successful enzootic arbovirus circulation if an infected bird and suitable numbers of susceptible birds were to be among those present in the area (Brown et al. 2014). However, this situation would be unlikely to persist with longer periods of drought as larval habitats dry up completely (Snow & Medlock 2006). Post-drought re-wetting of habitats in the USA has been observed to lead to rapid increases in the populations of *Anopheles quadrimaculatus* Say 1824 which are able to develop faster than their conspecific competitors and predators (Chase & Knight 2003).

In addition to influencing the ecology of indigenous mosquito species in the UK, climate change could increase the suitability of the UK for the establishment of exotic mosquito species (Vaux & Medlock 2015). Five invasive species, *Ae. aegypti*, *Aedes albopictus* Skuse 1894,

Aedes atropalpus Coquillet 1902, *Aedes japonicus* Theobald 1901 and *Aedes koreicus* Edwards 1917 are currently established in Europe (Medlock et al. 2015). Figure 1.3 shows the distribution of two of these, *Ae. aegypti* and *Ae. albopictus* as of July 2015.

These exotic species pose a serious biting nuisance but can also serve as arbovirus vectors. A DENV outbreak on the island of Madeira, Portugal in 2012 (Sousa et al. 2012) was facilitated by *Ae. aegypti* populations established there only six years before (Almeida et al. 2007). *Aedes albopictus* has been responsible for nuisance biting reports across many parts of Europe and has facilitated recent autochthonous outbreaks of both DENV (Marchand et al. 2013) and CHIKV (Delisle et al. 2015) in France. The UK climate is not considered suitable for the establishment of *Ae. aegypti* until at least 2100 (Thomas et al. 2011). However, short-term adult activity may be possible in the context of the historical transmission of YFV highlighted previously, where an "almost tropical heat" was reported, with temperatures of up to 35°C recorded during the month of the outbreak (Buchanan 1866). Additionally, the concentration of heat in so-called 'urban heat islands' which may increase temperatures in cities like London to 8.9°C above that in surrounding rural areas (Kolokotroni & Giridharan 2008) could lead to permissive conditions for temporary establishment.

In contrast to *Ae. aegypti*, large areas of southern UK are considered to be suitable for the establishment and between 4-6 months of adult activity of *Ae. albopictus* (Medlock et al. 2006). This species has experienced a rapid range expansion in mainland Europe, with, for example, rapid northward dispersal in France from initial incursion points in the south (Roche et al. 2015). In 2015, *Ae. albopictus* has been reported as far north as Paris, shortly after the production of the distribution map in Figure 1.3 (Promed 2015). Factors related to increased globalisation are most likely to facilitate the accidental import of this mosquito into the UK in future. The global trade in used tyres is a potentially important mechanism of entry; as of 2002 at least 17 mosquito species, including the five exotic *Aedes* mentioned above, have been detected in used tyres in Europe (Schaffner 2003). The trade in plants such as lucky bamboo

(*Dracaena* sp.) has also been associated with the import of *Ae. albopictus* into the Netherlands (Schaffner et al. 2004) and presents a further potential entry mechanism.

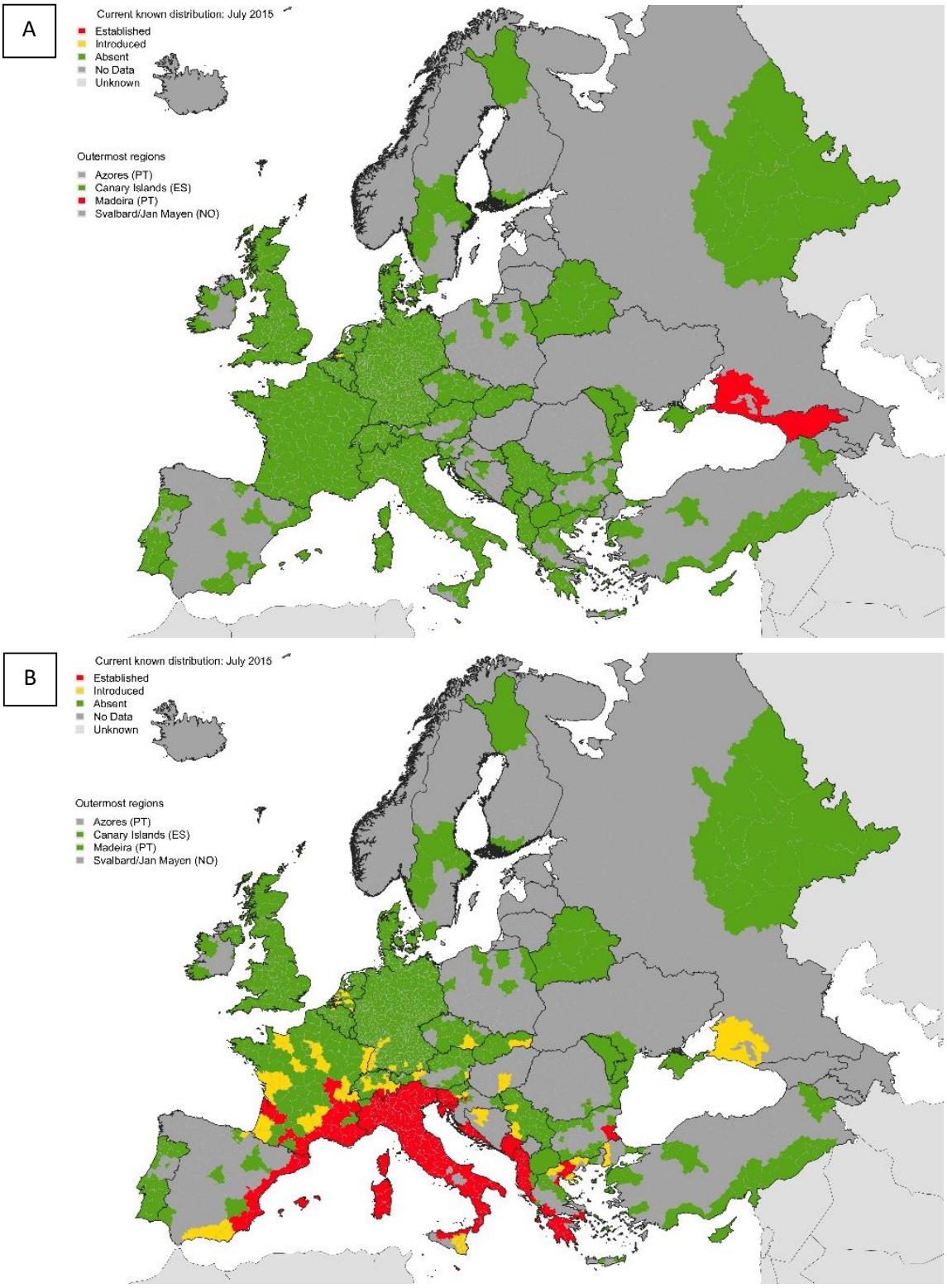


Figure 1.3: (A) European distribution of *Aedes aegypti* and (B) *Aedes albopictus* as of July 2015. Source: ECDC-EFSA 2015/VECTORNET

Intentional and unintentional anthropogenic land use changes may also influence the availability of aquatic habitat suitable for mosquito colonisation and thus the associated risk of nuisance biting and vector populations nearby (Medlock & Vaux 2011). Historically, large areas of marshland in England were drained which inadvertently contributed to the decrease in indigenous malaria (reviewed in Hutchinson (2004)). Wetlands now only occupy only 10% of their estimated original area in the UK (Hulme 2008). At present, extensive reclamation of arable farmland is taking place for the purposes of restoring wetlands in the east of England, such as those that are part of the Great Fen project[2]. This project aims to enhance biodiversity resources and mitigate flood risk by connecting and consolidating patchworks of existing wetland (Hulme 2008). In like manner, wetlands in or adjacent to urban environments are also being created with particular focus on local biodiversity, public wellbeing and sustainable sewage treatment, the latter by incorporation of reed beds which lead to the breakdown of pollutants such as phosphorus and ammonia (Medlock & Vaux 2014a; Soil Association 2006). Evidence indicates that wetland creation can increase the number of available habitats, particularly for *Cx. pipiens s.l./torrentium*, both transiently during the construction phase and more permanently, particularly in reed bed areas with reduced water turbulence and nutrient enrichment from sewage (Medlock & Vaux 2014a). Newly created fenland in close proximity to human habitation and livestock holdings is a very real possibility; obtaining an understanding of mosquito feeding patterns on farm-associated hosts is therefore important.

[2] See http://www.greatfen.org.uk/

1.4 Overview of PhD aims, objectives and hypotheses

This thesis aims to understand the biting and feeding behaviour of farm-associated mosquitoes on vertebrate hosts within farm environments in the UK using a combination of field and laboratory studies. Specific objectives and hypotheses for each chapter are presented below.

Chapter 2 – Methodological development

Details a pilot trapping study conducted in 2012 with the objective of establishing the presence of mosquitoes on a selection of field sites and to facilitate the selection of farms for future intensive studies in this thesis. This chapter additionally details the optimisation of molecular techniques for species-level identification of mosquitoes and for blood meal analysis, both of which are used throughout the thesis.

Chapter 3 – Mosquito biting patterns on humans

Describes a multi-collector human landing catch study conducted on four farms during July and August 2013. The objectives of this study were firstly to determine the highest anthropophilic biting rates experienced during the summer by farm-associated mosquito population assemblages and secondly to determine the influence of meteorological variables on the human biting rate within this period.

Hypothesis 1: Meteorological variables, including wind speed and air temperature, will significantly influence the biting rate on humans within farms.

Hypothesis 2: The mosquito biting rate and species composition responsible for biting humans will differ between farms.

Chapter 4 – Mosquito biting patterns on birds

This chapter examines the construction and operation of two chicken-baited traps at four UK farms between June and October 2013. The objective was to identify the seasonal ornithophilic host-seeking patterns of farm-associated mosquito population assemblages at two heights,

approximately 1 m from the ground and at 4 m from the ground, at the height of tree cover. Six chickens were used as bait and as proxies of bird-biting behaviour occurring on wild and domestic avian hosts.

Hypothesis 1: The biting rate and species assemblages of mosquitoes on birds will differ between trap heights.

Hypothesis 2: The mosquito biting rate and species composition responsible for biting birds will differ between farms.

Chapter 5 – Molecular species identification, host preference and detection of myxoma virus in the Anopheles maculipennis complex (Diptera: Culicidae) in southern England, UK

Describes the development and application of a tripartite approach with the objective of maximising the data that can be obtained from a single blood-fed *Anopheles maculipennis* complex specimen. Following a single DNA extraction step from the abdomen, the vertebrate origin of the blood meal is identified, the mosquito is identified to species level and those specimens identified as having fed on rabbits (*Oryctolagus cuniculus*) were tested for the presence of myxoma virus.

Hypothesis 1: A single DNA extract from the abdomen of a mosquito yields sufficient mosquito DNA, vertebrate blood meal DNA and myxoma virus DNA for detection.

Hypothesis 2: Mosquitoes feed on both myxoma virus-infected and uninfected, healthy rabbits.

Chapter 6 – The host selection and feeding preferences of farm-associated mosquitoes

Details the systematic collection of blood-fed mosquitoes from a modified resting box design and other artificial resting sites at one high mosquito-abundance farm. The objective of this study was firstly to understand the influence of meteorological variables and within-site location on the number of mosquitoes collected by the resting boxes. Secondly, the host range and

feeding preferences of farm-associated mosquitoes were determined by molecular blood meal analysis of engorged specimens followed by comparison with host abundance data. The final objective was to determine the likelihood of successfully identifying blood meal host depending on the stage of blood meal digestion within field-caught mosquitoes classified according to the Sella scale of oogenesis.

Hypothesis 1: Meteorological variables over the 12 hours preceding collections from the resting boxes significantly influence the number of blood-fed mosquitoes collected.

Hypothesis 2: Mosquitoes display feeding preferences for specific vertebrate hosts.

Hypothesis 3: The stage of blood meal digestion within blood-fed mosquitoes will influence the rates of success for identifying blood meal host.

Chapter 7 – Final discussion

This chapter summarises the findings of the thesis and provides suggestions for future work.

Chapter 2 – Methodological development

2.1 Introduction and rationale

At present no quantitative data exist that characterises mosquito population assemblages associated with livestock farms in the UK. Therefore, an important first step in this thesis was to identify, by way of pilot trapping studies, a selection of farms supporting populations of potential mosquito-borne pathogen vector species for use during further intensive studies. In addition to the presence of potential vector species, the logistical feasibility of conducting trapping studies, the seasonality of mosquito populations and the epidemiological relevance of farm-associated vertebrate hosts were assessed during selection of sites for future study. Furthermore, molecular assays enabling taxonomic identification of mosquito species and the origin of vertebrate blood meals in engorged mosquitoes were trialled. Several of the 34 mosquitoes recorded in the UK are known to exist as members of morphologically cryptic species complexes (*Anopheles maculipennis* s.l.) (Linton et al. 2005); as sibling species only separable on the basis of the structure of the male genitalia (*Culex pipiens s.l./Culex torrentium*, *Aedes cinereus/geminus*) (Medlock & Vaux 2009; Cranston et al. 1987); or as morphologically identical 'ecoforms' which exhibit only slight genetic variations by which their separation is possible (*Culex pipiens f. pipiens/Culex pipiens f. molestus*) (Danabalan et al. 2012). Preliminary adult mosquito collections were therefore essential to determine which of the cryptic mosquito species were present, their relative abundance to each other and thus which molecular assays required development in order to facilitate separation to species level. Molecular identification to the level of species is important when conducting mosquito behavioural studies as closely related mosquito species may exhibit clear differences in feeding behaviour with important implications for pathogen transmission cycles (see Takken & Verhulst (2013) for a review). Blood meal analysis was designed to provide data on the host selection and preference of farm-associated mosquito populations. It was necessary to develop and optimise the whole sample processing workflow, including sample transport and storage, DNA extraction and running the blood meal assay itself prior to conducting intensive blood-fed mosquito collections in the field.

The first part of this chapter details the initial recruitment of seven farms to this project, followed by the methodology and results of the pilot mosquito collection study conducted on them to investigate the presence of larval habitats, adult populations and any human biting species assemblages. This is followed by the details of the molecular methodologies developed for species identification as guided by the results of the pilot study, and the development of an optimised work flow for the identification of blood meal host. Finally, the discussion draws together the results of the pilot study in informing the selection of farms for future work. Detailed site maps including trap locations for all the studies conducted in this thesis are provided at the end of the chapter.

Aim of the chapter

To characterise, in a pilot study, the mosquito species diversity, abundance and seasonality on seven farms to facilitate the selection of sites for future intensive mosquito behaviour studies and to optimise appropriate molecular techniques for mosquito species identification and blood meal analysis.

Objectives

1. To identify mosquito larval habitats present on seven farms and to determine the mosquito species supported by them by conducting presence/absence larval dipping
2. To identify seasonal trends in the abundance of mosquito species present as adults on each of the seven farms by conducting light trapping at three different time points
3. To identify if and at what time of day human biting by farm-associated mosquitoes occurs by conducting human landing catches in the morning, afternoon and evening
4. To optimise appropriate molecular techniques for mosquito species identification depending on the specimens collected in the preliminary trapping
5. To optimise a molecular assay for the identification of vertebrate blood meal origin in blood-fed mosquitoes

2.2 Selection and mapping of farm sites

Recruitment and initial selection of farms

Eight farms were recruited from existing contacts held by the Entomology Group at The Pirbright Institute (TPI) and through online searches according to defined selection criteria (see below). Initial contact with the farms was made via email or telephone followed up with a preliminary visit to obtain consent for the study. An informed consent form was provided to each farm and signed prior to commencement of the work.

Farms were initially selected according to the following criteria:

1. **Location.** All farm sites were selected from southern England due to the epidemiological relevance and logistical feasibility of sampling. Risk assessment exercises have identified this region of the UK to be at greatest risk of incursion by vector-borne pathogens including West Nile virus (WNV) (Bessell et al. 2014), *Plasmodium vivax* malaria (Lindsay et al. 2010) and the establishment of exotic vector species such as *Aedes albopictus* (Medlock et al. 2006). Additionally, bluetongue virus (BTV) was first detected in the south of England in Ipswich, Suffolk, with wind-borne movement of infected vector *Culicoides* from continental Europe considered to be the likely route of entry (Gloster et al. 2008). In addition, the sites used were located within two hours driving distance from TPI which improved the feasibility of transport of materials and live hosts for study (chickens; chapter 4). Limits for transport of chickens were discussed with regard to ethical concerns with a Home Office inspector and subsequently limited travel to four hours/day.
2. **Livestock species present (cattle, sheep and/or horses).** For the preliminary trapping in this chapter farms were deemed acceptable if they kept livestock species including sheep, cattle, pigs or poultry; subsequently (Chapters 3, 4, and 5) sites were selected that all contained cattle in order to introduce a level of standardisation of host availability and associated resources (e.g. barns, feeding and watering troughs). While

other livestock species and farm-associated animals (e.g. dogs) were noted, they did not form part of the selection criteria.

3. **No active mosquito control activities.** Sites used did not employ routine spraying of sampling areas with insecticides (although residual exposure from crop spraying could not be entirely excluded). Selection did not consider the use of personal protection measures such as repellent use or bed nets.
4. **Logistical feasibility.** Willingness to allow full access to farm property for the planned duration of mosquito collection studies was assessed via interview prior to initiation of studies. Access was also examined and the issue of use outside 9am-5pm was discussed along with the risk of lone working.

Production of site maps

Inter- and intra-farm differences in host and habitat availability were systematically characterised at each holding. Ordnance Survey (OS) base maps were obtained for each farm site from the OS online Getamap service (https://www.ordnancesurvey.co.uk/shop/os-maps-online.html) (see site maps at the end of the chapter). Each map shows the boundaries of the farm highlighted in red (for large sites where the boundaries extend beyond the limits of the map these outlines are omitted), a 'mini-map' in the top left indicating the farm's location in the southern part of the UK, a north arrow, a scale bar and information on whether fields are used for grazing or arable purposes. Digital photographs of trapping locations and potential larval mosquito habitats were taken for reference purposes as appropriate.

In addition to standard photographs, 360° panoramic photographs were taken using Google Photosphere™, an android smartphone application (native to the Google Nexus smartphone range, models 4 and above). The resulting panoramic 'photospheres' can be viewed either on the phone itself or uploaded to Google maps to be shared. The benefit of the photospheres is that they provide a complete view of a given point, for example a trapping location, which shows all the environmental features in the immediate vicinity. This provides

more information than standard unidirectional photographs. Public uploading to Google maps is currently the primary method of sharing photospheres (short of lending the phone), however a recently developed online viewer at http://photosphereviewer.net enables a photosphere file to be uploaded and viewed without compromising the privacy of the farms. Photosphere files are included on CD with this thesis to provide additional information on trap locations for Chapters 3 and 5.

Description of farms

The seven farms initially selected are listed below with the following information to facilitate site comparison with any future studies: location (co-ordinates); approximate land area of the holding (acres); Nomenclature of Territorial Units for Statistics 3 (NUTS3); and soil type descriptions (categories 1-27, see appendix A2) according to Cranfield University's Soilscapes maps (http://www.landis.org.uk/soilscapes/). Approximate land cover information is provided according to the National Land Use and Cover database (after Harrison (2006); see additional information in appendix A3) as estimated using Google earth and http://www.mapdevelopers.com/area_finder.php (Table 2.1). Additionally, approximate numbers of livestock animals maintained on site is provided, together with observations on the wildlife on each site. Finally, as an estimate of the human presence on the farms, human activity on site was classified as either low (access limited mainly to farm owners/workers on site), or high (large amounts of public access to the sites). Finally, logistical information concerning the ease of access to the farms for the collections is provided.

ADAS Arthur Rickwood

ADAS is a private business offering agricultural and environmental consultancy and research services. The Arthur Rickwood site covers approximately 0.4 km^2 and maintains 800-1000 sheep within secure grazing areas on fenland near Ely, Cambridgeshire (52.422560, 0.098302, NUTS3: UKH12, soilscape category 23). The flock of sheep is maintained as a 'high-

health-herd' with strict biosecurity measures, requiring that mosquito traps be set up on the perimeter of the farm and meaning that access to the sheep unit complex itself was only possible after passing through a secured area with disinfectant shoe dips. In accordance with the strict biosecurity measures and restricted access to the public, human activity on site is classified as low. Wildlife observed included British wild birds of the order Passeriformes and European rabbits (*O. cuniculus*).

Coombelands Farm

Coombelands Farm is a mixed livestock farm of approximately 0.3 km^2 containing small numbers (<100) of high-health sheep, cattle and pigs plus <10 horses maintained as part of Animal and Plant Health Agency (APHA) property in New Haw, Surrey (51.360242, -0.499163, NUTS3: UKJ25, soilscape category 6). Access to the site is restricted to those employed by the APHA Weybridge site and relevant on-site subsidiaries and therefore human activity is considered to be low, although staff members with general access do technically have access to the farm site. Wildlife observed included British wild birds of the orders Passeriformes and Columbiformes and European rabbits.

Church Farm

Church Farm is a mixed livestock farm of approximately 1.7 km^2 in Oxfordshire comprising sheep (~1400), cattle (~90) and horses (~8) situated near the village of Northmoor, Oxfordshire (51.715807, -1.380813, NUTS3: UKJ14, soilscape category 20) and surrounded by other agricultural holdings. Access to the site is restricted by the owners and human activity is therefore classified as low, limited to those living, working or visiting the property. Wildlife observed included British wild birds of the orders Passeriformes and Columbiformes, red foxes (*Vulpes vulpes* L.) and European rabbits.

Northney Farm

Northney Farm is a dairy (120 head Aryshire cows) and arable farm of approximately 2.2 km^2 in the north part of Hayling Island, Hampshire (50.828166, -0.962151, NUTS3: UKJ35, soilscape categories 6 and 22). Publically accessible areas include a tearoom close to the cattle barns and public bridleways which were observed to be heavily used for dog walking; accordingly the site is classified as having high levels of human activity. The UK's longest-running mosquito control program takes place on Hayling Island, run by Havant Borough council; however control measures which consist of periodic application of *Bacillus thuringiensis* var. *israelensis* (*Bti*) to larval breeding sites in publically-accessible areas do not take place on farm property. Wildlife observed included British wild birds of the orders Passeriformes, Columbiformes and (owing to the coastal location), numerous Charadriiformes such as gulls (Family: *Laridae*).

Elmley Nature Reserve

Elmley National Nature Reserve (henceforth referred to as Elmley) is a coastal freshwater grazing marsh covering an area of 12 km^2 in the Thames estuary region of Kent (51.377587, 0.783954, NUTS3: UKJ43, soilscape categories 18 and 21). Cattle and sheep are maintained across the whole site, with between 30-100 cattle kept specifically in the fields within 2 km of the central area as part of 750 maintained site-wide. Approximately 100 sheep are moved into this same area in November but are normally in fields more than 2 km from the central area. Public access is available to the site year round and accessed from the central area where the majority of farm buildings and living quarters are situated. Elmley is popular primarily for bird-watching but also hosts events in the on-site barn and visitors for so-called 'glamping' in three shepherds' huts; human activity is therefore classified as high. Approximately 20 chickens are kept close to the housing in two coops and many British and migratory birds are known to use the marsh to breed or as a migration stopover to more northerly regions. Nesting birds are protected from mammalian predators such as foxes by means of a boundary fence

spanning land and water, with an additional annual winter relocation programme of hedgehogs (which pose a threat to ground-nesting birds) back to the mainland. The Thames estuary region is known to have been a historical hotspot for indigenous *P. vivax* malaria transmission. Although no transmission currently occurs, houses on the farm site are still equipped with mosquito bed nets to reduce nuisance biting by mosquitoes.

Glendell Livery and riding school

Glendell Livery is located in a mixed woodland area of Pirbright, Surrey (51.290499, -0.652256, NUTS3: UKJ25, soilscape category 15) occupying an area of approximately 0.1 km^2. Ten-fifteen horses are kept on the site, which are visited on a daily basis by their owners and members of the riding school. Public access is possible by means of public bridleways crossing the site and, in combination with the maintenance of the horses, the site is classified as having high levels of human activity. Wildlife observed included British wild birds of the orders Passeriformes and Columbiformes and European rabbits. This site has been used for several prior entomological studies at TPI, primarily targeting *Culicoides* spp.

Mudchute Farm

Mudchute Farm is the largest urban farm within inner London and one of the largest in Europe, encompassing 0.13 km^2 of land (51.491737, -0.009367, NUTS3: UKI42, soilscape category 21). The site is a fully working farm with small numbers (<10) of each of cattle, sheep, llamas, goats and pigs. Additionally, the farm hosts a stables and a riding school which houses 25 horses. Public access to Mudchute is either direct, through visits to the farm or riding school, or indirect by use of thoroughfares passing through the farm grounds. The latter are open throughout the night and are heavily utilised by the public. Accordingly, the site is classified as having high levels of human activity. Observed wildlife mainly comprised British wild birds of the orders Passeriformes and Columbiformes although a red fox was also observed.

Numerical category	Description	Land cover per farm (%)						
		ADAS Arthur Rickwood	Coombelands Farm	Church Farm	Elmley	Glendell Livery	Mudchute Farm	Northney Farm
C011	Field crops	0	0	0	0	0	0	50
C021	Improved grass	64	80	95	<1	90	85	20
C022	Unimproved grass	7	4	<1	5	1	0	<1
C023	Recreational grass	0	0	<1	<1	0	<1	<1
C033	Broadleaved woodland	1	0	0	<1	5	0	<1
C061	Standing water	<1	<1	<1	<1	<1	<1	<1
C062	Running water	10	<1	2	2	<1	0	1
C063	Freshwater marsh	0	0	0	80	0	0	20
C073	Salt marsh	0	0	0	10	0	0	5
C080	Building	4	7	<1	<1	<1	2	2
C091	Metalled roadway	4	5	<1	<1	<1	0	<1
C093	Pathway	<1	<1	<1	<1	<1	3	<1
C094	Other made surface	5	4	<1	<1	<1	2	<1
C101	Multiple surface	4	0	<1	<1	0	7	<1

Table 2.1: Estimated land cover (%) on the farms according to the categories of The National Land Use and Cover Database (see supplementary material in appendix A3 for further information on land cover categories).

2.3 Pilot mosquito collection study methods

Trapping schedule

An initial visit between March and April 2012 was made to each farm in order to meet with the farm owners and to establish the logistics of sampling on the sites. The trapping schedule consisted of three collection visits in 2012 during the period considered to encompass the peak vector season (April-October) in the UK for surveillance purposes (Phipps et al. 2008; Brugman et al. 2013). The first trapping visit was conducted in May/June ('early season'), the second in July/August ('mid-season') and the third in September ('late season'). To keep meteorological variables as consistent as possible across trap days, collections were restricted to days with forecasted weather conditions of <1mm rain and average wind speeds of <10 miles per hour according to www.xcweather.co.uk.

Trap positioning and logistics

The first consideration for selecting sites was the positioning of traps in relation to animals which might inadvertently damage or be harmed by the traps. In the majority of cases, traps were situated in places inaccessible to livestock, such as in field margins or in fenced-off areas set up within grazed grassland. Similar considerations were also employed to select sites for the human landing catch study in Chapter 3 and chicken-baited traps in Chapter 4. The primary concern was to avoid a contact and biasing influence on the experimental procedures due to overlap with livestock and humans for experimental and safety purposes. It was not possible to position traps to entirely avoid potential interference with wild animals, although no evidence of interactions (such as damage to traps) was found.

The logistics of transporting and setting up the traps on each site were also considered. All sites had varying degrees of vehicular access, however in many cases it was not possible to drive directly to specific trap locations. In these cases, equipment had to be carried by hand, limiting the range of sampling. Additionally, trapping locations in areas experiencing high

volumes of farm traffic were also avoided, along with access areas for the public (e.g. footpaths) to reduce the chances of trap interference and bias by humans.

2.3.1 Identification of larval mosquito habitats and larval sampling

All accessible water bodies that could potentially serve as larval mosquito habitats were noted and sampled on each of the three visits and assigned to the 11 habitat classifications of Laird (1988). For the purposes of this work, ponds were considered to be "water bodies between $1m^2$ and 20 $000m^2$ in area which may be permanent or seasonal, including both man-made and natural water bodies" (Biggs et al. 2005). Potential habitats included permanent water bodies such as ponds and drainage ditches and transient water bodies including hoof prints, container habitats and tree-holes up to eye level (it was not considered practical or safe to climb trees to look for tree holes above this height). It was observed on preliminary site visits that potential breeding sites varied considerably in the volume of water they contained therefore no attempt was made to quantify immature stages present per volume. Instead, potential breeding sites were sampled on a presence/absence qualitative basis. Up to three consecutive dips were made where possible using a 500ml larval dipping pot attached to the end of a 1m wooden pole. One minute was left between dips to allow the water to settle and any disturbed immature stage mosquitoes present to return to the surface. Dipping was conducted by carefully submerging one side of the dipping pot into the water, allowing water to fill the pot, then removing the pot from the water when filled. Dips were conducted towards the edge of water bodies or close to floating vegetation as larvae were observed to shelter mainly in these areas on preliminary site visits. Ponds were sampled with up to three dips at 15 points around the circumference of the pool. Dipping was stopped after the first positive dip (i.e. immature stage mosquitoes present); after three negative dips (no immatures present) the dip point was considered to be free of mosquitoes. For smaller water bodies (such as containers), available water was sampled using a hand-held 500ml plastic beaker and/or a 5ml pastette with truncated tip.

2.3.2 Adult mosquito sampling

CDC light trapping

Adult mosquitoes were collected using CDC 512 miniature light traps (John W Hock, Florida, USA; Figure 2.1) baited with CO_2 in the form of 2kg of dry ice placed in an igloo (John W Hock). The CDC light traps, their 6V batteries and igloos containing dry ice were placed at fixed locations situated a minimum of 50m apart. Four CDC traps were used on all farms with the exception of Mudchute Farm where three were used. Traps were hung 5-6 feet (152-183 cm) above the ground on either tree branches or 'Shepherd's hooks' (Gardman, Peterborough, UK). Traps were set up approximately one hour before sunset and run for approximately 14 hours until collection the following morning. Sunset times were obtained from www.timeanddate.com.

Human landing catches

Human landing catches (HLCs) were also conducted on each collection visit by the author. Mosquitoes alighting on one exposed lower leg were collected into a cardboard pillbox (Watkins and Doncaster, Herefordshire, UK) using a mouth aspirator (Model 612; John W Hock). The HLC location was kept the same on each collection visit to each farm and was situated a minimum of 50m away from the CDC light traps. Three HLCs were made per visit: one 30-minute collection in the morning (09:00-11:00), a second 30-minute collection in the mid-afternoon (14:00-16:00) and a final 90-minute collection starting 30 minutes prior to sunset.

2.2.3 Transport, killing and storage of specimens

Larvae were transferred from the dipper into small white pots using a truncated 5 ml pipette, transported to TPI and killed and stored in 70% ethanol at room temperature until processing. Adults were transported in a cooler containing dry ice sourced from either Green Gases (Hampshire, UK) or All About Ice (Hertfordshire, UK) to TPI, sorted into 5ml bijous and stored at -20°C until identification.

2.3.4 Morphological identification of larval and adult specimens

Adults and larval mosquitoes were identified on the basis of morphological features following the key of Snow (Snow 1990) with additional reference to the key of Cranston (Cranston et al. 1987). Pupae were also allowed to emerge and were then identified as adults. For species that were morphologically indistinguishable as larvae all potential species are listed (e.g. *Culiseta alaskaensis/annulata/subochrea*). Similarly, where adult morphological traits differ only slightly and the keys expressed caution in the reliability of identification, all potential species were included in the identification results e.g. *Ochlerotatus cantans/annulipes*.

2.3.5 Analysis of pilot data

Data were stored, and tables and graphs were produced, in Microsoft Excel. Image and graphical editing was carried out using The GNU Image Manipulation Program (GIMP) version 2.8.14 and Inkscape version 0.91. Mosquito species diversity for each farm was estimated using Simpson's Index of Diversity (Simpson 1949) using the formula $1 - (D = \Sigma \left(\frac{n}{N}\right)^2)$ in Excel (where *D* = Simpson's index, *n* = the number of specimens of a given mosquito species and *N* = total number of mosquitoes from the collection visit) using pooled CDC light trap data from the three visits to each farm. The resulting value (0-1) is the probability that that two randomly selected individuals from the habitat are from different species; the closer the value lies to 1 the greater the diversity.

Figure 2.1: A CDC light trap baited with CO_2 provided by ~2kg of dry ice in an igloo, *in situ* next to the cattle sheds at Coombelands Farm.

2.4 Pilot mosquito collection study results

2.4.1 Larval mosquito habitats and larval species collected

Larval mosquito habitats fitting into seven of the 11 categories of Laird (1988) were identified across the seven farms, of which five were found to contain mosquitoes (Table 2.2). Between two (Mudchute Farm) and seven (Elmley, Northney Farm) habitat categories were found on each farm, of which between one and five contained mosquitoes when sampled. Intermittent ephemeral puddles (category 7) and artificial containers (category 9) were the only two larval mosquito habitats found on every farm, however mosquitoes were found only in the latter, a trend consistent across farms. Container habitats were very varied in type, size and volume of water observed in them (see the photographs accompanying Table 2.2 for examples). Many of the containers can be considered farm-specific, for example water troughs, empty feed containers and buckets used for feed and cleaning purposes. Flowing streams (category 1) were found on all farms except Mudchute and took the form of freshwater or saline ditches which became slow-flowing or stagnant in places (ponded streams, category 2) when water levels were low or when blockage by vegetation occurred. Elmley and Northney Farm were the only two sites with marshy habitats (category 4), both of which were found to contain mosquito larvae.

Habitat categories according to Laird (1988)	Church Farm	Northney Farm	Elmley	Mudchute Farm	ADAS Arthur Rickwood	Coombelands Farm	Glendell Livery
1. Flowing streams	◧	◧	◧		◧		◧
2. Ponded streams	◧	■	■		◧		◧
3. Lake edges							
4. Swamps and marshes		■	■				
5. Shallow permanent ponds	■	■	■		■	◧	■
6. Shallow temporary pools	■	■	■		■		
7. Intermittent ephemeral puddles	◧	◧	◧	◧	◧	◧	◧
8. Natural containers							
9. Artificial containers	■	■	■	■	■	■	■
10. Natural subterranean waters							
11. Artificial subterranean waters							
Total # habitats per farm | total habitats with mosquitoes	**6 | 3**	**7 | 5**	**7 | 5**	**2 | 1**	**6 | 3**	**3 | 1**	**5 | 2**

Table 2.2: Larval mosquito habitats on the farms, classified according to Laird (1988). Half a blue cell indicates the presence of the habitat on a given farm but no larvae collected from it, a full blue cell indicates that larvae were also collected. Example photographs of the habitat categories are given at left.

Overall, ten species or species groups (morphologically indistinguishable as larvae) were collected from the water bodies present on the seven farms (Table 2.3). According to Laird (1988), hoof prints are considered to fall under whichever habitat category they are found in (for example prints on lake edges would be category 3) but they are included as an additional category in Table 2.3 to provide more detailed information. Three species, *An. maculipennis* s.l., *Cx. pipiens s.l./torrentium* and *Ochlerotatus punctor*, were found in hoof prints, the former two at Elmley and the third at Glendell Livery. *Culex pipiens s.l./torrentium* was collected from each of the habitat types found to contain mosquitoes whilst *Culiseta alaskaensis/annulata/subochrea* was collected in all but two habitats (Table 2.3). *Anopheles plumbeus* and *Oc. punctor* were each collected in only one habitat type each, artificial containers (category 9) and hoof prints (falling under category 2), respectively.

Mosquito species	Laird (1988) categories					
	2	4	5	6	9	n/a
	Ponded streams	Swamps and marshes	Shallow permanent ponds	Shallow temporary pools	Artificial containers	Hoof prints*
Anopheles claviger	CF, NF		CF, E, NF		CF	
Anopheles maculipennis s.l.	NF		CF, E	AAR, E	CF	E
Anopheles plumbeus					GL	
Culex modestus	E	E	E	E		
Culex pipiens s.l./torrentium	AAR, E, NF	E, NF	AAR, CF, E, NF	AAR, E, NF	ALL	E
Culiseta alaskaensis/annulata/subochrea	AAR, CF, E, NF		AAR, E, GL, NF	AAR, CF, E, NF	CF, GL	
Ochlerotatus cantans/annulipes	NF		AAR			
Ochlerotatus caspius/dorsalis/leucomelas	NF	NF		NF		
Ochlerotatus detritus	E, NF	NF		NF		
Ochlerotatus punctor						GL

Table 2.3: Mosquito larvae species distribution in the five habitat types (according to Laird 1988) in which they were collected. The presence of a particular species is indicated by blue fill. Initials for each farm are as follows: AAR = ADAS Arthur Rickwood, CF = Church Farm, COF = Coombelands Farm, E = Elmley, GL = Glendell Livery, MF = Mudchute Farm, NF = Northney Farm, ALL = all farms. *****Hoofprints are included as an extra category, with example photographs showing them falling under different habitat categories: (1) Freshwater hoofprints at the edge of a freshwater pond (category 5) at Elmley, (2) Hoofprints on the edge of a ponded saline ditch (category 2) at Northney farm, (3) freshwater hoofprint in field at Glendell Livery (category 6).

Across the seven farms, the greatest number of species/species groups (seven) was collected during the mid-season visit, as compared to six in the early and late season collections (Table 2.4). The total number of species collected was greatest in the mid-season visit for three farms specifically, ADAS Arthur Rickwood, Elmley and Northney farm. Five species/species groups, *An. maculipennis* s.l., *Cs. alaskaensis/annulata/subochrea*, *Cx. pipiens s.l./torrentium*, *Oc. caspius/dorsalis/leucomelas* and *Oc. detritus*, were collected on at least one farm during each of the three collection visits. *Cx. pipiens s.l./torrentium* was collected the most consistently with only the early season collection at ADAS Arthur Rickwood negative for this (and indeed all) species. Glendell Livery yielded two species collected in one collection visit only: *Oc. punctor* in the early season visit and *An. plumbeus* on the mid-season visit.

Mosquito species	early season							mid-season							late season						
	AAR	CF	COOF	E	GL	MF	NF	AAR	CF	COOF	E	GL	MF	NF	AAR	CF	COOF	E	GL	MF	NF
Anopheles claviger														■		■		■			■
Anopheles maculipennis s.l.				■				■	■		■			■		■		■			
Anopheles plumbeus												■									
Culex modestus											■							■			
Culex pipiens s.l./torrentium		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Culiseta alaskaensis/annulata/subochrea		■		■	■		■	■	■		■	■		■	■	■		■			■
Ochlerotatus cantans/annulipes							■	■													
Ochlerotatus caspius/dorsalis/leucomelas							■							■							■
Ochlerotatus detritus							■				■			■				■			■
Ochlerotatus punctor					■																
Total no. of species collected from each farm	0	2	1	3	3	1	5	4	3	1	5	3	1	6	2	4	1	6	1	1	5
Total no. of species, all farms combined	7							9							7						

Table 2.4: Mosquito species collected as larvae at each of the seven farms on each of the three site visits (early, mid and late season); the presence of a particular species is indicated by a blue fill. Early season=May/June, mid-season=July/August and late season=September. For species where morphological separation at this stage is not possible, all potential species names are presented. AAR = ADAS Arthur Rickwood, CF = Church Farm, COF = Coombelands Farm, E = Elmley, GL = Glendell Livery, MF = Mudchute Farm, NF = Northney Farm.

2.4.2 Adult mosquitoes collected

CDC light traps

A total of 950 adult mosquitoes of 12 species/species groups were captured using CDC light traps baited with CO_2 across all seven farms (Table 2.5). The total number of mosquitoes collected on each individual farm ranged from 18 (9 females, 9 males) at Church Farm through to 259 (245 females, 14 males) at Northney Farm. Fewer males than females were collected across farms and visits, with the overall total collection comprising 854 females and 96 males (approximately a 9:1 ratio) with the mean number of mosquitoes captured (per farm, per collection visit) ranging from 3.0 – 81.7 for females and from 0.0 – 19.3 for males. Seven of the 12 species collected in the light traps were represented by females alone. The numbers collected of each species varied considerably, with the most numerous species group, *Cx. pipiens s.l./torrentium*, totalling 553 (465 females and 88 males), followed by *Cs. annulata* (179 females, 5 males). The numbers collected of several of the remaining species were in single figures: *An. plumbeus* (9), *An. maculipennis* s.l. (8), *An. claviger* (7) and *Oc. cantans/annulipes* (4). Only a single specimen of *Aedes cinereus/geminus* was collected.

Species	ADAS Arthur Rickwood	Church Farm	Coombelands Farm	Elmley	Glendell Livery	Mudchute Farm	Northney Farm	Total per species
Ae. cinereus/geminus	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	1 (0)
An. claviger	3 (0)	2 (0)	0 (0)	1 (0)	0 (0)	0 (0)	1 (0)	7 (0)
An. maculipennis s.l.	7 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	7 (1)
An. plumbeus	0 (0)	0 (0)	1 (0)	0 (0)	2 (0)	6 (0)	0 (0)	9 (0)
Cq. richiardii	3 (0)	0 (0)	10 (0)	9 (0)	0 (0)	0 (0)	1 (0)	23 (0)
Cx. pipiens s.l./torrentium	83 (56)	6 (8)	21 (6)	115 (0)	20 (0)	10 (7)	210 (11)	465 (88)
Cs. annulata	32 (1)	1 (1)	68 (0)	1 (1)	17 (0)	52 (0)	8 (2)	179 (5)
Oc. cantans/annulipes	0 (0)	0 (0)	0 (0)	1 (0)	3 (0)	0 (0)	0 (0)	4 (0)
Oc. caspius/dorsalis	3 (0)	0 (0)	0 (0)	21 (0)	0 (0)	0 (0)	5 (0)	29 (0)
Oc. detritus	7 (0)	0 (0)	0 (0)	7 (0)	0 (0)	0 (0)	18 (1)	32 (1)
Oc. flavescens	1 (0)	0 (0)	1 (0)	54 (1)	0 (0)	0 (0)	0 (0)	56 (1)
Oc. punctor	0 (0)	0 (0)	0 (0)	0 (0)	29 (0)	0 (0)	0 (0)	29 (0)
Oc. spp. female	1	0	0	10	0	0	2	13
Total per farm	140 (58)	9 (9)	101 (6)	219 (2)	72 (0)	68 (7)	245 (14)	854 (96)
Mean catch/visit/farm	46.7 (19.3)	3 (3)	33.7 (2)	73 (0.7)	24 (0)	22.7 (2.3)	81.7 (4.7)	40.7 (4.7)

Table 2.5: The total number of adult female and male (in brackets) mosquitoes collected by CDC light traps baited with CO_2 on each farm over the three site visits. The mean catch is calculated as the total number of mosquitoes collected on a given farm divided by three visits; value given to one decimal place.

The overall abundance of mosquitoes collected using light traps was greatest in the early season collection at four farms: Coombelands Farm, Elmley, Glendell Livery and Northney Farm (Figure 2.2). At the remaining three farms, ADAS Arthur Rickwood, Church Farm and Mudchute Farm, the greatest abundance was collected in the mid-season visit. Late season collections yielded either equivalent or lower abundances of mosquitoes on each farm than the preceding visits. The relative abundance of each species collected per farm varied depending on the collection period, indicating the existence of seasonal differences in adult mosquito populations.

The early and mid-season collections of mosquitoes were most diverse with ten species/species groups represented, compared to seven for the late season collection (Table 2.6). *Culex pipiens s.l./torrentium* and *Cs. annulata* were recorded most commonly on more than one collection visit. The greatest number of species/species groups were collected at ADAS Arthur Rickwood (eight) followed by Elmley (seven) and both farms shared the greatest number of species/species groups recorded on a single visit (seven) on the second and first visit to each farm respectively. Two mosquito species, *Cx. pipiens s.l./torrentium* and C*s. annulata*, were collected at all farms, whereas other species were collected from a more limited number. For example, *Oc. punctor* was collected only from Glendell Livery. The value obtained for Simpson's index of diversity for total CDC collections per farm was the highest for Glendell Livery (0.70) followed by Elmley (0.65), Coombelands Farm (0.52), ADAS Arthur Rickwood (0.48) and Mudchute (0.46). The farms with the lowest index of diversity were Church Farm (0.37) and Northney Farm (0.26).

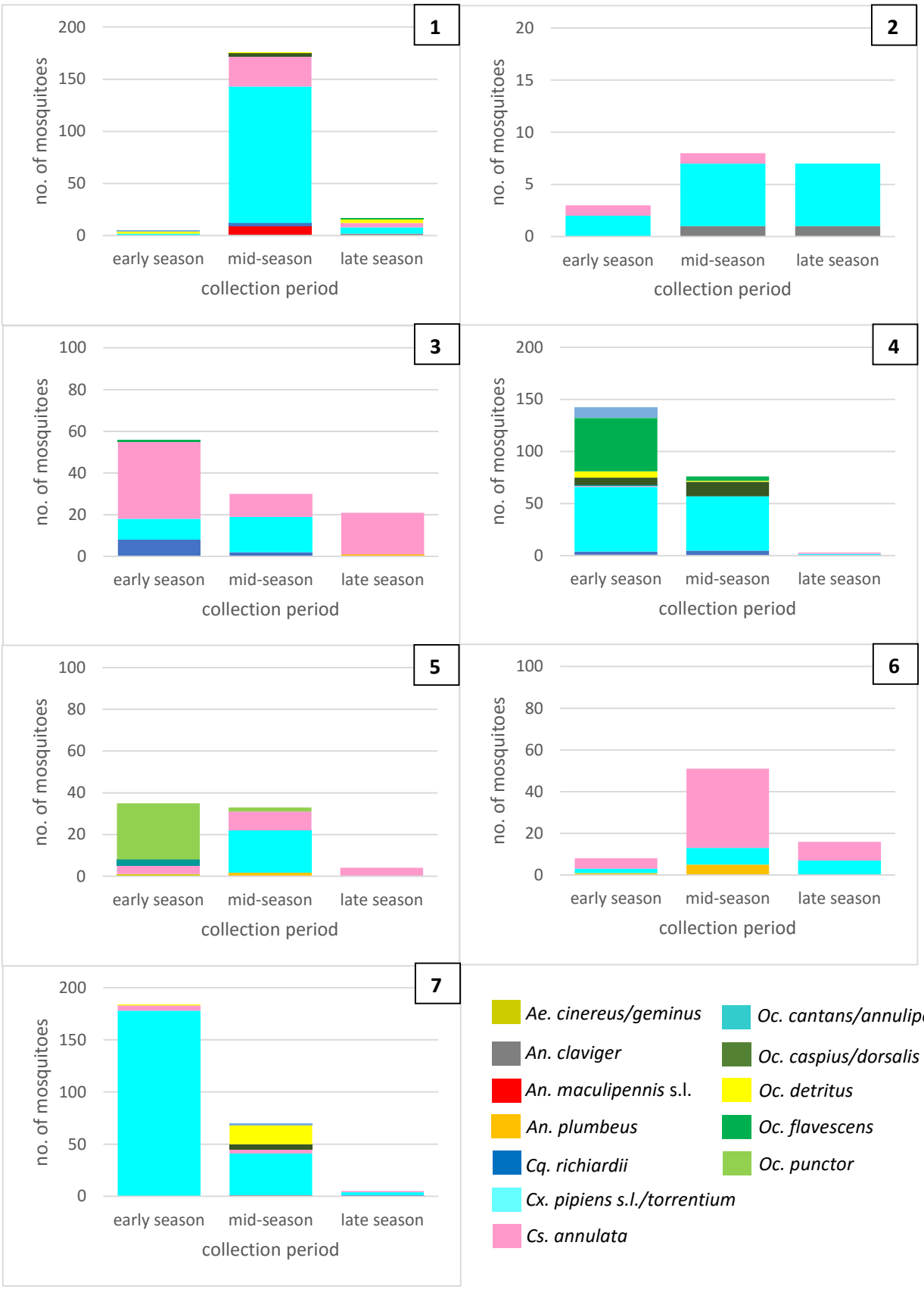


Figure 2.2: Mosquito species assemblages collected using CDC + CO_2. The key is given at bottom right. 1 = ADAS Arthur Rickwood, 2 = Church Farm, 3 = Coombelands Farm, 4 = Elmley, 5 = Glendell Livery, 6 = Mudchute Farm, 7 = Northney Farm. Note different scales on the axes.

Mosquito species	early season							mid-season							late season						
	AAR	CF	COOF	E	GL	MF	NF	AAR	CF	COOF	E	GL	MF	NF	AAR	CF	COOF	E	GL	MF	NF
Aedes cinereus/geminus					■																
Anopheles claviger								■	■					■	■	■					
Anopheles maculipennis s.l.								■													
Anopheles plumbeus						■						■	■				■				
Coquillettidia richiardii			■	■				■		■	■							■			■
Culex pipiens s.l./torrentium	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■		■		■	■
Culiseta annulata		■	■	■	■	■	■	■	■	■		■	■	■	■		■	■	■	■	■
Ochlerotatus cantans/annulipes				■	■																
Ochlerotatus caspius/dorsalis				■				■			■			■							
Ochlerotatus detritus	■			■			■	■			■			■	■						
Ochlerotatus flavescens			■	■							■				■						
Ochlerotatus punctor					■							■									
Total no. of species from each farm	2	2	4	7	4	3	3	7	3	3	5	4	3	5	5	2	2	3	1	2	3
Total no. of species, all farms combined	10							10							7						

Table 2.6: Mosquito species captured by CDC light traps baited with CO_2 at each farm on each of the three site visits; the presence of a particular species is indicated by green fill. Early season=May/June, mid-season=July/August and late season=September. *Ochlerotatus spp.* that could not be identified owing to damage to specimens are not included. AAR = ADAS Arthur Rickwood, CF = Church Farm, COF = Coombelands Farm, E = Elmley, GL = Glendell Livery, MF = Mudchute Farm, NF = Northney Farm.

Human landing catch results

A total of 140 mosquitoes were collected by HLC across all seven farms (Table 2.7). Host-seeking female mosquitoes were collected on all farms, with total numbers ranging from one (ADAS Arthur Rickwood and APHA Weybridge) or two (Church Farm) mosquitoes to 48 (Northney Farm) and 69 (Elmley). The most abundant species collected was *Oc. detritus* (63), which was three times greater in number than the second most numerous species, *Cq. richiardii* (23). The Elmley and Northney Farm sites were responsible for the majority of mosquitoes collected, with the numbers collected at the other farms being considerably lower, in most cases below a total of ten individuals. The majority of mosquitoes were collected in the evening collection period across the farms, with none collected in the 30-minute morning collection, only one mosquito collected in the afternoon collection, and the remaining 139 mosquitoes collected in the evening (Table 2.7). This pattern is reflected by the estimated hourly biting rate for each collection period (combining all mosquito species) of zero, 0.7 and 30.9 for morning, afternoon and evening collection periods respectively. Only three farms, Elmley, Northney Farm and Mudchute, yielded estimated hourly biting rates of at least one mosquito.

Ten species/species groups were collected by HLC across the seven farms (Table 2.8). The number of human-landing mosquito species collected per farm ranged from one (three farms) to seven (Elmley). The mid-season visit yielded nine human-landing species in total across the seven farms, compared to seven in the early season visit and one in the late season visit. The greatest number of species was collected during the mid-season visit to all farms, with the exception of ADAS Arthur Rickwood where the only mosquito caught, *Oc. detritus*, was collected in the early season visit, and Glendell Livery where the early season visit yielded two species, *Oc. cantans/annulipes* and *Oc. punctor*, compared to the one, *Cs. annulata*, collected during mid-season.

Farm	Morning (30 minutes)					Afternoon (30 minutes)					Evening (90 minutes)					Total
	early	mid	late	total (mean per visit)	est. biting rate	early	mid	late	total (mean per visit)	est. biting rate)	early	mid	late	total (mean per visit)	est. biting rate	
ADAS Arthur Rickwood	0	0	0	0 (0)	0	0	0	0	0 (0)	0	1	0	0	1 (0.3)	0.2	1
Coombelands	0	0	0	0 (0)	0	0	0	0	0 (0)	0	0	1	0	1 (0.3)	0.2	1
Church Farm	0	0	0	0 (0)	0	0	0	0	0 (0)	0	0	2	0	2 (0.7)	0.5	2
Elmley	0	0	0	0 (0)	0	0	1	0	1 (0.3)	0.7	24	44	0	68 (22.7)	15.1	69
Glendell Livery	0	0	0	0 (0)	0	0	0	0	0 (0)	0	3	1	0	4 (1.3)	0.9	4
Mudchute	0	0	0	0 (0)	0	0	0	0	0 (0)	0	9	6	0	15 (5)	3.3	15
Northney Farm	0	0	0	0 (0)	0	0	0	0	0 (0)	0	0	32	16	48 (16)	10.7	48
Total, all farms	**0**	**0**	**0**	**0 (0)**	**0**	**0**	**1**	**0**	**1 (0.3)**	**0.7**	**37**	**86**	**16**	**139 (46.3)**	**30.9**	**140**

Table 2.7: Mosquitoes (all species) captured by human landing catch on each of the three visits in the morning, afternoon and evening collection periods at each of the seven farms. The estimated biting rate per hour shows the expected number of bites in an hour calculated by either doubling the mean value obtained for the mosquitoes collected in 30 minutes (morning and afternoon) or by dividing the mean value by 1.5 (evening collection period).

Mosquito species	early season							mid-season							late season						
	AAR	CF	COOF	E	GL	MF	NF	AAR	CF	COOF	E	GL	MF	NF	AAR	CF	COOF	E	GL	MF	NF
Anopheles claviger																					
Anopheles maculipennis s.l.																					
Coquillettidia richiardii																					
Culex pipiens s.l./torrentium																					
Culiseta annulata																					
Ochlerotatus cantans/annulipes																					
Ochlerotatus caspius/dorsalis																					
Ochlerotatus detritus																					
Ochlerotatus flavescens																					
Ochlerotatus punctor																					
Total no. of species from each farm	1	0	0	5	2	2	0	0	1	1	6	1	3	3	0	0	0	0	0	0	1
Total no. of species, all farms combined	7							9							1						

Table 2.8: Mosquito species captured by human landing catch at each farm on each of the three site visits; the presence of a particular species is indicated by red fill. Early season=May/June, mid-season=July/August and late season=September. AAR = ADAS Arthur Rickwood, CF = Church Farm, COF = Coombelands Farm, E = Elmley, GL = Glendell Livery, MF = Mudchute Farm, NF = Northney Farm.

2.4.3 Summary of mosquito species collected

Across the seven farms, 13 species/species groups were collected either as larvae or as adults in CDC light traps (Table 2.9). Larval dipping collected one species, *Culex modestus* at Elmley, which was not collected in the CDC light traps. CDC light traps collected three species/species groups, *Ae. cinereus/geminus*, *Coquillettidia richiardii*, and *Ochlerotatus flavescens*, that were not collected as larvae.

Species/species group	**Larvae**	**Adults**
Aedes cinereus/geminus	no	yes
Anopheles claviger	yes	yes
Anopheles maculipennis s.l.	yes	yes
Anopheles plumbeus	yes	yes
Coquillettidia richiardii	no	yes
Culex pipiens s.l./torrentium	yes	yes
Culex modestus	yes	no
Culiseta alaskaensis/annulata/subochrea	yes	yes
Ochlerotatus cantans/annulipes	yes	yes
Ochlerotatus caspius/dorsalis/leucomelas	yes	yes
Ochlerotatus detritus	yes	yes
Ochlerotatus flavescens	no	yes
Ochlerotatus punctor	yes	yes

Table 2.9: Overall summary of species assemblages present across all seven farms, collected by larval dipping (left column) and/or as adults by CDC light trapping (right column) (yes/no).

2.5 Molecular methodologies

The pilot study (section 2.4 above) collected thirteen species/species groups on the seven farms studied including two, *Cx. pipiens s.l./torrentium* and *An. maculipennis* s.l., for which published molecular assays exist for species-level identification. End-point PCR assays are available for the separation of *Culex pipiens s.l./torrentium* and *Culex pipiens f. pipiens/Culex pipiens f. molestus* and a PCR-sequencing approach is available for identification of the members of *An. maculipennis* s.l. Mosquitoes collected in the pilot study were not identified by molecular methods for reasons of cost, however as part of Chapters 3, 4, 5 and 6, identification to species level has been conducted according to the techniques detailed below.

2.5.1 Culex pipiens s.l./Culex torrentium

The sibling species *Culex pipiens* s.l. and *Culex torrentium* were separated using a duplex PCR assay (Manley et al. 2015), using the ACEtorr and ACEpip forward primers and B1246s reverse primer. Primers are based on sequence differences in the nuclear acetylcholinesterase-2 (*ace-2*) gene and have been adapted from an original multiplex assay (Smith & Fonseca 2004). PCR reactions were carried out using a 2720 Thermocycler (Applied Biosystems, Paisley, UK) in the following reaction mix: 3 µl of DNA template, 0.4 µl nuclease-free H_2O, 5 µl TopTaq Mastermix (Qiagen), 0.2 µl $MgCl_2$ (Qiagen), 1 µl CoralLoad concentrate (Qiagen), 0.1 µl of each forward primer and 0.2 µl of the reverse primer (each at 10 pmol/µl). The thermal profile consisted of an initial denaturation step at 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, followed by a final elongation step at 72°C for 10 minutes. Products were separated on a 1.5% agarose gel using a phiX174 ladder (Thermo Scientific, Loughborough, UK) with negative (sterile water) and positive controls (*Culex pipiens* s.l. and *Culex torrentium* DNA (kindly provided by Dr Lara Harrup at TPI)). The assay produces a product of 610 base pairs (bp) in size for *Culex pipiens* s.l. and 416 bp for *Culex torrentium* (Figure 2.3).

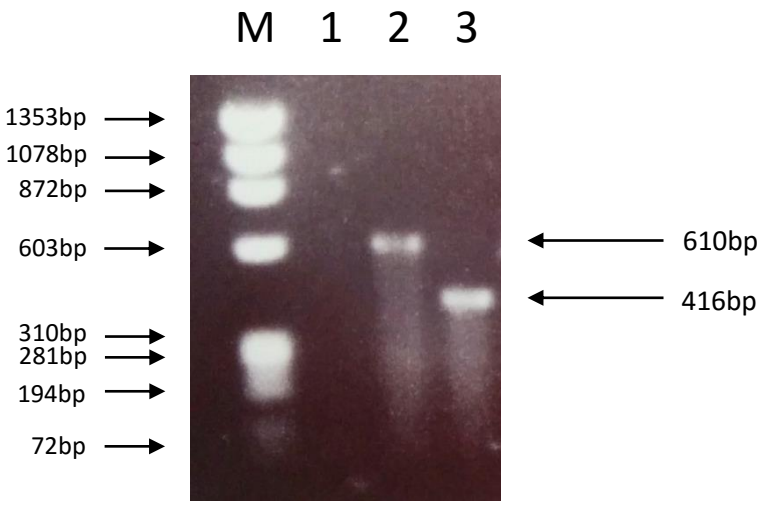


Figure 2.3: Gel image showing product sizes for (Lane 2) *Culex pipiens* s.l. (610bp) and (Lane 3) *Culex torrentium* (416bp) with comparison to (Lane 1) negative control (sterile water) and phiX174 marker (Lane M).

2.5.2 Culex pipiens f. pipiens/Culex pipiens f. molestus

The two ecoforms of *Culex pipiens* s.l. were separated using a duplex PCR assay using the forward primer CQ11F and reverse primers molCQ11R and pipCQ11R, based on the CQ11 microsatellite locus (Bahnck & Fonseca 2006; Fonseca et al. 1998). PCR reactions were carried out using a 2720 Thermocycler (Applied Biosystems) in the following reaction mix: 3 µl of DNA template, 0.325 µl nuclease free water, 5 µl TopTaq Mastermix (Qiagen), 0.2 µl $MgCl_2$ (Qiagen), 0.075 µl bovine serum albumin (New England Biolabs, Hertfordshire, UK), 1 µl CoralLoad concentrate, 0.15 µl of CQ11F, 0.15 µl of molCQ11R and 0.1 µl of pipCQ11R (each at 10 pmol/µl). The thermal profile consisted of an initial denaturation step at 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, followed by a final elongation step at 72°C for 10 minutes. Products were separated on a 1.5% agarose gel using a phiX174 ladder (Thermo Scientific) with negative (sterile water) and positive controls (*Culex pipiens f. pipiens*, *Culex pipiens f. molestus* and *Culex pipiens f. pipiens/molestus* hybrid

DNA (kindly provided by Dr Lara Harrup at TPI)). The assay produces products of 180 bp for *Culex pipiens f. pipiens*, 250 bp for *Culex pipiens f. molestus*, with both bands present in the case of *Culex pipiens f. pipiens x f. molestus* hybrids (Figure 2.4).

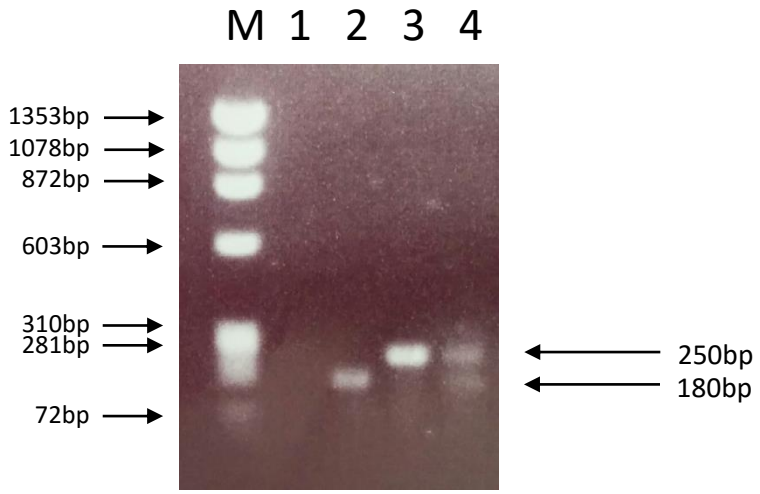


Figure 2.4: Gel image showing product sizes produced by the duplex assay separating (Lane 2) *Culex pipiens f. pipiens* (180bp), (Lane 3) *Culex pipiens f. molestus* (250bp) and (Lane 4) *Culex pipiens pipiens/molestus* hybrids (180bp and 250bp) with comparison to a phiX174 marker (Lane M) and (Lane 1) negative control (sterile water).

2.5.3 Anopheles maculipennis complex

Species level identification of the *Anopheles maculipennis* complex was achieved by amplification of a 435 bp region of the *ITS-2* ribosomal gene using the 5.8SF and 28SR primers of Collins and Paskewitz (1996). PCR products were obtained using a real-time PCR assay in a Mx3000P real-time PCR system (Stratagene, Agilent Technologies, Cheshire, UK) in the following reaction mix, final volume 40µl: 2 µl of DNA template, 14 µl H_2O, 20 µl SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, Dorset, UK) and 2 µl of each primer (each at 10 pmol/µl). The thermal profile consisted of an initial denaturation step at 94°C for 10 minutes followed by 35 cycles of: 94°C for 30 seconds, 53°C for 30 seconds, 72°C for one minute, followed by a final elongation step of 72°C for 10 minutes. PCR products were separated on a 1.5% agarose gel, and samples showing bands of the correct size were sequenced unidirectionally using the ABI

PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Paisley, UK) (all sequencing performed by the in-house APHA sequencing facility). Where necessary, sequences were edited using Lasergene version 12.1 (DNASTAR) and assigned to a particular mosquito species when agreement was ≥98% to sequences of known species in GenBank. Example sequences are presented in appendix A4A.

2.5.4 Molecular identification of blood meal origin in blood fed female mosquitoes

DNA extraction from mosquito abdomens

Abdomens of engorged mosquitoes were separated from the rest of the body on a chilled plate using forceps, and placed into individual 1.5 mL Eppendorf tubes containing 200 µl phosphate-buffered saline (PBS). The abdomens were pressed against the wall of the tube using the forceps to release the blood meal. The remaining head and thorax of each mosquito was stored at -20°C for morphological reference. Forceps were cleaned between specimens using a three stage wash to avoid cross-contamination. The first wash consisted of 5% decon, the second of 100% ethanol and the third of sterile water, at which point all liquid excess was wiped off with task wipes (Kimtech Science, Roswell, Georgia, USA). Each sample was incubated with 20 µl proteinase K (Qiagen) and 200 µl buffer AL (Qiagen) for 30 minutes at 56°C in a water bath. DNA extraction was carried out using DNeasy Blood and Tissue Kit (Qiagen), following manufacturer's spin column-protocol. All DNA extractions were stored at 4°C until processing.

Identification of blood meal host

The assay for blood meal identification consisted of a M13-tailed, triple primer cocktail (VF1_t1 + VF1d_t1 + VF1i_t1/VR1_t1 + VR1d_t1 + VD1i_t1) targeting an approximately 685 base pair (bp) region of the *cytochrome c oxidase I* (*COI*) gene (Ivanova et al. 2007). The final PCR reaction mix of 50 µl consisted of: 28.075 µl H_2O, 5 µl GeneAmp 10X PCR buffer I (Applied Biosystems), 1 µl dNTPs (at 0.2 mM/µl), 1.5 µl of each primer (at 10 pmol/µl), 0.25 µl AmpliTaq Gold DNA Polymerase (10 units/µl), 0.675 µl dimethyl sulfoxide (DMSO), 1 µl

tetramethylammonium chloride (TMAC) and 5 µl extracted DNA. All reactions were carried out on a 2720 Thermocycler (Applied Biosystems). The thermal profile consisted of an initial denaturation step at 94°C for 10 minutes followed by 40 cycles of: 94°C for 30 seconds, 53°C for 30 seconds, 72°C for one minute, followed by a final elongation step of 72°C for 10 minutes. PCR products were separated on a 1.5% agarose gel and samples producing products of the correct size (~685 bp) (Figure 2.5) were purified and sequenced. Sequencing was performed using M13 primers (Ivanova et al. 2007) at 1 pmol/µl. Amplification products were sequenced bidirectionally (chapter 5) or unidirectionally (chapter 6) using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and sequences compared to sequences in GenBank via a BLAST search and assigned to a particular vertebrate species when agreement was ≥98%. Example sequences obtained during the thesis are given in appendix A5.

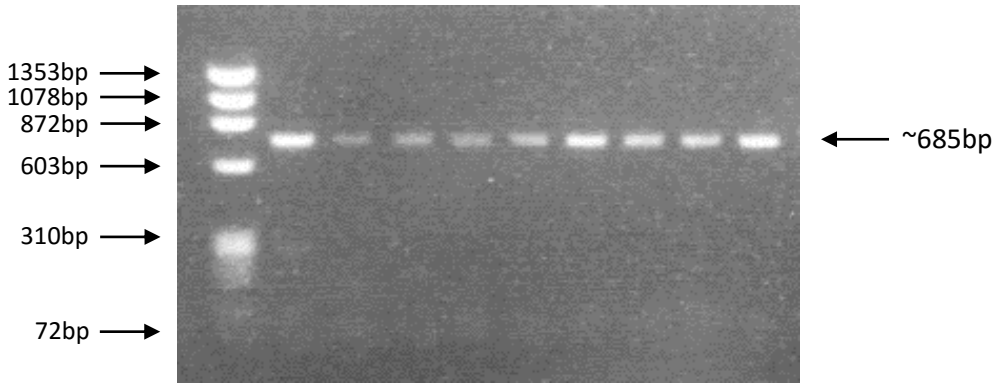


Figure 2.5: Gel image showing ~685bp products produced from the *COI* universal PCR (Ivanova et al. 2007). Lane M: phiX174 marker, lane 1: negative control, lanes 2-6 blood-fed mosquito samples.

Sensitivity testing of blood meal assay

The sensitivity of the blood meal assay was tested using two sets of serial ten-fold dilutions of whole horse blood (TCS Biosciences) and human blood (Cambridge Bioscience, Cambridge, UK). DNA was extracted from the whole blood using the DNeasy Blood and Tissue kit according to manufacturer's guidelines. The initial DNA concentration following extraction

was 16.4 nanograms (ng)/µl for horse blood and 14.8ng/µl for human blood as estimated by a Nanodrop 2000 spectrophotometer (Thermo Scientific). The PCR reaction was conducted as per protocol and products visualized on a 1.5% agarose gel. Products of the correct size were visible until the fourth ten-fold dilution for human blood (DNA concentration 0.00148 ng/µl) and until the third ten-fold dilution for horse blood (DNA concentration 0.0164 ng/µl).

Host-range validation of blood meal assay

To assess the range of vertebrate hosts the blood meal PCR assay could successfully identify, the protocol was tested using DNA extracted from whole human blood (Cambridge Bioscience), whole animal blood (TCS Biosciences), or the abdomens of blood-fed colony *Culex pipiens* s.l. fed via a Hemotek membrane feeder (Discovery workshops, Accrington, UK). Additional vertebrate DNA sources were provided as cDNA extracts produced in the course of WNV surveillance (see Brugman et al., (2013); Phipps et al., (2008)). DNA extracted from an unfed female *Culex pipiens* s.l. from TPI colony was also included as a negative control. DNA sequencing was omitted for cost-saving purposes. All 24 vertebrate DNA sources tested produced bands of the correct size (~685 bp) when separated on a 1.5% agarose gel, whilst the DNA from the unfed mosquito did not produce any visible product (Table 2.10). These results indicated that the chosen PCR assay was likely to be able to identify vertebrate blood meal origin whilst avoiding amplification of mosquito DNA.

Time-course blood-feeding study

The likelihood of successfully identifying the origin of mosquito blood meals decreases with time after blood-feeding due to digestion within the insect (Kent 2009). A time-course study was conducted to assess how long after feeding the chosen molecular approach was able to successfully identify vertebrate blood meal origin. Approximately 100 *Culex pipiens* s.l. from both colony lines at TPI, maintained solely on 10% sucrose following emergence, were offered defibrinated horse blood (TCS Biosciences) at 37°C for two hours via the Hemotek membrane

feeder (Discovery Workshops). Following this period the Hemotek feeder was removed and this point considered 'time zero'. Five blood-fed mosquitoes were collected by manual aspiration every 12 hours until 96 hours post-feeding, and killed by freezing at -20°C. DNA was extracted from three of the five mosquitoes from each time point according to standard protocol and samples were then subjected to the standard PCR protocol. Interestingly, all samples produced products of the correct size (~685bp) when visualized on a 1.5% agarose gel, however when sequenced, horse blood was only correctly identified in mosquitoes up to 24 hours post-feeding. After this time point samples were identified as the mosquito host in GenBank.

DNA source	Order	Family	Species name	Common name
Blood-fed mosquito	Artiodactyla	*Suidae*	*Sus scrofa*	Pig*
Blood-fed mosquito	Lagomorpha	*Leporidae*	*Oryctolagus cuniculus*	Rabbit*
cDNA	Anseriformes	*Anatidae*	*Cygnus* spp.	Swan*
cDNA	Anseriformes	*Anatidae*	not specified	Duck*
cDNA	Anseriformes	*Anatidae*	not specified	Goose*
cDNA	Passeriformes	*Corvidae*	*Corvus* spp.	Crow*
cDNA	Passeriformes	*Corvidae*	*Pica pica*	Magpie*
cDNA	Passeriformes	*Fringillidae*	*Fringilla coelebs*	Chaffinch*
cDNA	Passeriformes	*Turdidae*	*Turdus merula*	Blackbird*
cDNA	Passeriformes	*Turdidae*	*Turdus pilaris*	Fieldfare*
cDNA	Columbiformes	*Columbidae*	*Columbus* spp.	Pigeon/Dove*
cDNA	Strigiformes	*Strigidae*	not specified	Owl*
cDNA	Charadriiformes	*Laridae*	not specified	Gull*
cDNA	Charadriiformes	*Alcidae*	*Uria aalge*	Guillemot*
cDNA	Charadriiformes	*Alcidae*	*Alca torda*	Razorbill*
cDNA	Charadriiformes	*Haematopodidae*	*Haematopus ostralegus*	Oystercatcher*
cDNA	Procellariiformes	*Procellariidae*	*Puffinus puffinus*	Manx Shearwater*
Whole blood	Galliformes	*Phasianidae*	*Gallus gallus*	Chicken*
Whole blood	Artiodactyla	*Bovidae*	*Bos Taurus*	Cow*
Whole blood	Artiodactyla	*Bovidae*	*Capra hircus*	Goat*
Whole blood	Artiodactyla	*Bovidae*	*Ovis aries*	Sheep*
Whole blood	Carnivora	*Canidae*	*Canis lupus familiaris*	Dog*
Whole blood	Primates	*Hominidae*	*Homo sapiens*	Human*
Whole blood	Perissodactyla	*Equidae*	*Equus caballus*	Horse*
Pirbright colony	Diptera	*Culicidae*	*Culex pipiens* s.l.	Mosquito

Table 2.10: DNA source and species used for validating the host range blood meal PCR assay (Ivanova et al. 2007). As cDNA was obtained from West Nile virus surveillance submissions and the identification made by the submitting body, on occasion full identification beyond Family or Genus was not provided. * indicates a visualised band of the correct size (~685bp).

2.6 Discussion and selection of sites for future work

The pilot study confirmed that the seven farms all supported mosquito population assemblages, with thirteen species/species groups collected across the farms including those that are potential or confirmed vectors of mosquito-borne pathogens. Larval and adult mosquito collection results provided evidence of seasonality in mosquito population assemblages across the sites, as was expected considering available data on UK mosquitoes (Service 1969a; Medlock & Vaux 2015b). The first two collection visits (early season and mid-season) yielded the greatest numbers of different species and the greatest abundance of adult mosquitoes collected in the light traps. Human landing catch (HLC) collections identified the presence of human biting mosquito species on each of the seven farms with landing activity primarily restricted to around sunset. The preliminary data gathered in the pilot study highlighted key aspects of mosquito species diversity, behaviour and fieldwork logistics which facilitated the subsequent selection of a subset of four farms for use in the further intensive studies contained within this thesis. Additionally appropriate molecular techniques were successfully optimised for use in species identification of mosquitoes and blood meal origin.

Larval habitat sampling and classification

Seven larval mosquito habitats as defined by Laird (1988) were identified across the seven farms. Of these, only two, intermittent ephemeral puddles (category 7) and artificial containers (category 9) were found on every farm of which only artificial containers contained mosquito larvae at every farm. Several containers, such as watering troughs, can be considered to be associated primarily with the farm environment and were found on every one the farms. The presence of container habitats is reflected by the larval species profiles collected across the farms with the two most frequently recorded, *Cx. pipiens s.l./torrentium* and *Cs. alaskaensis/annulata/subochrea*, known to breed in containers. Of the other two species collected from containers, of particular interest is the single specimen of *An. plumbeus* from a used tyre at Glendell Livery. This finding supports previous evidence from Europe (Dekoninck et

al. 2011; Townroe & Callaghan 2014), that in the absence of its preferred tree hole habitat this species is able to exploit other container-type habitats that are commonly associated with both urban areas and farms. This species can cause a considerable human biting nuisance (Dekoninck et al. 2011) and has also recently been shown to be competent for the transmission of the NF54 strain of *Plasmodium falciparum* (Schaffner et al. 2012).

The classification system of Laird (1988) differs somewhat from the classification used more recently in some of the UK mosquito literature (e.g. Medlock & Snow, (2008a), however it was chosen here as a simple and a more universally standardised method of characterising the sites. As Laird (1988) aimed to unify the numerous classification systems drawn up by many different prior authors the categories are quite broad, rather than precise, descriptions of every possible habitat variation. One difficulty encountered was in the categorisation of hoof prints (Table 2.3), a potential larval mosquito habitat strongly associated with livestock farms. According to Laird (1988), hoof prints are considered part of the habitat in which they are made; for example, a hoof print in a marsh is still categorised as being a marsh habitat (category 4). It was however observed that hoof prints often appeared in relative isolation from other habitats, for example in an otherwise dry field, likely following the drying of the surrounding habitat. In such a case it would be likely that the composition of the aquatic habitat may change so as to become more akin to, for example, a natural container habitat (category 8). This illustrates the overlapping boundaries of some of the categories as described by the author himself. Although beyond the scope of this thesis, future studies recording the pH and organic matter of water within hoof prints may enable a relationship between these factors and the mosquito species within them to be determined.

CDC light trap results

Mosquitoes from 12 species/species groups were collected using CDC light traps baited with CO_2 across the seven farms. Different species assemblages, and different associated species diversity indices (*1-D*) were associated with each farm which likely reflects the different larval

mosquito habitats available on each site. *Culex pipiens s.l./torrentium* and *Cs. annulata* were the only two species collected from each farm, in line with the results of the larval sampling, and on the majority of farms one or both of these species was the most abundant in the light traps. The CDC light trap was an appropriate trap to use in the pilot study to assess the presence of mosquito species assemblages. The trap is extensively used in mosquito and arbovirus studies across the world. Examples include India (Sadanandane et al. 2007), Nigeria (Amusan et al. 2005), Sweden (Lundström et al. 2013), Thailand (Khaklang & Kittayapong 2014), The Netherlands (Reusken et al. 2011) and the USA (Andreadis et al.; Ginsberg et al. 2010). In addition, one study in the UK considered the CDC light trap ideal for the rapid assessment of mosquitoes present on a site (Hutchinson et al. 2007). The traps were fairly easy to use on the farm sites however there were limitations on the number of available sites on which traps could be hung to be both sufficiently distant (50m) from one another and from interference from animals, especially as many sites lacked much tree cover. Additionally the need to recharge batteries every night was a potential logistical limitation to future studies. Mosquito Magnet Pro (MMP) traps, used in mosquito and arbovirus surveillance activities in the UK (Vaux & Medlock 2015; Vaux et al. 2015) were therefore used as control traps in future studies (Chapters 4 and 5). Despite being more cumbersome in the initial stages of transport and setup, MMP traps could be left on site for the entire season following a single battery charge, requiring only periodic changes of the propane gas cylinder and did not require a means of hanging.

Larval and adult mosquito collections

Using both larval and adult mosquito sampling for the collections resulted in the detection of more species than any one technique alone. Larval sampling alone did not detect the presence of three species, *Ae. cinereus/geminus*, *Cq. richiardii* or *Oc. flavescens*, whilst adult sampling did not detect *Cx. modestus* at Elmley. The larvae of *Cq. richiardii* use their modified siphons to affix themselves to the submerged roots and stems of aquatic plants and thus require specific methods to successfully sample them and thus their absence from the larval dipping

results was not a surprise (Snow 1990). The absence of *Ae. cinereus/geminus* and *Oc. flavescens* from the larval samples may be a result of having missed larval sites or missing larvae as only three dips per location were used. The presence of *Cx. modestus* as larvae at Elmley but not as adults suggests that the limited number of sampling visits led to the period of adult activity being missed. This species was only recently described at this site as larvae only (Golding et al. 2012), the first UK report since the 1940s, and thus little is known about its behaviour or adult activity periods in the UK.

Human biting activity

Collections by HLC yielded a total of 140 mosquitoes of ten species/species groups across the seven farms. These pilot data suggest that human landing (and therefore biting) activity primarily occurs in the evening period as opposed to during mid-morning or mid-afternoon, corresponding to existing published data (Service 1969a; Service 1971b). Together with anecdotal evidence of human biting collected in the course of conversations with farm workers and visitors, this indicates that humans do serve as hosts for mosquito biting within farm environments in the UK. The number of species collected by HLC was much higher in the early and mid-season collection visits overall, seven and nine respectively (Table 2.8), corresponding to existing information on peak mosquito activity periods (Service 1969a; Medlock & Vaux 2015b). Taken together, these preliminary results indicate that the intensive study investigating farm-associated human biting should be conducted sometime between May-August.

Molecular methodologies and rationale for choice of hosts for baited collections

Six species groups were collected in the course of the pilot study: *Ae. cinereus/geminus*, *An. maculipennis* s.l., *Cx. pipiens s.l./torrentium*, *Cs. alaskaensis/annulata/subochrea, Oc. cantans/annulipes* and *Oc. caspius/dorsalis/leucomelas*. Of these, published molecular assays for species-level identification exist for *An. maculipennis* s.l. and

Cx. pipiens s.l./torrentium. Interestingly, although neither *Cx. pipiens f. pipiens* nor *Cx. torrentium* are generally associated with human feeding, *Culex pipiens s.l./torrentium* were collected by HLC. This emphasises the importance of separating out these species when conducting future studies into host preference but also indicated that the bird-biting (ornithophilic) activity of farm-associated mosquitoes should be addressed in this thesis. Both these species are considered to be primarily ornithophilic in the UK literature (Snow 1990; Cranston et al. 1987) so from the perspective of arbovirus transmission, if they regularly feed on humans and birds, then these species would be appropriate bridge vectors for arboviruses such as West Nile virus. Thus bird-baited collections were also conducted in this thesis (Chapter 4). Of the remaining species groups, very few *Ae. cinereus/geminus* were collected and as little is known about the differential biology of these species – owing to the very recent discovery of *Ae. geminus* in the UK (Medlock & Vaux 2009) – these were not separated. By accompanying larval sampling with the collection of adults, results indicate that it is likely that only *Cs. annulata* was present on the farms, and that *Oc. caspius/dorsalis* are present rather than *Oc. leucomelas*. *Ochlerotatus caspius/dorsalis* and *Oc. cantans/annulipes* exhibit fairly similar ecologies within their respective groupings (Snow 1990; Cranston et al. 1987) and as no targeted molecular assays currently exist for their identification, these groupings remain throughout the thesis.

The blood meal assay (Ivanova et al. 2007) was able to successfully produce bands on a gel of the correct size (~685bp) for 24 different vertebrate hosts without producing a visible product for *Cx. pipiens* s.l. DNA (Table 2.10). In the time course experiment, bands of the correct size were detected from extracted DNA from mosquito abdomens until 96 hours post-feeding. However, when sequenced, horse blood was only correctly identified until 24 hours post-feeding, after which the sample was identified as mosquito in BLAST searches. This suggests that the PCR, although designed to target vertebrate DNA only, amplifies mosquito DNA as well to a level that is sufficient to outcompete blood meal sequences once the blood meal starts to become digested. This informed the approach for future intensive blood meal collections (Chapter 6). Firstly, mosquitoes would be classified according to blood meal digestion state using

the Sella scale (Detinova 1962) in order to relate the likelihood of successfully identifying blood meal host to the stage of digestion as performed in other studies (Martínez-de la Puente et al. 2013). Secondly it was important to collect as many blood-feds as possible as only a limited number would be likely to produce a result for blood meal host. Accordingly, weekly intensive collections using several collection methods were utilised to collect blood-feds.

Farm site selection for future work

The primary purpose of this pilot study was to assess whether the farms supported populations of potential mosquito vectors of mosquito-borne pathogens and to enable the selection of a smaller (and logistically feasible) number of sites for future intensive studies using host-baited collections. The four farms selected for future work were Church Farm, Glendell Livery, Elmley and Northney Farm. This selection was a result of several factors. Firstly, species diversity indices (*1-D*) indicated that Glendell Livery and Elmley had the highest mosquito species diversity as compared to Church Farm and Northney Farm which had the lowest. This difference was despite the fact that Elmley and Northney Farm yielded the greatest numbers of adult mosquitoes by CDC trapping whilst Church Farm and Glendell Livery yielded far fewer. Secondly, the four farms exhibited differences in the total numbers of human biting species collected. Elmley and Northney Farm yielded the greatest overall numbers of human biting mosquitoes (as collected by HLC) and higher (preliminary) mean biting rates, whilst Church Farm and Glendell Livery yielded much lower total numbers and estimated biting rates. These differences were expected to provide a contrast in the results of future host baited collection studies. Thirdly, Elmley and Northney farm are historically associated with mosquito activity (e.g. Marshall, (1938); Ramsdale & Snow, (1995)) and therefore collecting data at these sites would enable comparison with previous work over the past century. Finally, these four farms provided the most favourable logistics for mosquito trapping.

The high levels of biosecurity at ADAS Arthur Rickwood made this site difficult to access except on the periphery, making future host baited collections difficult. Similarly, Coombelands

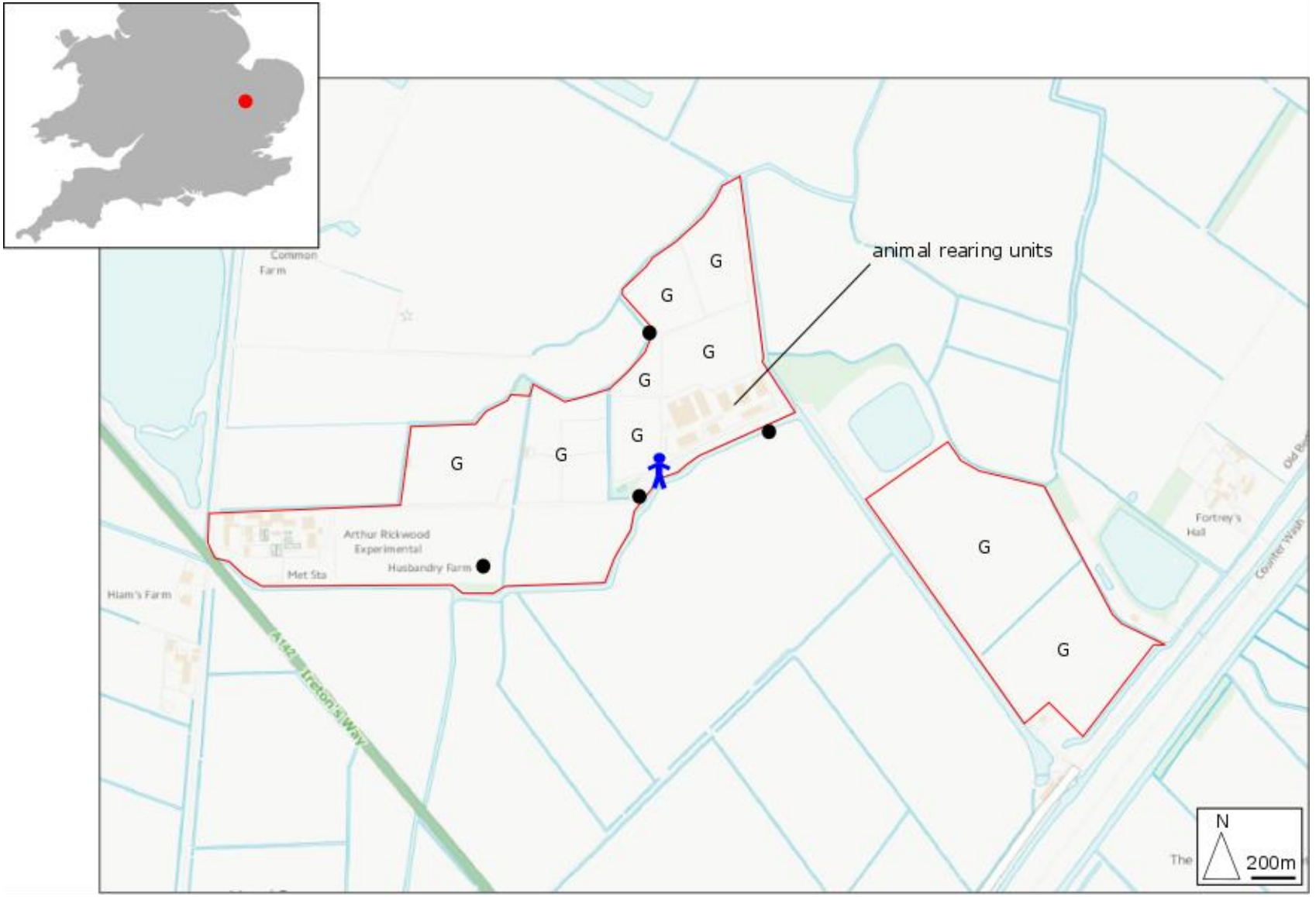
Farm required access through the main APHA Weybridge site, the gate of which was locked at sunset, making access in the evenings difficult. Although Mudchute Farm occupies an ecologically unique setting as a mixed animal farm surrounded by dense human habitation, the limited vehicular access to the site and the use of adjacent areas as a meeting point for groups of locals after dark precluded its use for evening host-baited collections.

Prior to the commencement of the studies in Chapters 3 and 4, Glendell Livery was replaced by a different, nearby site for several reasons. Firstly, an unexpected change of site ownership resulted in considerable building work to the stables area and some unforeseen access restrictions. Secondly, in re-visiting the pilot data, it became clear that a level of standardisation of livestock host availability should be introduced when conducting future studies and as the other three farms maintained cattle and this only horses, it should be replaced with a different site. Accordingly, White Lodge in Bisley, Surrey (51.322255, -0.637692, NUTS3: UKJ25, soilscape category 15/18), a farm of approximately 0.2 km^2 and maintaining approximately 50 beef cattle was introduced as a replacement. A preliminary visit identified White Lodge as having a similar habitat profile (including land cover and soil type) to Glendell Livery, similar levels of human activity and suitable site access to facilitate host-baited collections. A detailed map of the site is provided below alongside the other farms.

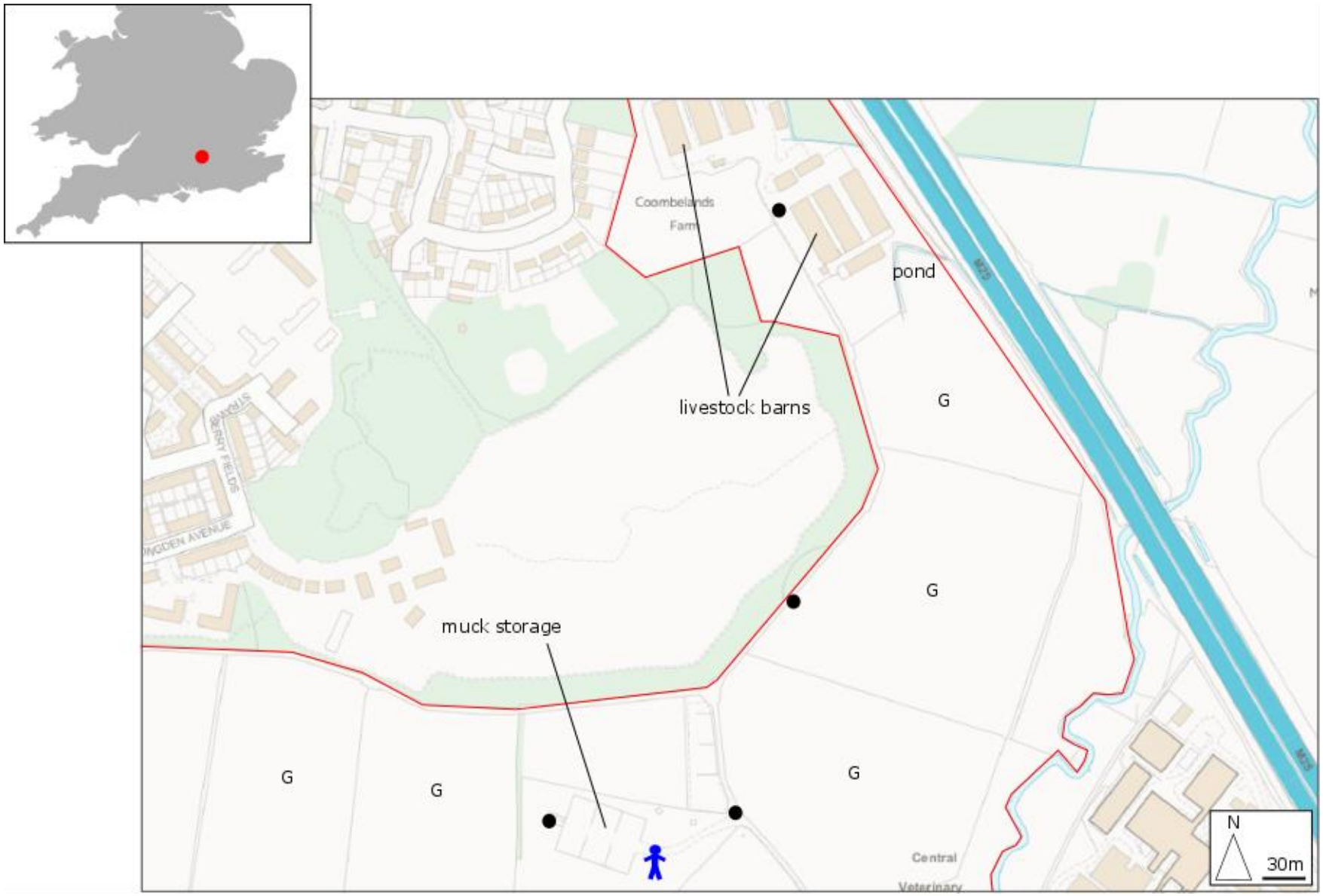
2.7 Maps of Farms

Key to map symbols	
	boundaries of farm
	CDC light trap + CO_2 locations, 2012 pilot study
	weather station location, 2013 or 2014 studies
	human landing catch location, 2012 pilot study
	human landing catch locations, 2013 human biting study, sites a-d
	chicken-baited trap (low and high) locations, 2013 avian biting study
	resting box location (five per location), 2014 study, Elmley only
A/G/AG	land use: Arable/Grazing/mixed usage

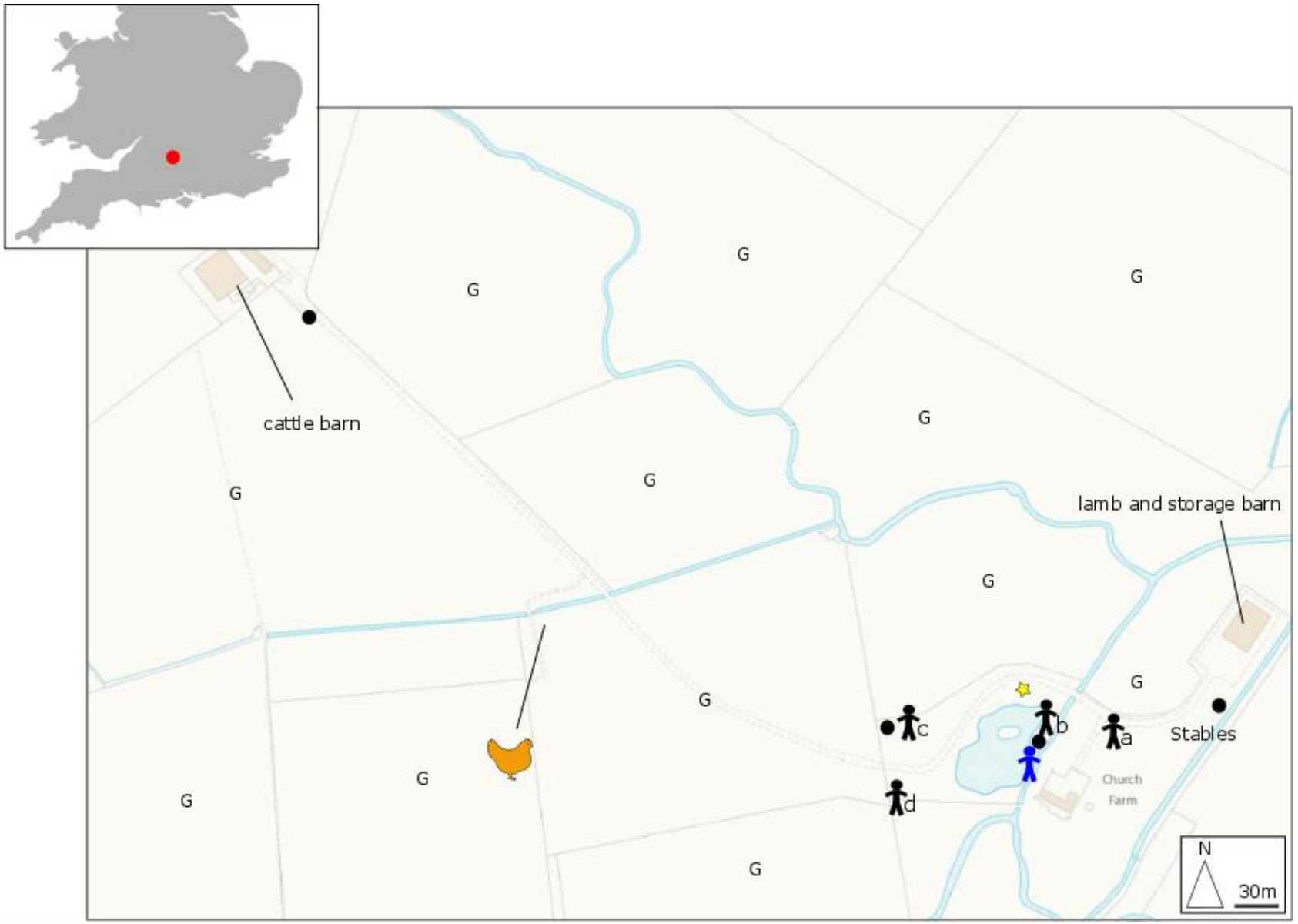
ADAS Arthur Rickwood, Mepal, Cambridgeshire



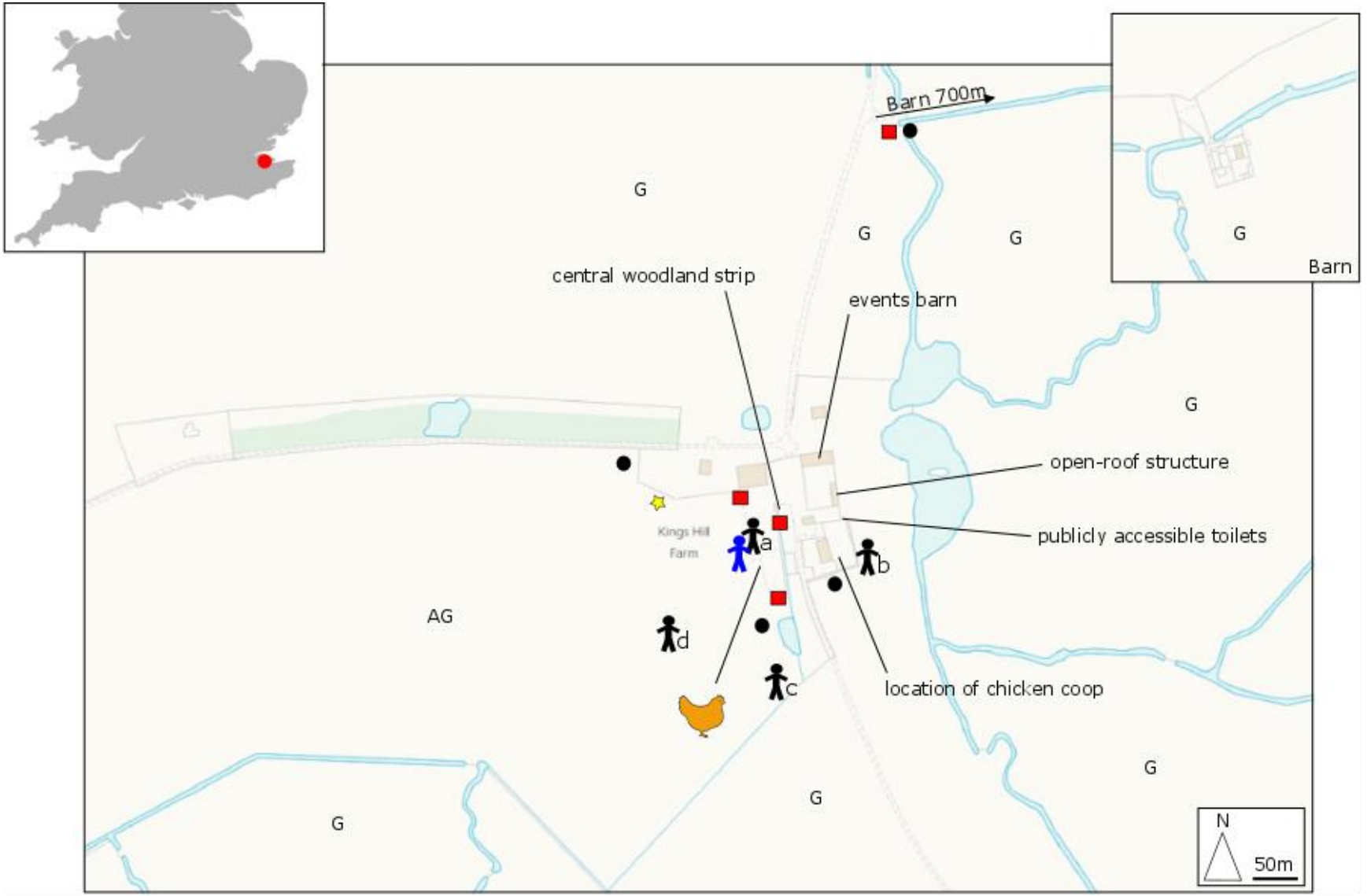
Coombelands Farm, APHA Weybridge, Surrey



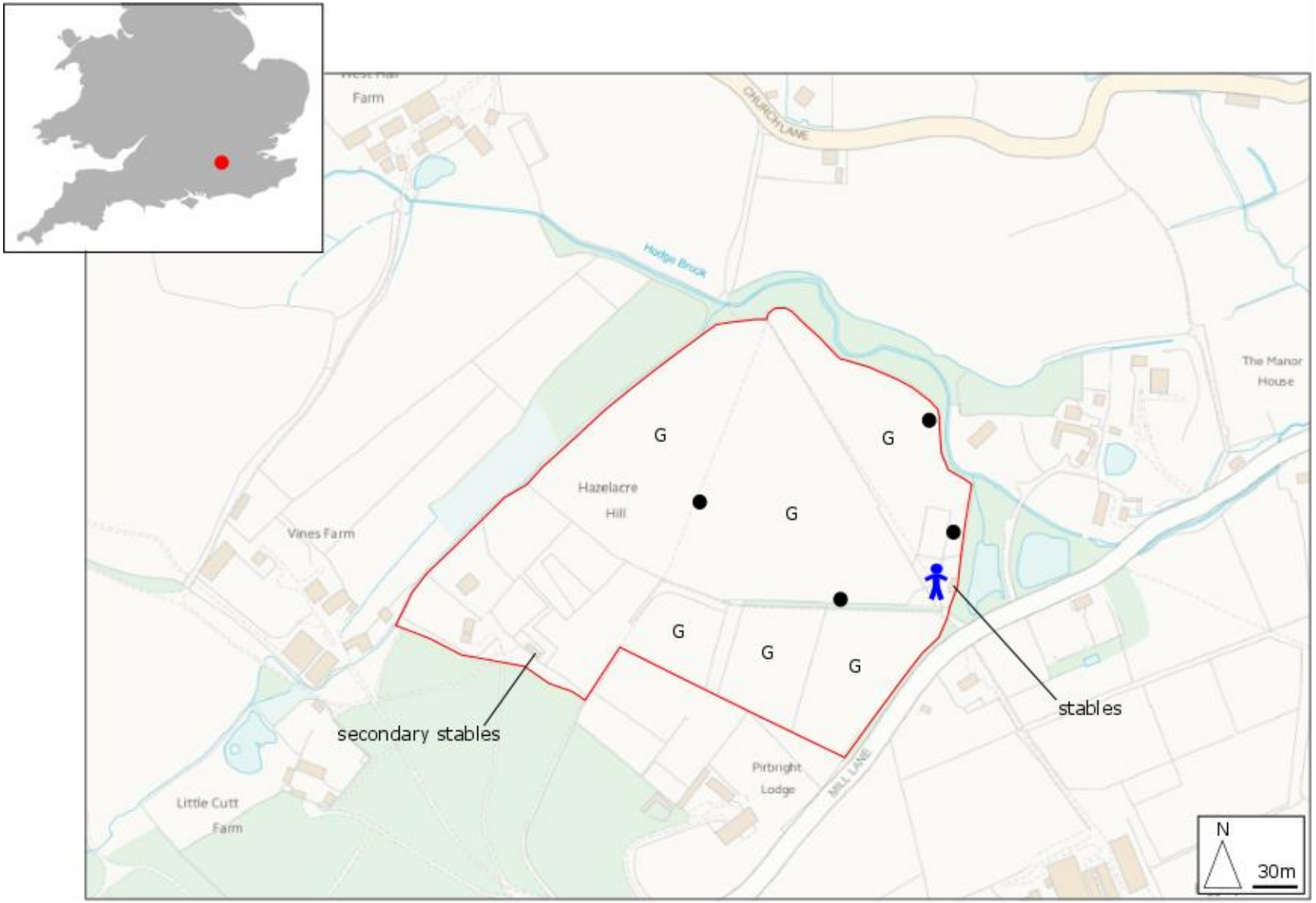
Church Farm, Northmoor, Oxfordshire



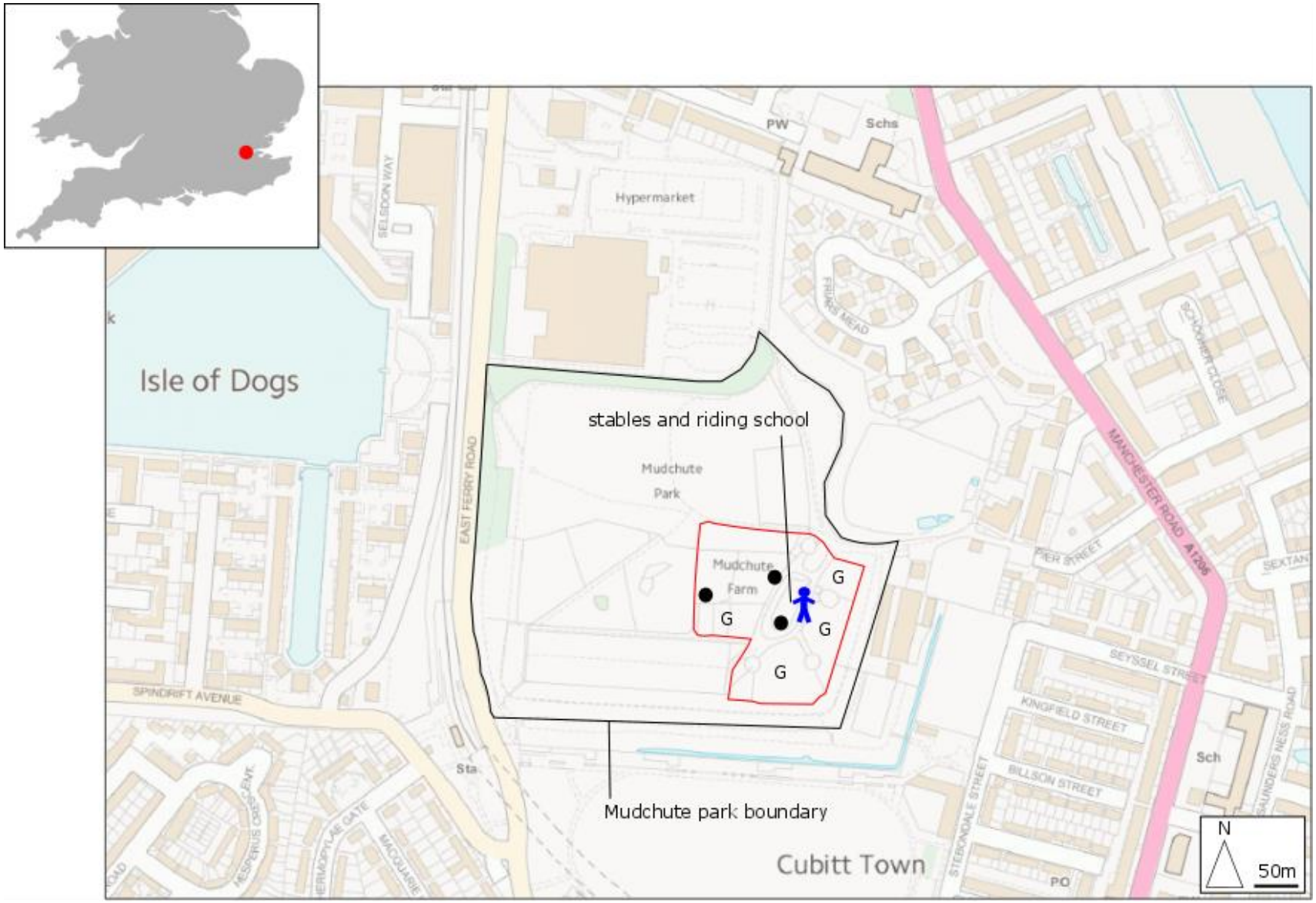
Elmley Nature Reserve, Kent



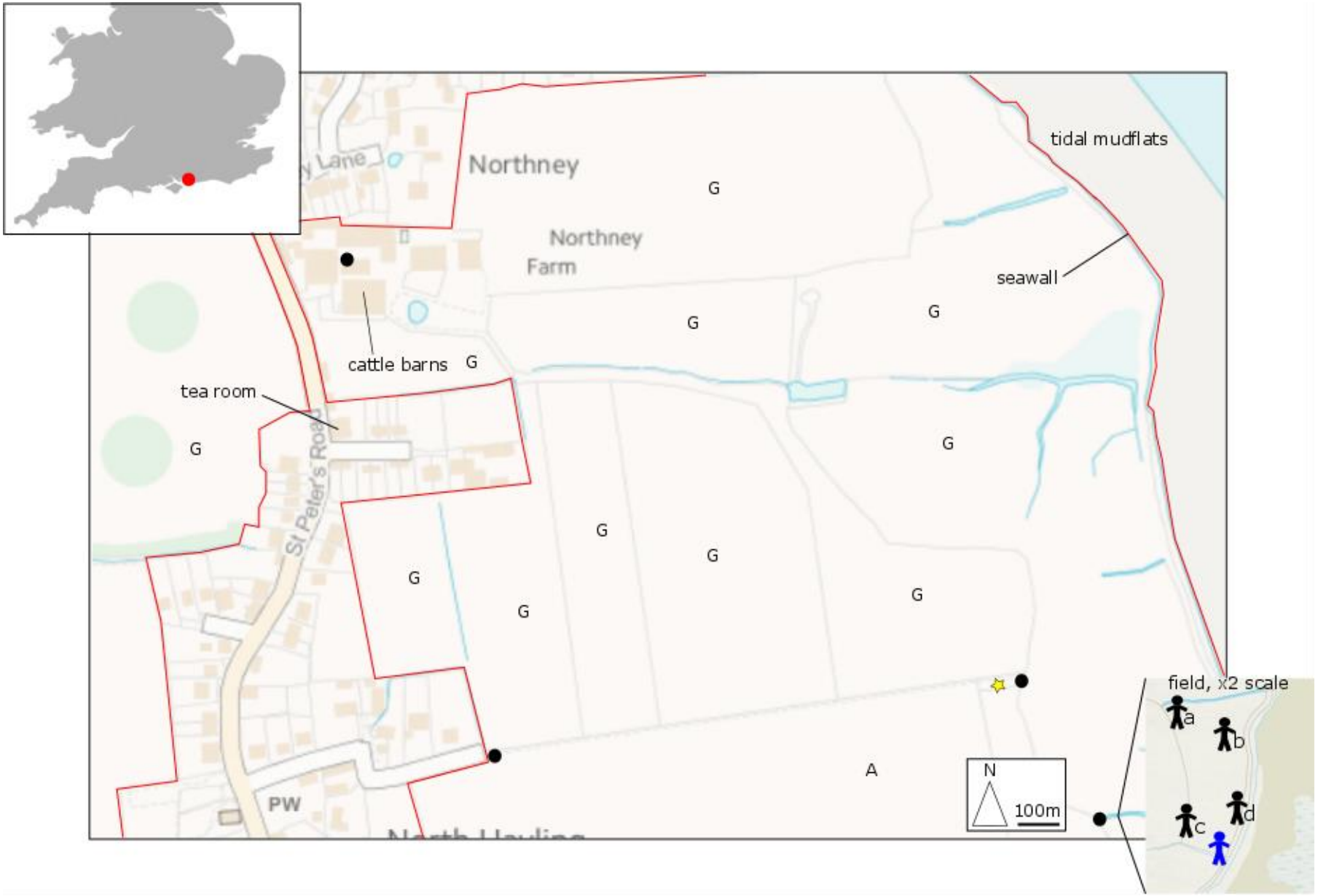
Glendale Livery and Riding School, Pirbright, Surrey



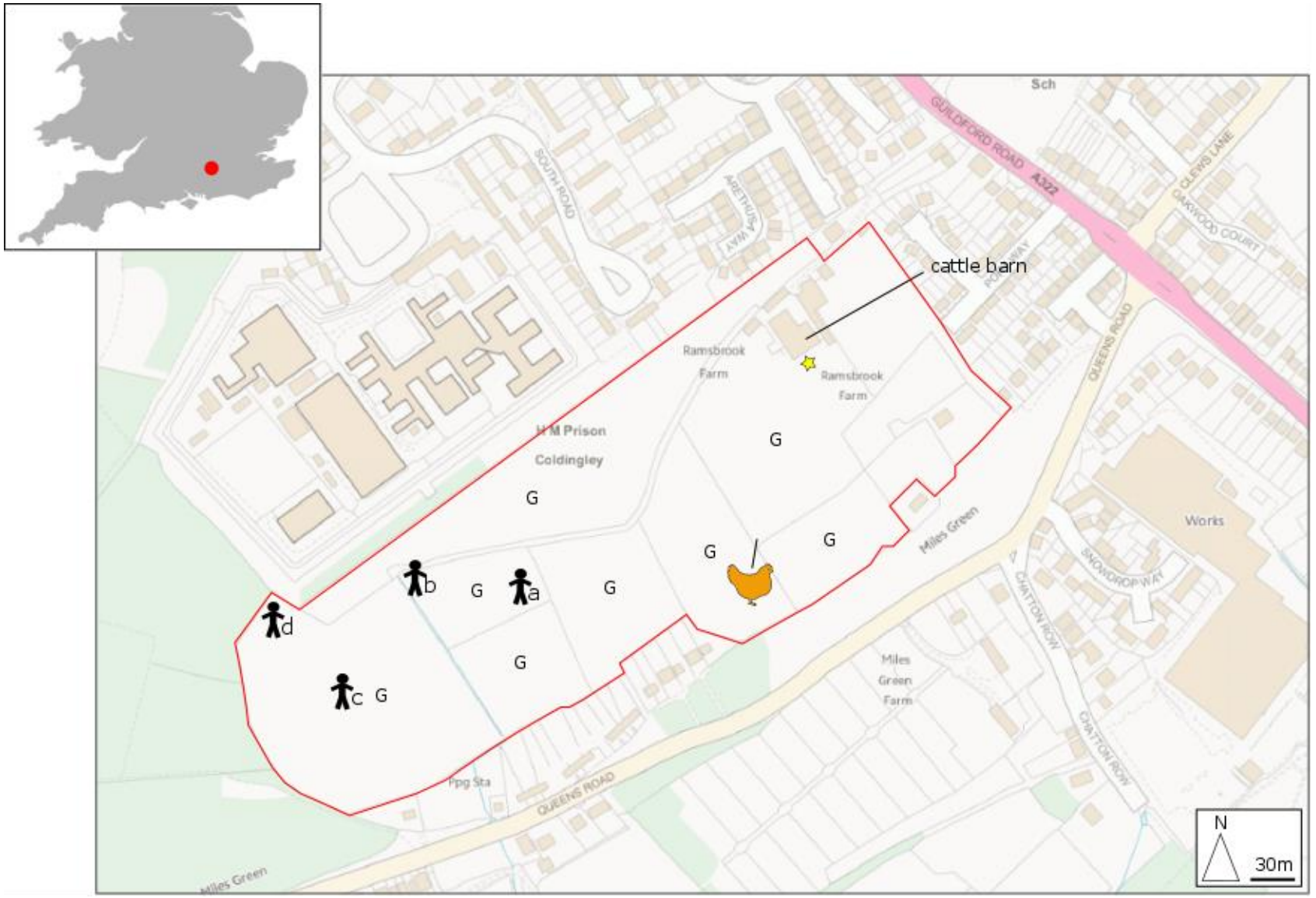
Mudchute Park and Farm, Isle of Dogs, London



Northney Farm, Hayling Island, Hampshire



White Lodge, Bisley, Surrey



Chapter 3 – Mosquito biting patterns on humans

3.1 Introduction

At least 24 of the 34 mosquito species in the UK have been recorded as feeding on humans (Table 3.1), of which five are responsible for the majority of nuisance biting reports: *Cs. annulata*, *Oc. detritus*, *Cx. pipiens* s.l. (attributed to the *molestus* ecoform), *Oc. cantans* and *An. maculipennis* s.l. (Medlock et al. 2012). As highlighted in Chapter 1, particular research focus in the UK has been given to understanding the biting behaviour of a limited number of species, particularly *Oc. cantans*, as part of ecological studies detailing life history traits including feeding behaviour and host preferences (Renshaw et al. 1994; Service 1977b). Focus has also been given to the studies of mosquito species assemblages present in sites favoured by key mosquito biologists, such as Brownsea Island in Dorset and Monks Wood in Cambridgeshire (Service 1969a; Service 1971b; Service 1994). However, the human biting behaviour of many of the remaining UK species have received far less attention and in many cases life history and behavioural information have been inferred from populations outside of the UK (Cranston et al. 1987). Furthermore, no behavioural field studies have been conducted in the UK since the addition of *Ae. cinereus/geminus* and *An. daciae* to the UK mosquito checklist (Medlock & Vaux 2009; Linton et al. 2005), and in areas known to support populations of potential arbovirus vector *Cx. modestus*, now believed to be more widespread across the south of England than was originally believed (Medlock & Vaux 2012; Golding et al. 2012).

Species	Published evidence for human-biting behaviour in the UK		
	Biting nuisance reports	Blood meal analysis	Host-baited collections
Aedes cinereus/geminus	✓[1]	✓[2,3]	✓[2,3,4]
Aedes vexans	✓[1]	-	-
Anopheles algeriensis	✓[5]	-	-
Anopheles claviger	✓[6,7]	-	✓[3]
*Anopheles maculipennis s.l.**	✓[1,8]	✓[9]ø	-
Anopheles plumbeus	✓[1]	✓[2,3]	✓[2,3,4]
Coquillettidia richiardii	✓[8]	✓[2,3]	✓[2,3,4]
Culex europeaus	-	-	-
Culex modestus	-	-	✓[10]¥
*Culex pipiens s.l.***	✓[6,7,8]	✓[2,3]	-
Culiseta annulata	✓[1,6,7,8]	✓[2,3]	✓[2,3]
Culiseta litorea	-	✓[2,3,14]	-
Culiseta morsitans	-	✓[2,3,14]	-
Culiseta subochrea	✓[1,8]	-	-
Dahliana geniculata	✓[1]	✓[3]	✓[2,3,4,11]
Ochlerotatus annulipes	✓[8]	-	✓[2]
Ochlerotatus cantans	✓[6,7,8]	✓[2,3,12]	✓[2,3,4,12,13]
Ochlerotatus caspius	✓[1,6,7]	-	✓[2]
Ochlerotatus communis	-	-	-
Ochlerotatus detritus	✓[1,6,7,8]	✓[2,3]	✓[2,3,4]
Ochlerotatus dorsalis	✓[1]	✓[3]	-
Ochlerotatus flavescens	✓[1]	-	-
Ochlerotatus punctor	✓[1,8,15]	✓[2,3]	✓[2,3,4]
Ochlerotatus rusticus	✓[6,7]	-	✓[2,4]

Table 3.1: UK mosquitoes with reported human-biting behaviour in the literature based upon Medlock et al. (2005) and collated from the following references (in superscript): (1) Marshall (1938), (2) Service (1969a), (3) Service (1971b), (4) Service (1971d), (5) Edwards (1932), (6) Snow (1987), (7) (Snow 1996), (8) Medlock, Hansford, Anderson, et al. (2012), (9) Danabalan et al. (2014), (10) Marshall (1945), (11) Yates (1979), (12) Renshaw et al. (1994), (13) Service (1977), (14) Service (1994), (15) Harold (1926). * Early studies were not able to separate the three members of *An. maculipennis* s.l. and others elected not to identify to species. Ø This study found evidence of human biting in all three members of *An. maculipennis* s.l.. ¥ Not a host-baited study *per se*, but an incidental collection of one specimen biting the collector ** Ecoforms of *Culex pipiens* s.l. not separated.

The method used most commonly to record biting mosquito density is the human landing catch, with variation in individual attractiveness to mosquitoes presenting a requirement to randomise collectors between trap times and locations (Silver 2007). The use of different trapping locations within a site may also influence the numbers and mosquito species assemblages collected according to distance to larval habitats and other factors. In the UK, mosquito biting activity during the day predominantly occurs in sheltered areas, largely owing to proximity to outdoor mosquito resting sites (Service 1969a; Service 1971d), although meteorological parameters including reduced wind speed and lower levels of solar radiation are likely to also play a role. Field studies on African *Anopheles*, *Culex* and *Mansonia* species found that flight activity ceased at wind speeds of between 1.2 m/s (Snow 1980) and 1.8 m/s (Gillies & Wilkes 1981). However, in noting that *Ochlerotatus punctor* in Sweden is able to continue flying at temperatures as low as 4°C (Jaenson 1988), Service (1980) highlights that species- and ecosystem-specific adaptations in flight and biting behaviour may exist. In the UK, the effect of meteorological variables on mosquito activity has not been studied in detail, with the exception of investigations attempting to understand the relationship between temperature and humidity and the indoor resting sites of hibernating *Cx. pipiens* s.l. (Service 1969a).

Although some studies have investigated human biting by mosquitoes in areas known to host livestock species, for example near Liverpool, UK (Renshaw et al. 1994) and in the Camargue, France (Balenghien et al. 2006), the use of the farm environment was largely incidental. Current evidence indicates that host availability, rather than specific host preferences, may drive the feeding behaviour of mosquitoes (Chaves et al. 2010). Although humans are by no means the most numerous available hosts on farms, the behavioural patterns of farm workers may expose them to mosquito biting at times of the day (dawn and dusk) and year (the summer) during which peak biting activity of mosquitoes would be expected, and in areas where alternative hosts may not be present. Additionally, routine movement of livestock populations on and off the site, or between fields within the same site, will alter the host availability locally and could therefore result in an increased probability of mosquitoes biting

humans. Some evidence of such opportunistic feeding patterns have been demonstrated in the UK, with partially blood-fed *Oc. cantans* disturbed mid-feed from cattle collected from human landing catches conducted nearby (Renshaw et al. 1994).

The present study aimed to measure the rate at which humans were bitten by mosquitoes on UK farms by means of standardised human landing catches. Unlike many such studies conducted worldwide, the experimentation was conducted across multiple farms and at multiple sites within these farms and used randomisation of collectors across a relatively large source of volunteers. In addition, environmental monitoring was used to specifically investigate the impact of diel periodicity, temperature, precipitation, relative humidity and wind speed on mosquito biting rates.

Study aim

To investigate the peak human biting rate on UK farms and determine the effect of environmental variables on the number of mosquitoes collected.

Objectives

1. To conduct standardised human landing catches using a pool of volunteers to determine the human biting rate on four UK farms during July and August 2013.
2. To determine which environmental variables are important in influencing the biting rate of mosquitoes on humans within farm environments by comparison of mosquito catch data with meteorological variables.

3.2 Materials and Methods

3.2.1 Study farms

Four farms: Church Farm, Elmley, Northney Farm and White Lodge, were selected for this study according to the criteria detailed in Chapter 2. Within each farm, four sites were chosen at which to conduct human landing catches (see the maps at the end of Chapter 2). Each site was situated a minimum of 50 m apart, within an overall area of no more than 500 m^2 and located in areas frequented by farm workers and livestock. Care was taken to minimise volunteer contact with farm animals or farm machinery in use. Photospheres are provided for each site except those at Church Farm (due to a camera fault), see enclosed CD-ROM.

3.2.2 Recruitment of collectors

Thirteen collectors, including the author, took part in the study. Collectors were recruited on a voluntary basis directly from TPI and the APHA. Volunteers were provided with details of the study, signed a consent form and were free to withdraw from the study at any point. The selection of volunteers was primarily based on their availability to commit to the time requirements of the project (a minimum of four nights), although those known to suffer severe reactions to insect bites were advised not to participate. No specific selection criteria for age (other than participants being at least 18 years old), gender or race were considered in this study. Ethical approval was obtained prior to the commencement of the study from the London School of Hygiene and Tropical Medicine (LSHTM) ethics committee, reference number 6446.

3.2.3 Requirements of collectors

Collectors were asked to refrain from washing with scented soaps within two hours of starting collections and were requested not to wear scented deodorants or perfumes on the trial days. Collectors were not subject to specific clothing requirements (e.g. colours), with the exception of being advised to take warm clothes that covered exposed skin whilst enabling one lower leg to be exposed to the knee to facilitate mosquito collections. No dietary restrictions were imposed. Smoking was not permitted during the study but was not considered as a factor

for volunteer recruitment. A trained first aider was part of the collection team on every trap evening and the fieldwork vehicle used for transport to the farms was equipped with a first aid kit.

3.2.4 Organisation of collectors and assignment to trapping visits

Due to volunteer availability two groups of volunteers were created, a 'primary' group consisting of five collectors available for 12 nights each and a 'secondary' group consisting of eight collectors, each available for four nights' collection only. Each collector from the primary group was required to visit each farm three times, whilst those in the secondary group were required to visit each farm only once. Each collector was provided with a rucksack containing: a manual aspirator (John W Hock, Florida, USA, Figure 3.1), cleaned after each use with sterile cleansing wipes; eight cardboard pillboxes (Watkins and Doncaster, Cranbrook, UK) with an insect mesh upper section for collection of mosquitoes; a walkie talkie to facilitate communication on site; a head torch with red filter; and a clipboard containing study information. The clipboard contained: a checklist and instruction sheet outlining equipment and methodological procedure; a laminated map detailing the four HLC sites on the specified farm; details of the allocation of collectors to sites for each collection period; results sheets to record the presence/absence of mosquitoes in each collection period and any other relevant observations. Four folding stools were provided and positioned at each collection site for the duration of the collection period.

Figure 3.1: Manual aspirator used for human landing catch collections, model 612 (John W Hock).

3.2.5 Human landing catch protocol

The study took place between July and August 2013 for a total of 24 collection nights, six at each farm. On a given trap evening, four collectors arrived at the farm approximately 30 minutes before the start of the collections. Collections ran for four hours starting two hours before sunset; sunset times were obtained from www.timeanddate.com. The four-hour period was split into eight collection periods of 25 minutes each, after each of which 5 minutes was available to allow movement of the volunteer to the next collection site. Collectors were assigned to a site for each collection period (1-8) by two sequential Latin Square randomisations, to control for differences in attractiveness to biting, using a script in R 3.2.0 (R Core Team 2015), kindly provided by Dr Lara Harrup (TPI) (see Table 3.2 for an example and appendix A6 for the R script). Landing catches were conducted by participants who sat on a stool and aspirated any mosquitoes alighting on one exposed lower leg after which the mosquitoes were transferred into a pillbox. Collectors were instructed to only aspirate mosquitoes when they had landed, but before they began biting. Mosquitoes flying around, alighting on, or attempting to feed on the collectors during the 5 minute transition period were not collected. The red-light head torch was used by collectors when natural light intensity was insufficient to carry out collections. At the end of each collection visit, volunteers placed the pillboxes containing collected mosquitoes into a polystyrene cooler where they were transported to TPI for killing and storage at - 20°C until processing.

Trapping site	*Collection period*							
	1st	*2nd*	*3rd*	*4th*	*5th*	*6th*	*7th*	*8th*
Site A	4	3	1	2	1	4	2	3
Site B	3	4	2	1	4	2	3	1
Site C	1	2	3	4	3	1	4	2
Site D	2	1	4	3	2	3	1	4

Table 3.2: An example of the allocation of collectors (1-4) to trap sites within each farm on a given trap evening by way of two randomised Latin square rotations per evening. The dotted line indicates the end of the first randomisation and the start of the second.

3.2.6 Collection of meteorological data

Evenings were judged to be suitable for mosquito collections if average forecasted wind speeds were below 3.1 m/s (approximately 7 miles/hour) and with minimal rainfall (< 1mm) as forecasted by www.xcweather.co.uk following the criteria used by Service, (1969a). Meteorological data were collected at hourly intervals from each farm using an automatic weather station (AWS) data logger model CR800 (Campbell Scientific, Loughborough, UK) (Figure 3.2); details on AWS locations are provided as stars on the maps in Chapter 2. Variables collected were air temperature (°C), relative humidity (%), wind speed (m/s), solar intensity (kJ/m²) and rainfall (mm). The hourly data points are a mean value calculated automatically from values recorded every minute. Data were stored on the on-board CR800 data logger, downloaded onto a laptop at the end of the study and stored as an Excel file. Due to failure of the weather station at Elmley, temperature, wind speed and rainfall data at hourly intervals were obtained from the nearest Met Office weather station located approximately 20 km away at Shoeburyness (data kindly provided by Noel Nelson, Met Office). The temperature/relative humidity probe at White Lodge failed shortly before collections commenced and therefore temperature data were collected using a TinyTag Plus2 datalogger (Gemini Data Loggers Ltd., West Sussex, UK) hung at the same height as the standard probe. To provide additional within-farm comparison, wind speeds on one day in November 2013 at each site (A – D) were recorded every minute for 15 minutes using a digital hand-held anemometer (Model ADC Summit, Silva, Sweden) and the average figure compared to the figure recorded by the weather station on each farm.

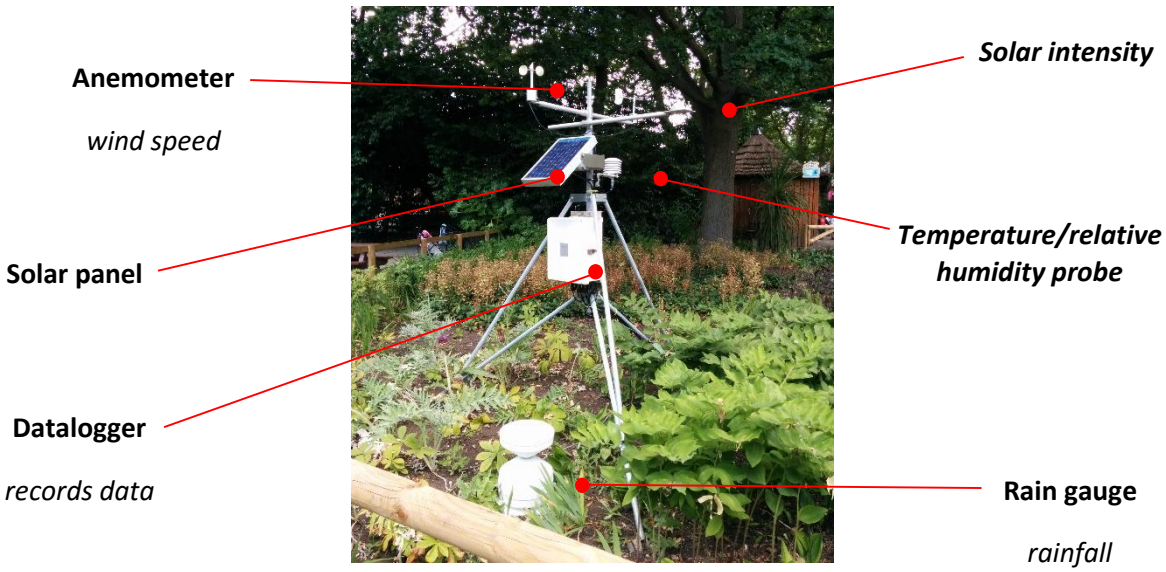


Figure 3.2: An automatic weather station (Campbell Scientific) for the recording of meteorological variables *in situ*, with annotations highlighting the functions of the attachments.

3.2.7 Identification of mosquitoes and blood meals

Mosquitoes were identified based on morphological features following published keys (Snow 1990; Cranston et al. 1987). Mosquitoes morphologically identified as *Culex pipiens s.l./torrentium* were identified using molecular methods to species level using the techniques detailed in sections 2.5.1 and 2.5.2. Mosquitoes identified morphologically as *Anopheles maculipennis* s.l. were identified using the techniques detailed in section 2.5.3. Collectors were instructed to collect mosquitoes after they landed but prior to their biting (see section 3.2.5 above), however any collected mosquitoes identified as containing blood were analysed to identify the blood meal origin. Identification of vertebrate blood meal host was performed using a PCR-sequencing assay as detailed in section 2.5.4.

3.2.9 Data Analysis

Data were stored and descriptive analyses performed using Microsoft Excel. To assess the effect of different variables on the human biting rate, a generalized linear mixed model (GLMM) was fitted to the data using the 'glmmadmb' function in the 'glmmADMB' package in R. Relative humidity and solar intensity were omitted from the analyses due to the weather

station failure at Elmley. The first model considered total biting pressure of all species. As the response variable (mosquito catch, all species, per 25-minute collection period) was in the form of count data, the initial model consisted of a Poisson GLMM with a log link function including *time to sunset*, *temperature* and *wind speed* as covariates, fitted by maximum likelihood with the Laplace approximation. *Farm* was included as a fixed effect in the model. *Site* within each farm was included as a random factor, fully nested within farm, and *collector* was included as a random effect. *Rainfall* was included as a fixed (presence/absence) factor. The Poisson model indicated that the data was overdispersed (residual deviances > degrees of freedom) therefore a negative binomial GLMM was fitted to the data. The goodness-of-fit of the models to the data was assessed by comparison of Akaike information criterion (AIC) values using function 'AIC' in the 'stats' package in R, with lower values indicating a better model fit. The final model was obtained by step-wise deletion of non-significant factors and variables on the basis of AIC values. An AIC value change of ≤ 2 units indicated a particular factor or variable did not significantly explain the response variable.

Two additional models were subsequently fitted to consider the factors influencing the human biting rate of two individual species collected in sufficient number (> 100 specimens) to permit separate modelling. Both species were collected at one farm site only and therefore farm was removed as a factor in the models. The intial models consisted of Poisson GLMMs with a log link function, including *time to sunset*, *temperature* and *wind speed* as covariates and *site* as a random factor, fitted by maximum likelihood with the Laplace approximation. As residual deviances > degrees of freedom, indicating overdispersion, negative binomial models were subsequently fitted to the data. Sequential model refitting to exclude non-significant fixed factors was conducted as above. The script for the three analyses are included in appendix A7 (A-C).

3.3 Results

Human biting rates

A total of 915 mosquitoes were collected in the study over 24 collection evenings (Table 3.3). The greatest number of mosquitoes was collected at Elmley (802), followed by Northney Farm (72) and White Lodge (41). No mosquitoes were collected from landing catches at Church Farm. The mean overall biting rate, (per collector, per 25-minute collection period, per trap evening) combining all mosquito species, was 4.18 (range 0 – 89 mosquitoes) at Elmley, 0.38 (0 – 9 mosquitoes) at Northney Farm and 0.21 (0 – 6 mosquitoes) at White Lodge. At Elmley, the species collected in greatest number in a single collection period was *Cq. richiardii* (67), followed by *An. maculipennis* s.l. (29) and *Cx. modestus* (23), with mean biting rates of 2.59 (range 0 – 67), 0.28 (range 0 – 29) and 1.04 (range 0 – 23), respectively (Table 3.3). With the exception of collector F who withdrew from the study after two collection visits, all visits were conducted according to plan (the author, collector A, took the place of collector F on the remaining two visits).

Human-biting mosquito species collected

Fourteen species/morphologically indistinguishable species complexes were collected (Table 3.3). Molecular separation of the 18 specimens identified morphologically as *Cx. pipiens* s.l./*torrentium* indicated that only *Cx. pipiens* s.l. was present. Of these, 16/18 were identified as *Cx. pipiens f. pipiens* whilst the assay did not produce a result for the remaining two specimens. Fifty-five *An. maculipennis* s.l. were collected, 50 of which were identified by molecular methods as *An. atroparvus* and five as *An. daciae/An. messeae*; the latter two species, collected only from Elmley, presented identical query results in BLAST searches thus precluding their separation. The three most numerous species collected overall were *Cq. richiardii*, 511 (55.8% of the total), *Cx. modestus*, 199 (21.7%) and *Oc. detritus*, 71 (7.8%). *Coquillettidia richiardii* was the only species to be collected from all three farms with Elmley

accounting for 498/511 (96.9%) of the collection; *Cx. modestus* was collected solely at Elmley and *Oc. detritus* was collected primarily at Northney Farm 68/71 (95.8%). Five species/species groups, *An. claviger*, *An. atroparvus*, *An. plumbeus*, *Cx. pipiens s.l.* and *Oc. detritus* were collected on at least two farms whilst the remaining species were collected from only one.

Species	***Elmley***			***Northney Farm***			***White Lodge***			***Total***
	UF	***BF***	***mean (range)***	***UF***	***BF***	***mean (range)***	***UF***	***BF***	***mean (range)***	
Ae. cinereus/geminus	0	0	-	0	0	-	2	0	0.01 (0 -1)	**2**
An. claviger	3	0	0.02 (0 - 1)	1	0	0.01 (0 - 1)	0	0	-	**4**
*An. maculipennis s.l.**	51	3	0.28 (0 - 29)	0	0	-	1		0.01 (0 - 1)	**55**
An. plumbeus	0	0	-	1	0	0.01 (0 - 1)	4	0	0.02 (0 - 1)	**5**
Cq. richiardii	495	3	2.59 (0 - 67)	1	0	0.01 (0 - 1)	12	0	0.06 (0 - 2)	**511**
Cx. modestus	198	1	1.04 (0 - 23)	0	0	-	0	0	-	**199**
*Cx. pipiens s.l.***	16	0	0.08 (0 - 4)	0	0	-	2	0	0.01 (0 -1)	**18**

Table 3.3: Total unfed (UF) and blood-fed (BF) mosquitoes collected at Elmley, Northney Farm and White Lodge over six visits to each. Church Farm is omitted from the table as no mosquitoes were collected there. *Ochlerotatus spp.* refers to specimens for which definitive morphological ID was not possible due to damage. Mean biting rates (range), refer to the average biting experienced by a collector in a single collection period (25 minutes), given to two decimal places. * *An. maculipennis* s.l. includes *An. atroparvus* and *An. daciae/messeae*. ** *Cx. pipiens* s.l. consists of *Cx. pipiens f. pipiens* and those specimens not identified to ecoform.

Species	*Elmley*			*Northney Farm*			*White Lodge*			*Total*
	UF	*BF*	*mean (range)*	*UF*	*BF*	*mean (range)*	*UF*	*BF*	*mean (range)*	
Cs. annulata	2	0	0.01 (0 - 1)	0	0	-	0	0	-	**2**
Da. geniculata	0	0	-	0	0	-	1	0	0.01 (0 - 1)	**1**
Oc. cantans/annulipes	0	0	-	0	0	-	9	1	0.05 (0 - 2)	**10**
Oc. detritus	3	0	0.02 (0 - 1)	59	9	0.35 (0 - 9)	0	0	-	**71**
Oc. flavescens	24	2	0.14 (0 - 6)	0	0	-	0	0	-	**26**
Oc. punctor	0	0	-	0	0	-	3	0	0.02 (0 - 1)	**3**
Oc. rusticus	0	0	-	0	0	-	6	0	0.03 (0 - 1)	**6**
Oc. spp.	1	0	0.01 (0 - 1)	1	0	0.01 (0 -1)	0	0	-	**2**
Totals per farm	**802**	**9**	**4.18 (0 - 89)**	**72**	**9**	**0.38 (0 - 9)**	**41**	**1**	**0.21 (0 - 6)**	**915**

Table 3.3 continued**.**

Blood meal analysis results

The majority of mosquitoes collected were unfed females, however 19 specimens of six species were found to contain blood in their abdomen: *An. atroparvus* (3), *Cq. richiardii* (3), *Cx. modestus* (1), *Oc. cantans/annulipes* (1), *Oc. detritus* (9) and *Oc. flavescens* (2) (Table 3.4). Blood-feeding hosts were successfully identified in 12/19 specimens (63%) (Table 3.4). Ten blood meals were identified as being of human origin, with two blood meals, one from each of *An. atroparvus* and *Cq. richiardii*, identified as originating from a cow (*Bos taurus* L.).

Species	***Total blood-fed***	***Total (%) positive for blood meal host***	***Blood meal hosts (number)***
An. atroparvus	3	1 (33%)	Cow, *Bos taurus* (1)
Cq. richiardii	3	3 (100%)	Human, *Homo sapiens* (2) Cow, *Bos taurus* (1)
Cx. modestus	1	1 (100%)	Human, *Homo sapiens* (1)
Oc. cantans/annulipes	1	1 (100%)	Human, *Homo sapiens* (1)
Oc. detritus	9	6 (67%)	Human, *Homo sapiens* (6)
Oc. flavescens	2	0 (0%)	n/a
Totals	**19**	**12 (63%)**	

Table 3.4: Results of blood meal analysis of engorged specimens collected by human landing catch at Elmley, Northney Farm and White Lodge.

Potential influences on the human biting rate

The total number of mosquitoes collected on each farm over the six collection visits (Figure 3.3, A1, B1 and C1) indicate that day-specific factors (such as meteorological conditions) may have influenced the number of mosquitoes collected. The total number of mosquitoes collected at the four sites on each farm also varies within each farm (Figure 3.3, A2, B2 and C2). At Elmley the total number collected at a single site ranged from 144 (site C) to 336 (site B); at Northney Farm the range was from 2 (site B) to 33 (site C); at White Lodge the range was 3 (site

A) to 25 (site D). these results indicate that there may be additional, within-farm factors or variables that may have influenced the numbers of mosquitoes collected. These differences were not explicitly tested for but are included as random factors contributing to the variation of the statistical models (below). According to measurements made using the hand-held anemometer, wind speeds at each site may differ considerably from those recorded by the main weather station on each farm (Table 3.5).

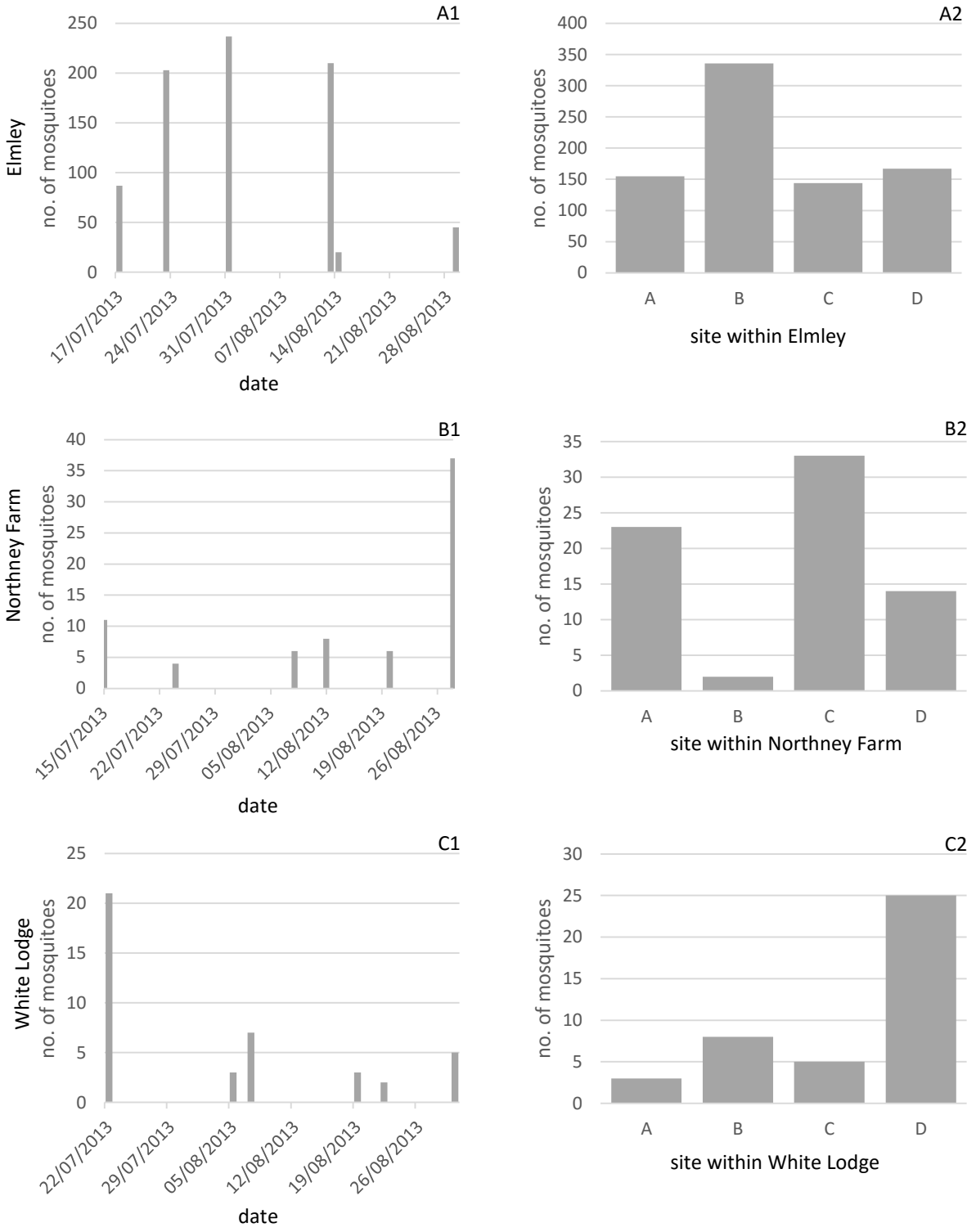


Figure 3.3: Graphs A1, B1, and C1 show the total mosquitoes collected on each of the six visits to each farm. Graphs A2, B2 and C2 show the total number of mosquitoes collected at the four sites within each farm. Note that there is no relationship between sites labelled with same letter at different farms.

Farm	Site	Average (range) hand-held anemometer wind speed, m/s	Weather station wind speed, m/s
Northney Farm	A	0.00 (0.00 – 0.00)	5.60
	B	5.12 (3.60 – 6.60)	5.66
	C	2.67 (0.50 – 4.90)	6.39
	D	3.81 (2.10 – 6.30)	5.42
White Lodge	A	0.67 (0.00 – 2.80)	1.60*
	B	0.29 (0.00 – 1.60)	1.60*
	C	1.03 (0.00 – 3.20)	1.43*
	D	0.06 (0.00 – 0.50)	1.43*

Table 3.5: Comparison of mean (range) wind speeds as recorded by hand-held anemometer at each human landing catch site at Northney farm and White Lodge. The values were derived from readings taken each minute for 15 minutes. * No 15-minute interval data was available from the weather station at White Lodge therefore the hourly average encompassing the 15-minute recording time is given. Church Farm is omitted owing to no mosquitoes being collected there and Elmley is also omitted due to the failure of the weather station.

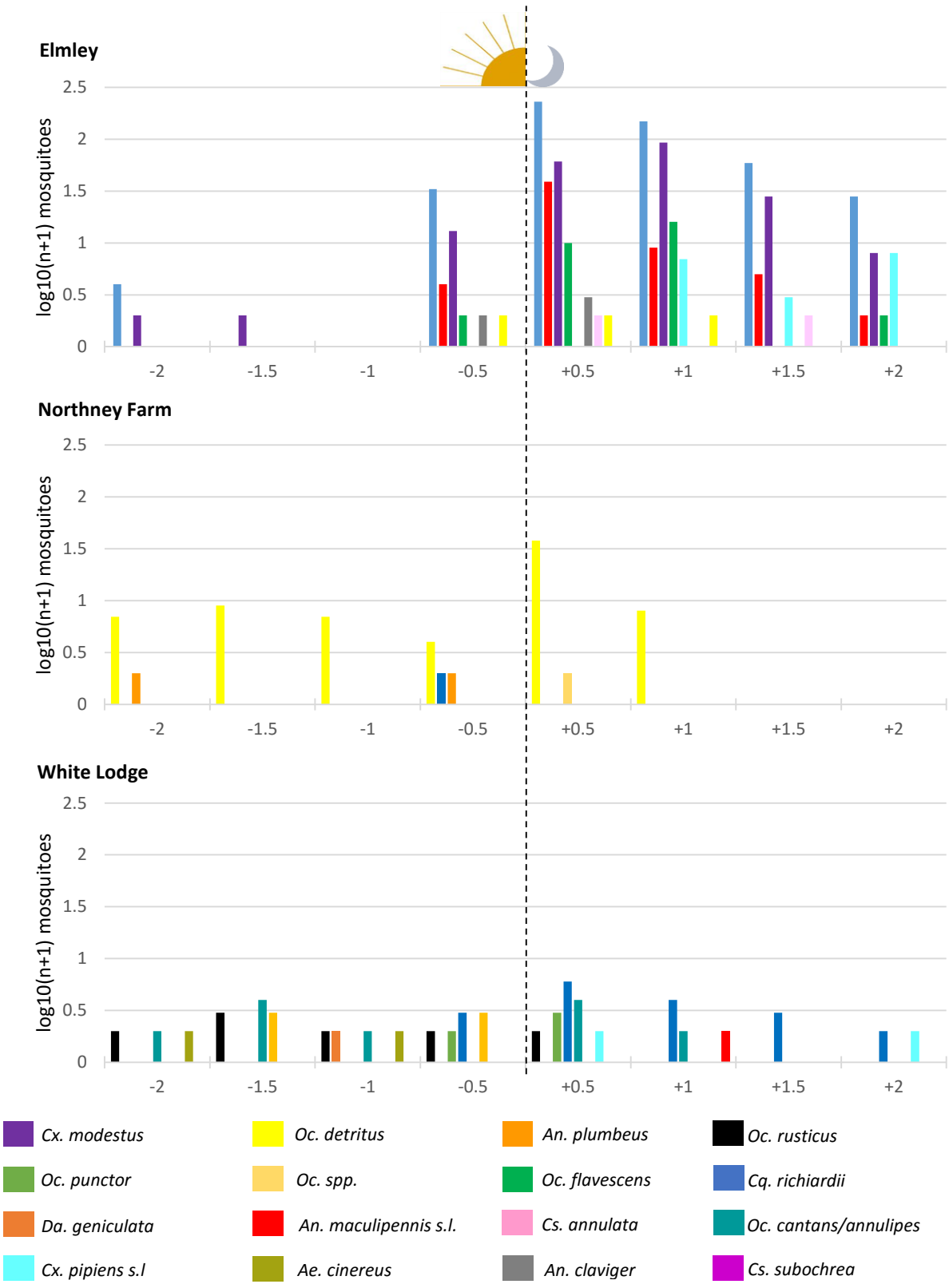


Figure 3.4: Bar charts showing the relationship between time relative to sunset and the $\log_{10}$ (n+1) of mosquito species collected by human landing catch at (from top) Elmley, Northney Farm and White Lodge, totalled over the six visits to each. The dotted line and sun/moon image represents sunset time. *Anopheles atroparvus/daciae/messeae* are presented as *An. maculipennis* s.l.

Biting rates relative to sunset

Several of the species collected display clear temporal trends in human biting as shown by plotting the $\log_{10}$ (n+1) totals of each species against time relative to sunset (Figure 3.4). At Elmley, the trend for biting just after sunset was most defined: very little biting occurred in the first three collection periods with a sharp increase in biting in the -0.5h period. The biting activity of *Cq. richiardii* and *An. claviger* peaked in the +0.5h period whilst *An. maculipennis* s.l., *Cx. modestus* and *Oc. flavescens* displayed peak biting activity in the +1h period after which their biting activities decreased steadily. At both Elmley and White Lodge *Cx. pipiens* s.l. started biting after sunset (+1h and +0.5h periods respectively) and continued until the final collection period with no increasing or decreasing trend evident. At Northney Farm, *Oc. detritus* was collected fairly consistently across all collection periods until peak biting after sunset (+1h period) after which no biting was recorded. The low numbers of other species collected makes it difficult to draw conclusions about their biting activity although approximately half of the human biting activity at White Lodge occurs in the collection periods preceding sunset.

Generalized linear mixed model results

Total biting pressure

The initial GLMM was fitted using all mosquito species across all farm sites in order to model the total biting pressure experienced by humans on each of the farms. Subsequently, data for the two most numerous species, *Cq. richiardii* and *Cx. modestus*, were analysed separately (see following sections); no other species were collected in adequate numbers (> 100 mosquitoes) to permit modelling. The initial total biting pressure model included two fixed effects (*farm* and *rainfall*: mm), two random effects (*collector* and *site*) and three continuous covariates (*time relative to sunset*: hours), *temperature* (°C) and *wind speed*: (m/s). As no mosquitoes were collected at Church Farm, quasi-complete separation in the data resulted and thus the model could not be fitted; this farm was therefore omitted from the model. The initial

Poisson model had a considerably higher AIC value (1868) than the negative binomial model (AIC = 1190), indicating that the latter was a better fit to the data. Farm was found to be a significant predictor of the human biting rate ($P \leq 0.001$) as were time relative to sunset ($P \leq 0.001$) and wind speed ($P \leq 0.001$). No significant effect on biting rate was observed for temperature ($P > 0.01$) or rainfall ($P > 0.01$) and AIC value comparisons indicated that these factors could be excluded from the final model. Total biting pressure was therefore best described by a negative binomial model including the fixed factors *farm*, *time relative to sunset* and *wind speed*, with *collector* and *site* as random factors (Table 3.6). Relative to the biting rate at Northney Farm, total biting activity was 1764% higher at Elmley ($P \leq 0.001$), with a non-significant difference in biting rate between Northney Farm and White Lodge. For every half an hour movement away from sunset, a 29% decrease in the biting rate would be expected ($P \leq 0.001$). An increase of 1 m/s in wind speed would be expected to lead to a 58% decrease in the total biting rate ($P \leq 0.001$).

Coefficients	**Estimate (95% CI)**	**Standard error**
(Intercept)	0.716 (-0.33; 1.76)	0.534
White Lodge	-1.160 (-2.40; 0.08)	0.631
Elmley	2.870 (1.72; 4.02) ***	0.589
Time from sunset	-1.236 (-1.65; -0.82) ***	0.211
Wind speed	-0.541 (-0.78; -0.30) ***	0.123

Table 3.6: Regression coefficients, with Wald 95% confidence intervals and standard errors, for fixed effects of the final, best-fit negative binomial model used to describe total biting pressure. *** $P \leq 0.001$.

Coquillettidia richiardii

As 495/511 (96.9%) of *Cq. richiardii* were collected from Elmley (Table 3.3), only these data (i.e. n = 495) were used for separate analysis. The GLMM was therefore modified so as to exclude *farm* as a factor from the model and thus focused on comparing the effects of meteorological variables and time relative to sunset on the biting rate of this one species. As no rainfall was recorded on the days this species was collected, *rainfall* was also excluded as a factor in the model. The initial Poisson GLMM therefore included two random effects (*collector* and *site*) and three continuous covariates (*time relative to sunset*: hours), *temperature* (°C) and *wind speed* (m/s). The best-fit model was a negative binomial model with an AIC value of 554, lower than that of the initial poisson model (996). Both wind speed and time from sunset were significant predictors of biting rate ($P \leq 0.001$) however temperature was not found to be a significant factor influencing biting rate of *Cq. richiardii* and AIC values indicated that this factor could be removed from the model. The biting rate of *Cq. richiardii* was therefore best described by a model including the fixed factors *time relative to sunset* and *wind speed*, with *collector* and *site* as random factors (Table 3.7). A 1 m/s increase in the wind speed would be predicted to lead to a 41% decrease in biting rate ($P \leq 0.001$). The model predicts that for every half an hour movement away from sunset, an 18% decrease in the biting rate would be expected ($P \leq 0.001$).

Coefficients	**Estimate (95% CI)**	**Standard error**
(Intercept)	4.440 (2.99; 5.89) ***	0.741
Time from sunset	-1.741 (-2.46; -1.02) ***	0.368
Wind speed	-0.888 (-1.25; -0.52) ***	0.189

Table 3.7: Regression coefficients, with Wald 95% confidence intervals and standard errors, for fixed effects of the final, best-fit negative binomial model used to describe the biting activity of *Coquillettidia richiardii*. *** $P \leq 0.001$.

Culex modestus

This species was only collected at Elmley and therefore, as with *Cq. richiardii*, the GLMM was modified so as to exclude *farm* as a factor from the model. *Rainfall* was also excluded as a factor in the model as no rainfall was detected on days on which this species was collected. The initial Poisson GLMM therefore included two random effects (*collector* and *site*) and three continuous covariates (*time relative relative to sunset*: hours), *temperature* (°C) and *wind speed* (m/s). The best-fit model was a negative binomial model with an AIC value of 418, lower than that of the initial poisson model (660). Only time from sunset was a significant predictor of the biting rate of *Cx. modestus* (Table 3.8) and therefore the biting activity of this species was best described by a model including only *time relative to sunset* as a fixed factor*,* with *collector* and *site* as random factors. The model predicts that for every half an hour movement away from sunset, a 21% decrease in the biting rate would be expected ($P \leq 0.001$).

Coefficients	**Estimate (95% CI)**	**Standard error**
(Intercept)	1.688 (0.58; 2.79) *	0.564
Time from sunset	-1.526 (-2.39; -0.66) ***	0.441

Table 3.8: Regression coefficients, with Wald 95% confidence intervals and standard error, for fixed effects of the final, best-fit negative binomial model used to describe the biting activity of *Culex modestus*. *** $P \leq 0.001$, * $P \leq 0.05$.

3.4 Discussion

This is the first study in Europe to describe the human-biting behaviour of farm-associated mosquitoes using a randomised, multi-collector study design. Mosquito populations biting humans were present at three of the four livestock farms (Elmley, Northney Farm and White Lodge) on which trapping was conducted. Fifteen mosquito species/species groups were responsible for biting overall with the most numerous species, *Cq. richiardii*, the only species to be collected at all three of the farms. Five species/species groups, *An. claviger*, *An. atroparvus*, *An. plumbeus*, *Cx. pipiens* s.l. and *Oc. detritus* were collected at two farms each. This study showed that *Cx. pipiens f. pipiens*, despite being described as being almost exclusively ornithophilic in the literature (Snow 1990; Cranston et al. 1987), does attempt to feed on humans when presented with the opportunity. *Culex modestus*, collected only at Elmley, was the second most numerous species from this site; this is the only study to describe the human biting activity of this species in the UK since a single report of a biting adult in the 1940s (Marshall 1945).

All the mosquito species collected in the current study have been reported previously as exhibiting biting of humans in the UK (Table 3.1). This includes the five species, *Cs. annulata*, *Oc. detritus*, *Cx. pipiens* s.l., *Oc. cantans* and *An. maculipennis* s.l., responsible for the majority of nuisance biting reports in the UK (Medlock et al. 2012). Here however, *Culex pipiens f. pipiens*, not the *molestus* ecoform, was collected by human landing catch at Elmley and White Lodge. This deviation from the almost exclusive ornithophilic behaviour reported in the literature from both the UK and Europe may either indicate that population-specific differences in feeding preferences exist or simply that this species displays a level of opportunistic feeding behaviour more frequently attributed to members of the genera *Aedes/Ochlerotatus*. From the perspective of potential arbovirus transmission, this could indicate, taken with caution, that the addition of *Cx. pipiens f. pipiens* to the list of potential bridge vectors in the UK is warranted. Biting rates for *Cx. modestus* appear to be comparable to or even exceed those reported in the

Camargue region in a similar coastal wetland habitat (Balenghien et al. 2006). Although the different experimental designs do not permit direct comparison, over 22 collection days 75 *Cx. modestus* were collected by human landing catches (15 minute exposures every 4 hours over a 24-hour period) in France, whereas in this study 199 specimens were collected in six visits of 4 hours, although the present study was conducted only in July and August.

It was not long after the establishment of the human landing catch as a standard method of collecting anthropophagic mosquitoes (Kerr 1933; Kumm & Novis 1938) that the need to minimise collector bias, resulting from differences between collectors' skill or attractiveness to mosquitoes, became important in study design (Haddow 1954). The principle remains important (Silver 2007) and accordingly, the present experimental design included randomised allocation of collectors to sites on each farm, with the aim of capturing natural human variation in attractiveness to mosquitoes rather than address specific differences between collectors. Thus, collector was included as a random factor in the GLMMs. There were also minimal restrictions placed on the volunteers in terms of their diets, smoking habits (other than not smoking during the collection period) or clothes they wore (in comparison, in Service (1969) the author wore the same clothes for all collections) as this was considered a more realistic situation to that which would be experienced by those working on farms. A key factor which most likely affected the number of mosquitoes collected was the experience of the collector. Although all volunteers were fully instructed on how to conduct the landing catches, very few of the volunteers had previously used the technique and thus the level of skill and success rate for capture would be variable. As a very loose estimate, the author would successfully collect on average nine of every ten mosquitoes alighting on his leg and it is likely that inexperienced collectors would collect mosquitoes at a lower success rate. Therefore, the human biting rates recorded in this study may represent slight underestimates of the true values.

The number of farms selected for study was in large part driven by the need to facilitate the organisation of multiple collectors within the short timeframe of the summer months. This

therefore resulted in the need to model farm as a fixed factor in the GLMM and limits the conclusions that can be drawn about variations in human biting patterns across the wider farm population in the UK. Nonetheless the biting rate varied significantly between farms, with Elmley displaying an average biting rate 11 times higher than at Northney Farm and nearly 20 times higher than at White Lodge, with up to 89 mosquitoes collected in a single 25-minute period at sunset. These results correspond with the results of the pilot study showing high mosquito abundance at Elmley and reports of human biting recorded in a survey conducted about a decade ago on the Isle of Sheppey (Hutchinson & Lindsay 2006b).

This study provides some evidence of within-farm variation in the biting rate. For example, the total number of mosquitoes collected at each of the four sites within each of the farms differs (Figure 3.3). This illustrates that biting patterns are not homogenous even over a relatively a small area, likely resulting from a combination of factors including changes in micro climate and proximity to outdoor resting sites as highlighted in previous UK studies (Service 1969a; Service 1971d). For statistical modelling purposes, the biting rate was first analysed both as total biting pressure of all mosquito species combined in order to understand the overall nuisance biting experienced by humans at the farms. Of the meteorological variables recorded, only wind speed was found to be a significant factor in influencing the total biting pressure. Overall, an increase in wind speed of 1 m/s would lead to a predicted 58% decrease in total biting pressure. Wind speed was also found to significantly influence the biting rate of *Cq. richiardii* when analysing the data for this species alone; an increase in wind speed of 1 m/s was predicted to lead to a 41% decrease in the biting rate. This contrasts with *Cx. modestus* however, for which only time from sunset was found to significantly influence the biting rate. This result may be as a result of the lower number of mosquitoes of this species having been collected, therefore reducing the ability of the model to pick out significant effects of this variable.

It is worthwhile noting that the hand-held anemometer readings indicate that the weather station situated on the same farm only a few metres away may not accurately reflect the wind speeds occurring at specific locations on site. To address such variation in future studies, each collector could be provided with a standardised means to collect or assess local meteorological factors, even at a rudimentary level by using a simple 1-5 numbered scale as used by Jaenson (1988). From the farm perspective, such local-scale differences could be important in influencing the biting rate experienced by farm workers when working in different areas of the farm. Rainfall and temperature were not found to be a significant influence on biting rate; this is unsurprising as collections were targeted to the warmest part of the year and biased to days considered 'ideal' for biting (loosely following the criteria of Service (1969a)) in order to determine the maximum or 'worst-case' biting situation. Future studies conducting more collection visits to include days with a wider range of weather patterns would enable better resolution of the effects of meteorological variables on the mosquito biting rate at these farms.

Preliminary work (chapter 2) guided the targeting of the present study to the evening crepuscular period. This targeting was necessary given the logistics and time constraints of the collectors. Such a targeted approach does, however, run the risk of missing atypical biting patterns; for example, 14-hour landing catches conducted in the Ivory Coast to study the (normally daytime) biting activity of *Ae. aegypti* found that biting occurred throughout the night with peak activity close to midnight (Diarrassouba & Dossou-Yovo 1997). In future therefore, depending on available time and resources, it would be preferable to conduct several 24-hour catches to capture the entire diel biting cycle. When looking at the results for time relative to sunset, the results of this study show that, overall, when considering total biting pressure, peak biting rates are closely tied to sunset, consistent with previous UK work (Service 1969a). The GLMM results for total biting pressure indicate that for every half an hour away from sunset, a 29% decrease in biting would be expected. However, in visualising the data per species (Figure 3.4), it is clear that this relationship with sunset is not clearly defined in all the species, particularly for those at White Lodge. Whilst too few specimens were collected from this site to

permit detailed analysis, *Ae. cinereus* was among those caught in the collection periods prior to sunset. This species is known to aggressively bite humans during the day when within shaded resting areas in reed beds, despite not actively host-seeking further from these areas during the daytime (Cranston et al. 1987). The biting activity of both *Cq. richiardii* and *Cx. modestus* show a significant association with sunset, with a predicted 18% and 21% decrease in biting rate for every half hour movement away from sunset. Although these analyses imply a symmetrical relationship around sunset, in visualising the data (Figure 3.4) there is some evidence that this might not be the case. At Elmley in particular, overall biting increased more sharply before sunset than it decreased afterwards. Whilst certain species, notably *Cq. richiardii*, *Cx. modestus* and *An. maculipennis* s.l. showed increased biting activity beginning shortly before sunset, *Cx. pipiens f. pipiens* began biting only after sunset. At all three farms on which mosquitoes were collected, some specimens were collected prior to sunset. This may be due to disturbance of the local resting population, a reason that led to some studies choosing to omit the first few minutes (generally five) of a collection when assessing human biting activity (Service 1969a). However, as the objective was to assess maximum biting rates in this study, such a methodology was not used. Mosquito biting activity at and around sunset during the summer months may be of considerable importance to farm workers, particularly on mixed-use (livestock and arable) farms, as time-critical activities such as cutting grass for hay/silage results in workers being outside for extended periods of time over sunset and late into the night. Indeed, such farm worker activity was observed at Elmley during at least one collection visit.

Although collectors were instructed to catch mosquitoes before they bit, some collectors reported that several mosquitoes did bite before they were collected and this was additionally confirmed by the presence of human blood in the abdomen of 19 specimens. Feeding on humans can therefore be confirmed for four species, *Cq. richiardii*, *Cx. modestus*, *Oc. cantans/annulipes* and *Oc. detritus* (Table 3.4) with the indication, but not confirmation, therefore that all mosquitoes collected in this study landed on the collectors with the intention of feeding rather than simply being attracted by generalist host-attractant cues (e.g. carbon

dioxide) and then subsequently choosing not to feed. *Anopheles atroparvus* and *Cq. richiardii* were found to contain cow blood which indicates that both these species are willing to feed on both cattle and humans; this reflects the findings of a previous study which observed *Oc. cantans* readily biting a human collector after evidently becoming disturbed mid-feed from a nearby cow (Renshaw et al. 1994).

The stationary HLC is a well-established method of collecting mosquitoes and owing to its widespread use in the literature, usefully allows for comparison between studies. Nonetheless a more realistic measure of the biting patterns experienced by a farm worker could be obtained by using a form of moving, or roving, landing catch technique such as that employed in the UK by Renshaw (1991) and by several studies further afield, for example early studies in Kenya to sample *Ae. aegypti* (Teesdale 1955) and in sampling a wide range of mosquito genera in Trinidadian forest (Aitken et al. 1968). Anecdotal observations from collectors in the present study indicated that they were sometimes bitten as they moved between sites on the farms; movement in this case would be providing an additional stimulus to mosquitoes as well as potentially disturbing resting mosquitoes and recruiting them to the subsequent collection site.

Although providing comparability to many studies, collecting landing mosquitoes exclusively from below the knee may have introduced bias into the collections. The selection of biting sites on the human body have been explored for several afrotropical mosquito genera, indicating that certain species show preferences for biting particular parts of the human body (see de Jong & Knols (1996) for a review). In field collections in Uganda, Haddow (1956) observed that *Eretmapodites chrysogaster* Graham 1909 bit standing humans almost entirely below the knee, whilst *Aedes simponsi* Theobald preferred to bite the head (Haddow 1946). In more recent, controlled laboratory experiments, *Anopheles atroparvus* and *An. albimanus* Wiedemann 1820 showed a preference for biting seated humans around the face and nose, in comparison to *An. gambiae* s.s. which preferred to bite the feet and ankles of a seated collector (De Jong & Knols 1995; Knols et al. 1994). The washing of feet with soap removed this

preference, leading to the conclusion that localised host odours were the primary factor in the selection of biting site for *An. gambiae* s.s., whilst human breath partly influenced the preference of the other two species toward biting the head area. A subsequent study however, found that by changing the orientation of the volunteer 'human bait' from being seated on stools to also include them sitting and lying on the ground (with and without feet elevated), the preference of *An. gambiae* s.s. for biting feet disappeared (Dekker et al. 1998). Furthermore, washing of the feet did not alter the preferred biting sites on all tested human volunteers. This led the authors to conclude that in addition to specific odours produced by different parts of the body, site-specific biting of *An. gambiae* s.s. was influenced by proximity of body parts to the ground, which they located using descending convection currents caused by heat from the volunteers' bodies (Dekker et al. 1998). Anecdotally in the present study, mosquitoes were observed to attempt to bite other parts of the body that were covered with clothes. This could be as a result of comparatively higher temperatures on covered body parts, as the skin temperature on the exposed leg may have dropped over the period of collection. Nonetheless, potential differences in feeding site preference on the body cannot be excluded as a factor playing a role here. There is unfortunately, to the knowledge of the author, no published evidence for variation in feeding site selection between UK mosquito species and thus the present methodology was considered to be the most appropriate.

In addition to the quantitative data gathered in this study, several behavioural observations were also made by the author or reported by other collectors. In agreement with previous UK work (Service 1971b), immediately after landing mosquitoes were observed to pause for several (≤5) seconds before beginning to probe. Attempts to aspirate mosquitoes before they started to probe often resulted in escape flights. Mosquitoes were also observed to frequently bite at the back of the knee, perhaps as a result of a comparative lack of hair compared to the front of the leg (at least in the case of the author). Several collectors also reported the arrival of mosquitoes in waves of several individuals at a time interspaced with several minutes of no arrivals, consistent with the observations of Service (1969). Male

mosquitoes (several identified visually as *An. maculipennis* s.l. at Elmley) were observed to start swarming above the head of collectors about half an hour before sunset and continuing into the night, although swarms had invariably disappeared by the time the final collection period (+2h after sunset) was completed. Finally, small numbers of other biting insects were observed attempting to bite the collectors although these were infrequently collected and therefore not included in the results. These insects included horseflies (Family: *Tabanidae*), blackflies (*Simulium* spp.) and biting midges (*Culicoides* spp.) and indicate that in future a combined investigation into biting insect assemblages as a whole could maximise the information gained about farm-associated insect biting populations.

This study demonstrated that humans are readily being bitten by mosquitoes within certain farm environments. However, it does not inform on the host preferences of the collected mosquitoes relative to other hosts present on site; information to this effect require direct host-choice experiments and/or the analysis of field-caught blood-fed specimens (see Chapters 5 and 6). Some potential interference with the collections by animals was observed during collections at White Lodge and Elmley, due to free-roaming cattle approaching the collectors at the former site and due to the occasional presence of a dog belonging to the site owners at the latter. It is difficult to assess the potential impact of these on collections, but the presence of an alternative host could have either increased biting by drawing more mosquitoes to the area, or reduced biting of humans if mosquitoes were preferentially attracted to the alternative host. These contrasting effects form the conceptual basis of zooprophylaxis and zoopotentiation respectively, in which the presence of domestic animals (primarily livestock) may either reduce or enhance biting and thus pathogen transmission to associated humans, although the efficacy of the former remains a subject of debate (Saul 2003; Bøgh et al. 2002). Of the various factors influencing which of these apply within a particular ecological setting, mosquito host preference plays a pivotal role (see Donnelly et al. (2015) for a review). Nonetheless, this multi-host (livestock and wildlife) situation realistically reflects what farm-associated workers or transient visitors to farms would experience within the farm environment.

Chapter 4 – Mosquito biting patterns on birds

4.1 Introduction

Birds play an important role as hosts in the transmission of several mosquito-borne pathogens of human and veterinary importance. This includes pathogens that are considered to present an incursion risk to the UK such as WNV and those endemically circulating which are poorly understood such as avian malaria. Mosquito host preference is important in the maintenance of arboviruses such as WNV in their avian enzootic (bird-mosquito-bird) cycles as well as in the occurrence of epizootic (mosquito-mammal) infection in mammalian hosts (Campbell et al. 2002). Avian enzootic transmission is usually reliant upon mosquitoes displaying a primarily ornithophagic host preference, a behavioural trait often exhibited by members of the genus *Culex* (Farajollahi et al. 2011). However, mosquitoes that exhibit limited selection between ornithophagy and mammalophagy are important in driving epizootic infection in mammals and host preference in these species is often less well defined (Kilpatrick et al. 2006).

Pathogens and their vectors (for example, *Borrelia*-infected ticks) can be dispersed from areas of endemic transmission to new areas by the movement of birds (see Reed et al. (2003) for review). Migratory bird movements in particular have the potential to drive introduction of mosquito-borne pathogens to new areas; a bird possessing an arbovirus viraemia, for example, may be bitten by an endemic competent mosquito species following arrival in a new region, which may subsequently result in the establishment of a new foci of transmission. Physiological changes associated with migratory restlessness may contribute to the reactivation of latent arbovirus infection within birds (Gylfe et al. 2000), which may facilitate this transmission. Migratory birds are considered important in the local spread of WNV both in the Old and New Worlds, owing to the isolation of the virus from these populations and the rapid spread of WNV down the east coast of the USA after 1999, closely matching domestic migratory flyways (Rappole et al. 2000; Malkinson et al. 2002).

Of the 34 mosquito species reported to occur in the UK, 15 are considered to display either primarily ornithophagy (2) or both ornithophagy and mammalophagy (13) (Medlock et al. 2005; Snow 1990; Cranston et al. 1987) (Table 4.1). Although the host feeding preferences of UK mosquitoes are considered to be well-established in the literature (Medlock et al. 2005; Cranston et al. 1987; Snow 1990), quantitative experimental or field data into bird-biting activity, conducted in the UK, are limited. A single bird-baited trapping study (Service 1969c) is supplemented by six studies that have used serological methods (precipitin testing or ELISA) (Service 1969a; Service 1971b; Renshaw et al. 1994; Onyeka & Boreham 1987; Curtotti 2009) or PCR-sequencing (Danabalan et al. 2014) to identify vertebrate blood meal origin. Some of these studies identified the blood meals of a very small number of samples only; for example, Onyeka & Boreham, (1987) did not aim to collect blood-fed mosquitoes specifically but decided to test the one engorged specimen they found. Whilst blood meal identification is effective in assessing the range of hosts on which a mosquito species will feed and in identifying host feeding preferences (see Chapters 5 and 6 for further discussion), it does not provide detailed information concerning the specific biting rate per unit time of different mosquito species on a particular host, an important parameter in models of mosquito-borne pathogen transmission cycles (see Chapter 1). Furthermore, the only study utilising bird-baited traps was conducted in the Poole Harbour area of Dorset; this was not a farm site and no mention of the alternative hosts present was made (Service 1969c).

Species	*Feeding preference (O, M, OM)*	*Published evidence for avian biting in the UK*	
		Blood meal analysis	*Host-baited traps*
Aedes cinereus	OM	✓[1]	-
Anopheles plumbeus	OM	✓[1,2]	-
Coquillettidia richiardii	OM	✓[1,2]	-
Culex modestus	OM	-	-
*Culex pipiens s.l.**	OM	✓[1,2,4]	✓[3]
(Culex pipiens f. pipiens)	O	✓[6]	-
(Culex pipiens f. molestus)	OM	✓[6]	-
Culex torrentium	O	✓[1,2]	-
Culex europaeus	OM	-	-
Culiseta annulata	OM	✓[1,2]	-
Culiseta litorea	OM	✓[1,2]	-
Culiseta morsitans	OM	✓[1,2]	✓[3]
Ochlerotatus cantans	OM	✓[1,5]	-
Ochlerotatus detritus	OM	✓[1,2]	-
Ochlerotatus punctor	OM	✓[1]	-
Orthopodomyia pulcripalpis	OM	-	-

Table 4.1: Mosquitoes with reported avian-feeding behaviour in the UK, after Medlock et al. (2005); ornithophagy is represented by (O), mammalophagy by (M), and feeding on both by (OM). Original references to papers providing data for avian feeding from either blood meal analysis or host-baited collections using birds are provided. *Early studies did not have the molecular means to separate the two ecoforms of *Culex pipiens* s.l.. Literature references (in superscript): (1) Service, (1971b), (2) Service, (1969a), (3) Service, (1969c), (4) Onyeka & Boreham, (1987), (5) Renshaw et al., (1994), (6) Curtotti, (2009).

There is considerable evidence demonstrating that mosquito population assemblages possess distinct vertical stratification in activity patterns in common with other arthropod groups (Ulyshen 2011). This is hypothesised to be driven by host preferences, with abundance gradients between those species feeding on birds that nest in tree canopies and those that feed on ground-nesting birds and/or mammals. This relationship is also modified by the availability of oviposition sites (most commonly tree holes) and the atmospheric boundary layer altitude

(Silver 2007). Within Europe, vertical stratification in mosquito species distribution and activity has been investigated in several countries including the Czech Republic (Černý et al. 2011), Italy (Bellini et al. 1997), Sweden (Lundstrom et al. 1996), France (Balenghien et al. 2006; L'Ambert et al. 2012) and the UK (Service 1969a; Service 1971c). Techniques to define vertical stratification of activity have not been standardised, however, and vary from the use of live avian bait (Balenghien et al. 2006; Service 1969c; Černý et al. 2011) to light/suction traps (Lundstrom et al. 1996; Service 1971c; Bellini et al. 1997) or a combination of both (L'Ambert et al. 2012). While this renders results difficult to compare across studies, certain species, such as *Culex pipiens* s.l., demonstrate a distinct preference for flight and host-seeking activity in the tree canopy. In the UK for example, 90% of *Cx. pipiens* s.l. collected by suction traps placed at different heights from the ground were collected in traps at 550 centimetres (cm) (Service 1971c). In the Czech Republic however, 68% of *Cx. pipiens* s.l. collected in bird-baited traps at different heights were in the traps set at 1.5 m above ground level (Černý et al. 2011). This indicates that geographically separated mosquito populations may exhibit different patterns of flight and host-seeking activity, important when considering that the range of available hosts at different heights may expose mosquitoes to different pathogens.

Worldwide, chickens have been widely used in studies investigating the avian host-seeking behaviour of mosquitoes due to their widespread availability, standardised genetic background and ease of handling. Studies have utilised chickens in bird-baited traps across the world including in the USA (Darbro & Harrington 2006; Savage et al. 2008; Nelson et al. 1976; Dow et al. 1957), Peru (Need et al. 1993), Japan (Scherer et al. 1959), The Czech Republic (Černý et al. 2011), The Gambia (Snow 1983), Malaysia (Chiang et al. 1986), Senegal (Diallo et al. 2010) as well as the one UK study (Service 1969c). The chicken-baited trap used in the UK collected *Cx. pipiens* s.l. and *Cs. morsitans* (Service 1969c); to date these remain the only two species to be collected by bird-baited collection methods in the UK. It is preferable to use multiple hosts when conducting bird-baited collections in order to minimise any individual biases in host attractiveness that might exist, although in contrast to wild birds or other domesticated fowl,

chickens have an extremely inbred genetic background that is likely to minimise this issue. Chickens are additionally easy to maintain and amenable to handling and thus multiple individuals could be maintained for use in the current study.

A comprehensive review of the use of other bird species and of various bird-baited trap designs is provided by Silver (2007). Traps frequently used include: variations on simple net traps, whereby animals are secured within one or two mosquito nets into which mosquito entry is permitted via the lower edges (e.g. Sasa & Sabin, 1950); stable-type traps, consisting of semi-permanent wooden hut-like structures with slits (with or without a baffle) to permit mosquito entry (e.g. (Loftin et al. 1997; Nelson et al. 1976); various wood or steel-framed structures covered with insect netting, often including a baffle-style entrance slit (Balenghien et al. 2006; Scherer et al. 1959; Chiang et al. 1986); and lard-can traps whereby the bait bird is placed within a metal cylinder with an inverted funnel entrance at either end through which mosquitoes can enter (Darbro & Harrington 2006; Diallo et al. 2010; Savage et al. 2008). This proliferation is largely driven by the logistical requirements of trapping using live hosts and the degree of permanency of the trapping site concerned.

Study aim

To identify the avian-biting mosquito species assemblages present on four livestock farms in the UK between June 2013 and October 2013 using a chicken-baited trap design and to investigate vertical differences in mosquito populations.

Objectives

1. To determine the seasonality of ornithophilic mosquito population assemblages present on four farms by collecting mosquitoes June-October 2013 using a chicken-baited, baffle-style trap modified from an original bird-baited trap design used in France
2. To investigate whether differences exist between ornithophilic populations found at two heights, ~1m and ~4m above ground level
3. To determine the efficiency of the chicken- baffle-style trap design in collecting mosquitoes using colony mosquitoes in a “free-flight, insect-proof tent”.

4.2 Materials and Methods

4.2.1 Chicken-baited trap design

Two identical chicken-baited traps were constructed by the author based on an original design by Balenghien et al. (2006). The trap design utilises a 'gutter-style' baffle entrance through which mosquito entry is facilitated but escape is difficult. Photographs of the original trap, approximate dimensions and advice on materials for construction were kindly provided by Thomas Balenghien. Several modifications (detailed below) were made from the original design based on the logistics of transporting the two traps in the fieldwork car (2004 Subaru Forester) and advice from the Home Office liaison at TPI to maintain high standards of chicken welfare, which required that the chickens were not exposed to mosquito biting. All materials used in the construction of the trap were purchased from either Homebase (Guildford, UK), B&Q (Guildford, UK) or Wickes (Woking, UK) unless specified otherwise.

The trap consisted of a frame made from untreated pine strip wood (W: 18 mm x D: 6 mm) covered with insect-proof mesh (Amber Lumite Screen, 0.965 mm hole width; BioQuip, Rancho Dominguez, California, USA) of exterior dimensions W: 500 mm x H: 650 mm x D: 500 mm (Figure 4.1). A single piece of exterior plywood (4 mm thickness) was used as a base for the trap. Construction used wood glue ('No More Nails', Unibond, Henkel, Hemel Hempstead, UK) and galvanised screws and the mesh was attached to the frame using a heavy-duty staple gun. The 'gutter' baffle entrance was built from two pieces of pine edging strip (W: 30 mm) attached using cross-struts, with a 30 mm-high gap extending from the base of the gutter and spanning its width. Mosquitoes entering the trap were collected using a manual aspirator (John W Hock) either from the top collection area or from the side sections with access via a Velcro section on the rear of the trap (Figure 4.1). To facilitate carrying, two wooden handles were affixed to the side of the trap. Exposure of the chickens to mosquito biting was prevented by an internal screen consisting of a chicken wire and mesh screen. The screened area containing the chickens consisted of a floor area (450 mm x 450 mm) with a central perch bar at 50 mm height. Chickens

were placed into the trap via a hinge door at the front of the cage and the door secured by means of a simple latch with a combination lock. Chicken feed and water were provided in pots attached to the internal mesh. Sheets of newspaper were placed on the base of the trap to facilitate cleaning of the trap after each use. One trap was fitted with additional cross-strut supports (Figure 4.2) in order to provide attachment for ropes to raise it into trees and to secure it to the ground to prevent the trap from swinging when hanging.

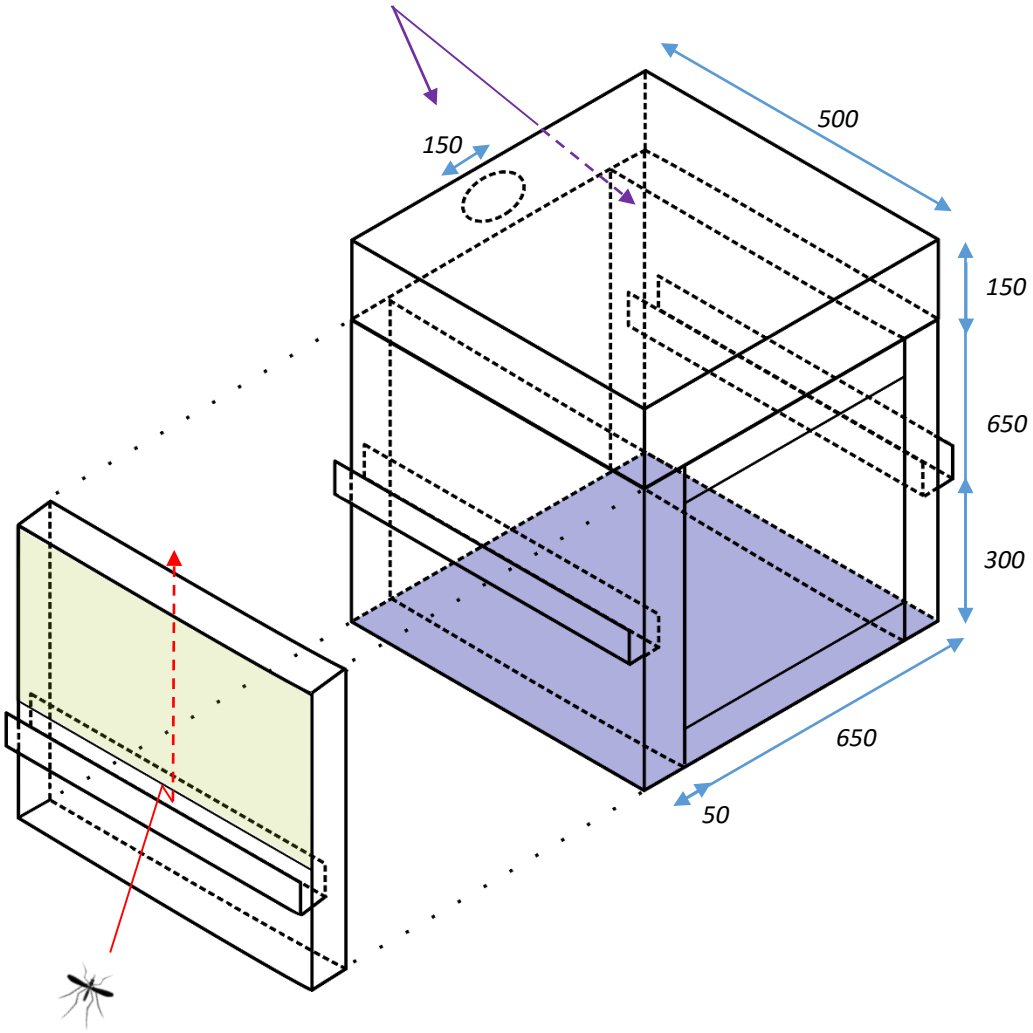


Figure 4.1: Isometric projection showing the primary features of the chicken-baited trap with dimensions (mm). One side has additionally been isolated to illustrate the route of mosquito entry into the collection area of the trap via the gutter baffle. The yellow section indicates the mesh that covers the outside of the trap which has been removed from the remainder of the diagram to allow all areas to be visible. The collection areas for mosquitoes are indicated by the purple arrows.

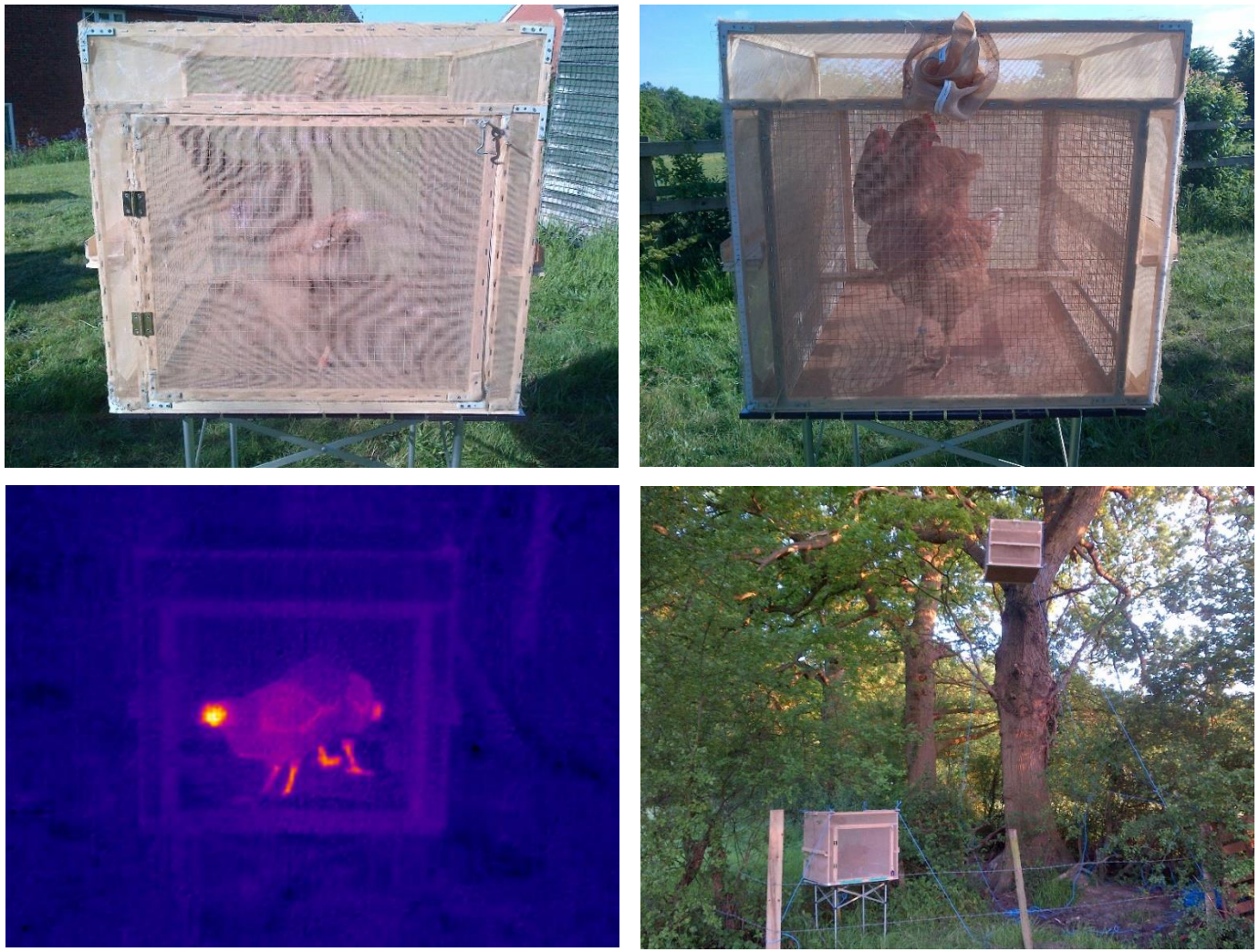


Figure 4.2: The chicken-baited trap. Clockwise from top left: (1) front view, with latch visible, (2) rear view, (3) 'high' and 'low' traps *in situ* at White Lodge, (4) thermal image of chickens *in situ* at Northney Farm, taken at night using a Testo 875-1 Thermal Imaging Camera.

4.2.2 Study chickens

Six point-of-lay (approximately 16 weeks old) ISA/Warren hybrid chickens were purchased in April 2013. The chickens were maintained at a private residence in Pirbright, Surrey, UK, on a standard diet of layer pellets and supplemented with poultry grit, mixed corn, fresh vegetables and mealworms. Food-based enrichment activities were regularly provided for the chickens to prevent boredom and stress. The chickens were not subjected to any insecticidal protection for the duration of the studies. To prevent the establishment of pests such as red mites, the enclosure was regularly cleaned, sprayed with detergent and, after drying, diatom powder added underneath the fresh bedding. No red mites or other pests (e.g. lice) were observed on the chickens or in the enclosure during the studies. Prior to the commencement of the study, the chickens were acclimatised to handling by the author and to transport in the fieldwork vehicle. The study received approval from the Home Office liaison at TPI and the trap design, with its modifications from the original design used in France, was not deemed to constitute a procedure and therefore a licence was not required. Following completion of the study, the chickens were retired in good health to a property in Cambridgeshire, UK.

4.2.3 Selection and transport of chickens to study farms

Two chickens were placed into each trap on each trial night. Chickens were randomly selected from the six individuals and allocated to each trap using the random number generator of www.random.org. This randomisation was modified to prevent any chicken being exposed to more than two consecutive nights of trapping to minimise stress. Chickens were transported in pairs within the traps and provided with feed and water for the duration of travel. The traps were so designed as to fit almost exactly into the rear space of the fieldwork vehicle, thus minimising movement of the cage and disturbance to the chickens. During the initial acclimatisation period it was determined that it was not necessary to cover the chickens (i.e. place them in darkness) during transport as they displayed no signs of distress when left uncovered.

4.2.4 Mosquito collection procedure

Four farms (Church Farm, Elmley, Northney Farm and White Lodge), were selected for inclusion in this study according to the pilot data and criteria detailed in Chapter 2. Nine collection visits were conducted at each farm, for a total of 36 collection visits, between June and October 2013. On each collection visit two chicken-baited traps (CBTs), each containing two chickens, were positioned at a fixed location on each farm (see maps in Chapter 2). One trap was placed at approximately 1 m (denoted as 'low') above the ground on an aluminium foldable table and the other within a 5 m radius from it at approximately 4 m in height, manually raised into a tree via ropes and a karabiner and secured in place using additional guy ropes to prevent the trap from swinging. A Mosquito Magnet Pro (MMP) trap (Midgetech, Stirling, UK) baited with a capsule containing 1-octen-3-ol was set up as a control at a fixed location a minimum of 50 m away (see maps). The CBTs and MMP were set up approximately one hour prior to sunset; times obtained from www.timeanddate.com. A one-hour human landing catch (HLC) was also conducted as a control starting 30 minutes prior to sunset at the same location as in the pilot study (Chapter 2) (except White Lodge which was not included in the pilot study; see maps for location). The location of the HLC was a minimum of 50 m away from the CBTs and MMP locations. Mosquitoes captured in the traps were collected the following morning (12-14 hours after setup), placed in a polystyrene cooler and transported to TPI for killing and storage at -20°C until processing. Collections were restricted to nights on which there was < 1 mm of rain and average wind speeds of < 3 metres/second were forecast (www.xcweather.co.uk).

4.2.5 Identification of mosquitoes

Mosquitoes collected in traps were identified based on morphological features following published keys (Snow 1990; Cranston et al. 1987). Additionally, mosquitoes morphologically identified as *Culex pipiens* s.l./*torrentium* were identified by molecular methods to species level using the techniques detailed in sections 2.5.1 and 2.5.2. Mosquitoes identified morphologically as *Anopheles maculipennis* s.l. were identified using the techniques provided in section 2.5.3.

4.2.6 Trap efficiency experiment

This study aimed to test the efficiency of the chicken-baited trap in allowing host-seeking mosquitoes to enter and subsequently prevent them from escaping. The study was conducted in the garden of a private residence in Pirbght, Surrey, UK, separate to the one where chickens were maintained, during August 2014. The experiment took place within a white, insect-proof mesh tent (henceforth, 'tent') (W: 1450 mm x H: 1520 mm) (Insectopia, Austrey, UK) and used one chicken-baited trap, the same six chickens as in the main avian biting study above and *Cx. pipiens f. pipiens* mosquitoes, line "Caldbeck" from TPI colony. The experiment was only conducted on nights where no rain was forecast. At 18:00-19:00 on a given night, between 45-50 mosquitoes were placed either directly into the 'capture' section at the top of the trap ("captive") or directly into the insect-proof tent ("free"), with two randomly selected chickens placed into the cage as per normal use ("bait"), or the cage left chicken-free as a control ("no bait"). The four treatments compared were (1) mosquitoes captive + bait, (2) mosquitoes captive + no bait, (3) mosquitoes free + bait, (4) mosquitoes free + no bait. The study was conducted over 16 nights with treatments randomly allocated to a night. Mosquitoes inside the traps and free inside the tent were collected by manual aspiration between 06:00 – 07:00 the following morning before being killed, counted and stored at -20°C.

4.2.7 Analysis, diagrams and photographs

Data were stored, cleaned and graphs produced using Microsoft Excel. Summary statistics were obtained using a combination of Microsoft Excel and R version 3.2.0 (R Core Team 2015). The normality of trap catch data for the chicken-baited traps was tested using the D'Agostino-Pearson omnibus test (D'Agostino & Pearson 1973) using the function 'dagoTest' in package 'fBasic' in R. This tests the null hypothesis that data were sampled from a normal distribution. Significant *P* values (≤ 0.001) were obtained, indicating that the null hypothesis should be rejected i.e. the data were not normally distributed. As a result, comparisons between the mosquitoes collected in the 'high' and 'low' position traps were made using a paired, two-tailed Wilcoxon signed rank test using function 'wilcox.test' in package 'MASS' in R, combining

all species collected and at a significance level of 0.05. The null hypothesis was that the median difference between pairs of observations (i.e. in the 'high' and 'low' traps on the same collection night) was equal to zero.

Results from the trap efficiency study were split into two parts for analysis, the 'trap entry' and 'trap escape' results, each of which was analysed using Fisher's exact test of independence using function 'fisher.test' in R. The null hypothesis for the 'trap entry' results was that the proportions of mosquitoes entering the trap was the same regardless of the presence of chickens in the trap. The null hypothesis for the 'trap escape' results was that the proportions of mosquitoes remaining in the trap was the same regardless of the presence of chickens in the trap.

4.3 Results

4.3.1 Chicken-baited trap collections

A total of 610 unfed female mosquitoes were collected during the study (Table 4.2). The chicken-baited traps collected a total of only 22 mosquitoes across all farms, of which 12 were collected in the 'low' trap and 10 in the 'high' trap. This difference was not found to be statistically significant (W = 16, *P* = 0.83). The majority of the mosquitoes (18/22) collected in the chicken-baited traps were collected at Elmley, with two collected at Northney Farm and one at White Lodge. No mosquitoes were collected in any trap at Church Farm. The mean (range) avian 'biting rate' (mosquitoes caught/night) for the 'low' trap as calculated for each positive farm was: Elmley 1.11 (0 –6), Northney Farm 0.11 (0 – 1) and White Lodge 0.11 (0 – 1). The mean (range) avian biting rate for the 'high' trap was: Elmley 0.89 (0 – 3), Northney Farm 0.22 (0 – 2) and White Lodge 0 (n/a). Human landing catches yielded 190 mosquitoes in total and the MMP baited with 1-octen-3-ol collected 398 mosquitoes. The greatest number of mosquitoes was collected at Elmley (n = 448), followed by White Lodge (n = 93) and Northney Farm (n = 69).

Farm	CBH *mean (range)*	CBL *mean (range)*	Human landing catch *mean (range)*	Mosquito Magnet Pro *mean (range)*	Total per farm *mean (range)*
Church Farm	0	0	0	0	**0**
	0 (0 – 0)	*0 (0 – 0)*	*0 (0 – 0)*	*0 (0 – 0)*	***0 (0 – 0)***
Elmley	8	10	114	316	**448**
	0.89 (0 – 3)	*1.11 (0 – 6)*	*12.67 (1 – 40)*	*35.11 (0 – 102)*	***49.78 (1 – 119)***
Northney Farm	2	1	33	33	**69**
	0.22 (0 – 2)	*0.11 (0 – 1)*	*3.67 (0 – 12)*	*3.67 (0 – 14)*	***7.67 (0 – 16)***
White Lodge	0	1	43	49	**93**
	0 (0 – 0)	*0.11 (0 – 1)*	*4.78 (0 – 23)*	*5.44 (1 – 26)*	***10.33 (1 – 49)***
Totals	**10**	**12**	**190**	**398**	**610**

Table 4.2: The total number of mosquitoes collected from the chicken-baited traps in the high (CBH) and low (CBL) positions, by human landing catch and Mosquito Magnet Pro trap baited with 1-octen-3-ol at each of the four farms, collated over the nine collection visits. Values for the mean catch per trap night and range are also presented. Note that the mean value for the human landing catch result is obtained from one-hour collections per trap night, whilst the mean values for the other traps represent overnight collections.

Seventeen species/morphologically indistinguishable species groups were collected overall, in all trap types, during the 36 visits across farms (Table 4.3). The chicken-baited traps collected three species/species groups, *Cq. richiardii*, *Cx. modestus* and *Cx. pipiens s.l./torrentium*. Of these, *Cq. richiardii* and *Cx. modestus* were collected only in the 'low' chicken-baited trap, and only at Elmley, whereas *Cx. pipiens s.l./torrentium* was collected at both trap heights and at all farms except Church Farm. *Culex pipiens s.l./torrentium* was also the most commonly collected species in the chicken-baited traps, accounting for 86% of the mosquitoes collected by this method. Human landing catches and the MMP collected fourteen species/species groups each, including all three species/species groups collected in the chicken-baited traps. The greatest number of mosquito species collected overall, in all trap types, was at

Elmley, 12, followed by White Lodge with eight and Northney Farm with four. Three species, *Cq. richiardii*, *Cx. modestus* and *Oc. flavescens* accounted for 76% and 87% of the total mosquitoes collected at Elmley by HLC and in the MMP respectively. The species most dominant in collections at Northney Farm was *Oc. detritus*, accounting for 98% and 91% of the collections by HLC and MMP respectively. *Ochlerotatus punctor* was the most collected species by HLC at White Lodge (81%), however the most common species collected in the MMP was *Cs. annulata* (49%).

Mosquitoes were collected in the 'low' position chicken-baited traps in every month (June-September) with the exception of October, whilst the 'high' position trap collected mosquitoes only in July and August (Figure 4.3). *Culex pipiens s.l./torrentium* was collected in the chicken-baited traps between June and August, whilst HLCs collected this species in June and July and the MMP between June and September. *Coquillettidia richiardii* was collected in the 'low' chicken-baited traps in July and August whilst it was collected by HLC in June and August and the MMP between June and September. A single specimen of *Cx. modestus* was collected from the chicken-baited traps in September, corresponding to the greatest abundance of this species collected by HLC and in the MMP, although it was also collected in both the latter trap types between June and October.

Thirty-seven of the forty mosquitoes identified morphologically as *Cx. pipiens s.l./torrentium* were identified by molecular methods as *Cx. pipiens f. pipiens* (Table 4.2). Two specimens, one collected by the MMP and one from the 'low' chicken trap at Elmley, failed to produce a result in either of the two multiplex reactions. A third specimen, an individual collected in the 'low' chicken trap at Northney Farm was identified as C*x. pipiens* s.l. but produced an ambiguous result in the multiplex reaction to separate the two ecoforms. All four specimens of *An. maculipennis* s.l. were identified by molecular methods as *An. atroparvus*.

Species	Elmley				Northney Farm				White Lodge				Total
	CBH	*CBL*	*HLC*	*MMP*	*CBH*	*CBL*	*HLC*	*MMP*	*CBH*	*CBL*	*HLC*	*MMP*	
An. atroparvus	0	0	4	0	0	0	0	0	0	0	0	0	**4**
An. claviger	0	0	7	1	0	0	0	0	0	0	0	0	**8**
An. plumbeus	0	0	0	0	0	0	0	0	0	0	1	1	**2**
Cq. richiardii	0	2	23	141	0	0	0	0	0	0	0	0	**166**
Cs. annulata	0	0	0	7	0	0	0	0	0	0	0	24	**31**
Cs. morsitans	0	0	0	1	0	0	0	0	0	0	0	0	**1**
Cs. subochrea	0	0	0	0	0	0	0	0	0	0	0	3	**3**
Cx. modestus	0	1	42	50	0	0	0	0	0	0	0	0	**93**
Cx. pipiens f. pipiens	8	6	3	14	2	0	0	2	0	1	0	1	**37**
Cx. pipiens s.l.	0	0	0	0	0	1	0	0	0	0	0	0	**1**
Cx. pipiens s.l./torrentium	0	1	0	1	0	0	0	0	0	0	0	0	**2**
Da. geniculata	0	0	0	0	0	0	0	0	0	0	2	0	**2**
Oc. cantans/annulipes	0	0	0	0	0	0	0	0	0	0	5	8	**13**
Oc. caspius/dorsalis	0	0	1	7	0	0	1	1	0	0	0	0	**10**
Oc. detritus	0	0	6	11	0	0	32	30	0	0	0	0	**79**
Oc. flavescens	0	0	22	80	0	0	0	0	0	0	0	0	**102**
Oc. punctor	0	0	0	0	0	0	0	0	0	0	35	9	**44**
Oc. rusticus	0	0	5	2	0	0	0	0	0	0	0	3	**10**
Ochlerotatus spp.	0	0	1	1	0	0	0	0	0	0	0	0	**2**
Totals per farm	**8**	**10**	**114**	**316**	**2**	**1**	**33**	**33**	**0**	**1**	**43**	**49**	**610**

Table 4.3: Mosquito species collected over the nine collection visits to each of the four farms by each of the trap types: chicken-baited traps 'high' position (CBH), chicken-baited traps 'low' position (CBL), human landing catch (HLC) and Mosquito Magnet Pro baited with 1-octen-3-ol (MMP). Light blue fill highlights the three species collected in the chicken-baited traps. Specimens of *Cx. pipiens s.l./torrentium* and *An. maculipennis* s.l. for which molecular species identification failed are presented as their respective species groupings. No mosquitoes were collected at Church Farm therefore this farm is not included.

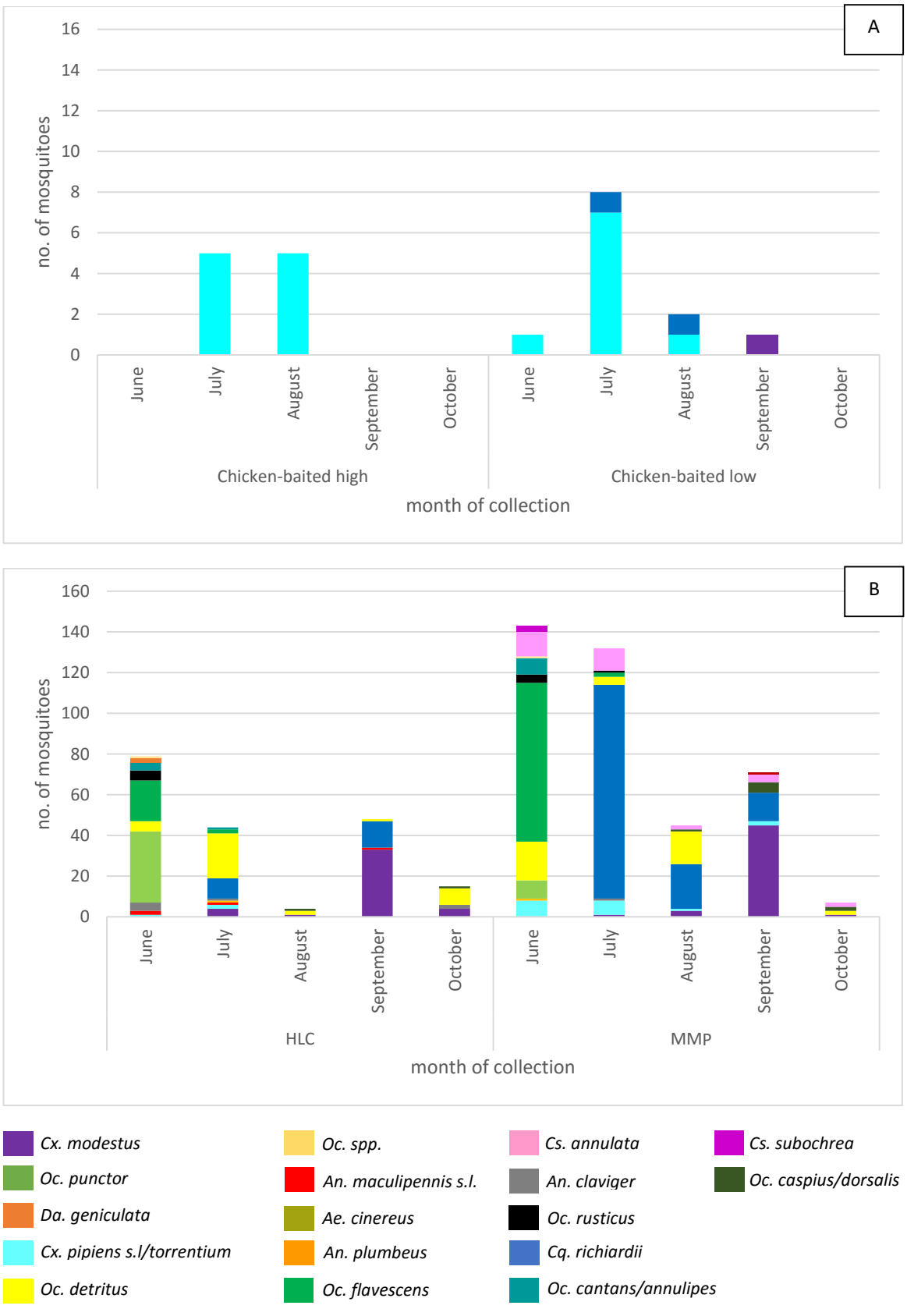


Figure 4.3: Stacked bar graphs showing (A) the seasonality of mosquitoes collected June-October in the high and low chicken-baited traps and (B) by human landing catch (HLC) and Mosquito Magnet Pro trap (MMP) (bottom graph). Note different scales on axes.

4.3.2 Trap efficiency experiment

The mean 'capture rate' of the trap with chickens ('bait') was 0.15, range 0 – 0.35 (0 – 35%), but when no chickens were present ('no bait'), no mosquitoes were found to have entered the trap (Table 4.4 (A), Figure 4.4 (B)). A significant association between the presence of chickens and entry into the trap was found ($P \leq 0.001$). For the 'trap escape' phase of the experiment the mean 'retention rate' of mosquitoes was 0.5, range 0.24 – 0.65 (24 – 65%) when bait was present in the trap, and 0.07, range 0 – 0.12 (0 – 12%) when no chicken bait was present (Table 4.4 (A), Figure 4 (B)). A significant association between the presence of chickens in the trap and the retention rate was found ($P \leq 0.001$).

A	Replicate #	Bait status	Total # mosq. added to tent	# entered trap	# remaining in tent	Capture rate
	1	Bait	48	17	31	0.35
	2	Bait	47	11	36	0.23
	3	Bait	47	0	47	0.00
	4	Bait	45	1	44	0.02
	1	No bait	50	0	50	0.00
	2	No bait	50	0	50	0.00
	3	No bait	50	0	50	0.00
	4	No bait	50	0	50	0.00
B	**Replicate #**	**Bait status**	**Total # mosq. added to trap**	**# remaining in trap**	**# escaped to tent**	**Retention rate**
	1	Bait	49	32	17	0.65
	2	Bait	50	27	23	0.54
	3	Bait	46	26	21	0.57
	4	Bait	50	12	38	0.24
	1	No bait	48	4	44	0.08
	2	No bait	49	6	43	0.12
	3	No bait	50	3	47	0.06
	4	No bait	50	0	50	0

Table 4.4: Results for the trap efficiency study showing (A) results for 'trap entry' where mosquitoes were placed into the insect-proof tent and allowed to enter the trap with or without chickens present and (B) trap escape where mosquitoes were placed directly into the capture section of the trap with and without chicken bait present to assess how many remained.

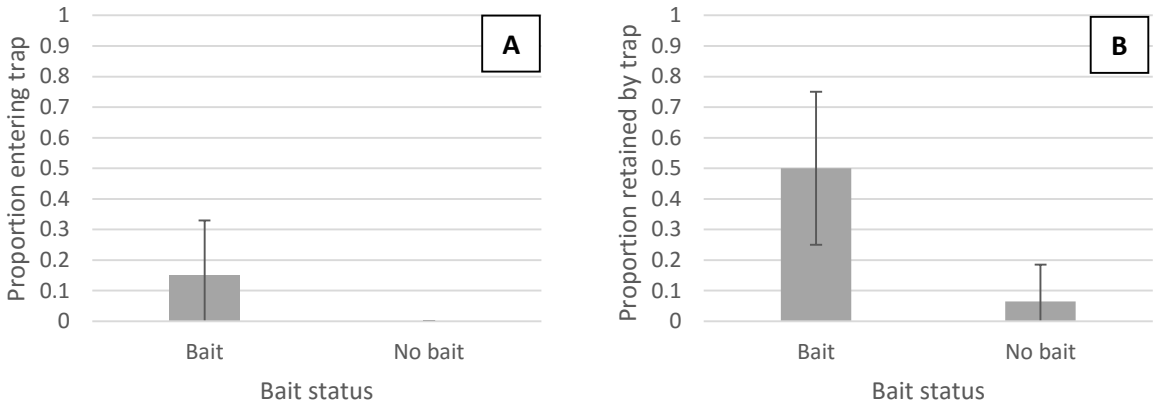


Figure 4.4: (A) mean (± standard error) proportion of mosquitoes entering the trap with and without chicken bait and (B) mean (± standard error) proportion of mosquitoes retained by the traps with and without chicken bait.

4.4 Discussion

Ornithophilic mosquitoes of three species were present on three of the four livestock farms on which trapping was conducted. The CBTs were successful in collecting mosquitoes of three species: *Cx. pipiens s.l./torrentium* from Elmley, Northney Farm and White Lodge, and *Cq. richiardii* and *Cx. modestus* from Elmley. This is the first study reporting the ornithophilic activity of *Cx. modestus* in the UK and the first to collect *Cq. richiardii* in a bird-baited trap in the UK. Mosquitoes were collected in the CBTs at both 'low' and 'high' positions with only *Cx. pipiens s.l./torrentium* collected from both trap positions and *Cq. richiardii* and *Cx. modestus* collected only from the 'low' traps. Mosquitoes were collected from the chicken-baited traps between June and September, broadly matching the period of time when the collected species were at their highest abundance according to HLC and MMP trap collections.

All but three mosquitoes identified morphologically as *Cx. pipiens s.l./torrentium* were identified by molecular methods as *Cx. pipiens f. pipiens*. This species is known to be mainly ornithophagic (Medlock et al. 2005), with only occasional and limited feeding on humans (Cranston et al. 1987). In this study this species was also collected by HLC, supporting the results in Chapter 3 that demonstrated landing activity of this species on humans at two farms. Additionally, *Cx. pipiens f. pipiens* was collected in the MMP trap, although at a relatively low abundance as compared with other species such as *Cq. richiardii* and *Oc. flavescens* when present during the same month. This reflects the results of other studies using MMP traps in the UK; in 292 trap nights in Cambridgeshire fenland only 19/14 025 mosquitoes (0.14%) were *Cx. pipiens s.l./torrentium* (Medlock & Vaux 2015b). That relatively few *Cx. pipiens f. pipiens* were collected in the MMP may reflect a low preference for feeding on mammals, as the lure in the MMP was 1-octen-3-ol, a volatile compound originally isolated from cattle (Hall et al. 2011). Despite positioning the MMP traps a minimum of 50 m from the CBTs, there is a possibility that they could have drawn mosquitoes away from the chickens. Owing to the poor rates of collection of *Cx. pipiens s.l./torrentium* in the MMP in the literature however, this is unlikely to have

occurred for this species, but could have played a role for other species. If such an effect did occur, it would also very much depend on local factors such as wind direction influencing the dispersal of the odour plume, that were not controlled for in this study. Other animals (livestock and wildlife) moving near to the chicken traps could also have influenced the collections. Traps were positioned (with fencing/electrical tape as appropriate) such that livestock were prevented from interfering with them directly, however the presence of animals in close proximity to the traps could not be controlled.

As regards the other two species collected in the CBTs, *Cq. richiardii* is known to bite both birds and mammals in the UK (Medlock et al. 2005; Service 1969a; Service 1971b) and therefore its collection in a bird-baited trap is novel to the UK but not unexpected. The low number of specimens collected of this species (two) reflects the results of bird-baited collections in the Camargue, France, where only three specimens were collected in weekly trap visits between May and October at two sites (Balenghien et al. 2006). This could perhaps indicate a generally low feeding preference for birds despite bird-feeding being reported from studies of blood meals. *Culex modestus* was collected from the CBTs and by HLC (thus supporting the results from Chapter 3), only from Elmley. The ornithophilic and anthropophilic behaviour of this species also fit with data from the Camargue, France, where it was collected using bird-baited traps and HLCs (Balenghien et al. 2006).

Of the species not collected in the chicken-baited traps but present in HLC and MMP collections (Table 4.2 and Figure 4.3), eight are considered to be bird-biting in the literature (Medlock et al. 2005): *Cs. annulata*, *Oc. detritus*, *Oc. punctor*, *An. plumbeus*, *Ae. cinereus*, *Cs. morsitans*, *Da. geniculata* and *Oc. cantans/(annulipes[3])*. The majority of these species were represented by one or only a few specimens at the farms indicating a low background

[3]*Oc. annulipes* is considered to be primarily mammalophagic (Service 1971b; Medlock et al. 2005).

abundance, however, *Cs. annulata* and *Oc. punctor* were the two most abundantly collected species at White Lodge by MMP and HLC respectively, whilst *Oc. detritus* constituted over 90% of the total collection at Northney Farm. That these species were not collected in the chicken-baited traps may be as a consequence of trap design, trap location, a preference for feeding on alternative hosts, or a combination of factors.

The number of mosquitoes collected in the chicken-baited traps was very low overall in comparison to effort of trapping and considerably fewer were collected than in the MMP which was run for the same period of time overnight. There has been little standardisation between bird-baited trap designs used to date to collect mosquitoes worldwide, with different studies utilising different designs, in part dependent on the environment in which they were working and the bird and mosquito species targeted (see chapter 5 of Silver (2007) for further discussion). Furthermore, some authors provide little information on the methods of construction of their traps or how the bait were contained within them thus making it difficult to replicate their trap or experimental designs. This study attempted to follow the design of Balenghien et al. (2006), with necessary modification, to allow comparison of results between the studies. In France, the original trap design collected 2341 mosquitoes (1084 and 1257 in traps set at 8 m and 1 m respectively) (Balenghien et al. 2006). It was important to conduct the trap efficiency study given that a 'control' trap without bait was not run during the main study due to time and logistical limitations. Studies elsewhere have used empty control traps as a comparison to bird-baited traps, for example Buescher & Scherer (1959) who compared collections of *Culex tritaeniorhyncus* Giles 1901 on Black-crowned night herons in Japan, collecting 4170 mosquitoes as compared to none collected in the control trap. The results of the trap efficiency study show that significantly more mosquitoes were captured and retained by the chicken-baited trap when chicken bait was present within it, although both the capture and retention rates varied considerably. This evidence indicates that the mosquitoes collected in the trap were attracted to the bait chickens in the trap rather than being a result of 'accidental' capture, although, as emphasised by Service (1969b), caution needs to be taken when

interpreting the results of animal-baited traps where feeding on the bait animal is not permitted. Thus, this study provides evidence that the mosquito species collected in the chicken-baited traps are ornithophilic but not necessarily ornithophagic, i.e. they are attracted to but not necessarily feeding on birds; evidence of the latter requires blood meal analysis as conducted in Chapter 5.

In accordance with welfare considerations as discussed with the Home Office liaison at TPI, an additional screen, not present in the original design, was added to the trap to prevent mosquitoes from feeding on the chickens inside. This meant that no blood-feeding could take place, which may have resulted in the mosquitoes trying to exit the trap to seek an alternative blood meal source after unsuccessfully trying to reach the bait chickens. Indeed, Service (1977a) notes that mosquitoes that are prevented from feeding remain active and thus are more likely to escape a baited trap. In support of this, a study in the USA utilising a lard-can style chicken-baited trap found that a greater proportion (74%) of mosquitoes escaped from the trap when feeding on the chickens was prevented than when they were allowed access to the birds (54%) (Darbro & Harrington 2006). A further result of preventing feeding on the bait chickens in this study meant that no blood-fed specimens could be collected to confirm that the chickens had been fed upon, as was performed in France (Balenghien et al. 2006). Allowing mosquitoes to feed on the bait animal also provides additional information on success rates of achieving a blood meal, which in most cases is considerably lower than the numbers captured in the traps. For example, despite a large number of *Culex portensi* Sénevet & Abonnenc 1941 being captured in suction traps baited with the grass mouse *Akodon urichii* in Trinidadian forest, only 6.08% were found to have fed on the mouse (Davies 1978). In like manner, only a maximum of 2.3% of *Culiseta morsitans* and *Culex pipiens* s.l. collected in rabbit-baited traps in the UK had fed on the rabbits, indicating either an unwillingness or inability to feed on the rabbits (Service 1969c). In contrast, up to 88% of *Culex quinquefasciatus* Say 1823 collected in modified stable traps baited with chickens in New Mexico, USA, were blood-fed, a higher proportion than in dog-baited traps, despite the dog-baited traps collecting greater numbers of mosquitoes (Loftin et al. 1997).

Mosquitoes may also have been 'put off' by the trap design, precluding their entry into it, perhaps viewing the trap as an artificial structure requiring them to feed in an 'indoor' situation. Mosquito species which feed outdoors (exophagy) may therefore not enter the trap and even those with reported indoor (endophagic) feeding behaviour may choose to focus on alternative, outdoor hosts. This applies regardless of the bait animal used. For example, rhesus monkeys were readily bitten by *Aedes africanus* Theobald in Ugandan forest but when the monkeys were placed in cages the mosquitoes did not bite (Haddow & Smithburn 1948). Monkeys used for sentinel surveillance for the Yellow fever virus therefore had to be tethered in the open to permit mosquito biting. Both *Cx. pipiens* s.l. and *Cq. richiardii* are known to exhibit indoor feeding behaviour (Service 1969a; Service 1971b; Medlock et al. 2005) which may have contributed to their collection in the chicken-baited traps in the present study. Although very little is known about the feeding behaviour of *Cx. modestus* in the UK, this species was collected in the original trap design on which this trap was based and thus was expected to be able to enter the trap (Balenghien et al. 2006). A further potential reason is the design of the gutter baffle entry point itself. Upon hitting the outer mesh of the trap, the mosquitoes would need to fly around the trap until they located the gutter and then fly downwards into it in order to enter into the trap (Figure 4.1). Anopheline species, such as *Anopheles gambiae* s.l., are known to fly upwards upon reaching a vertical surface; such flight behaviour facilitates their entry into huts through the eaves (Snow et al. 2009). Flight behaviour like this would likely result in the mosquito missing the gutter entrance and therefore not being collected. Notably, the bird-baited trap design used in France did not collect any *An. maculipennis* s.l., despite its collection in significant numbers in the horse- and human-baited traps from the same area. Whilst this was interpreted as a result of host feeding preferences, without free-flight tests of the trap using this species, an effect of trap design cannot be excluded. Increasing the width of the baffle entrance could be a method of permitting the entry of a greater number of mosquitoes into the trap. However, in his review of animal-baited trap designs, (Service 1977a) emphasises that there is

always a trade-off between facilitating mosquito entry into baited traps and preventing them from escaping.

The location of the chicken-baited traps on each farm site may also have affected the collection rates. For logistical reasons, the traps could only be set up where vehicular access was possible and where there was a suitable tree on which to hang the 'high' chicken trap. Spatial differences reflecting different environmental conditions including shade have been shown to influence trap collections within even a small area (Crepeau et al. 2013) and thus the location of the traps may have been in areas not favourable to mosquito activity. In like manner, in Japan, differences in the catch of *Culex tritaeniorhynchus* and *Cx. pipiens* s.l. using identical bird-baited traps were thought to result from variations in the local microhabitat of each trap location (Scherer et al. 1959). The presence of other hosts in close proximity to the chicken-baited traps could also have influenced the numbers collected in the bird-baited traps. Reid (1961) working in Malaysia considered 45 m to be the minimum acceptable distance between different bait animals or traps to avoid interference, but recognised that too great a distance between traps would mean that, effectively, the traps would be sampling different habitats. Taking Elmley as an example in the present study, the chicken traps were placed directly next to central woodland strip in which several species of bird were observed during the trapping period. Upon encountering the trap as a barrier to feeding on the bait chickens, mosquitoes initially attracted to these hosts may have gone on to feed on other more accessible avian hosts instead of entering the trap.

The chicken traps in this study were placed in locations within 1 km of where human-biting populations had been collected in the pilot study (Chapter 2). Furthermore, a large-scale study in Australia found significant autocorrelation in trap counts for certain mosquito species within a range of 3-4.5 km, suggesting that one trap could successfully represent un-sampled areas within this range (Ryan et al. 2004). Now that this study has shown that avian-biting mosquitoes can be collected using this trap design, future studies using several of these traps

on one site would be preferable, assuming an appropriate number of personnel and vehicles to conduct such a study were available.

Finally, it must be considered that the bird-biting rate (or more specifically, the chicken-biting rate) may simply be very low at the farms on which trapping was conducted in this study. The greatest numbers of mosquitoes collected in the chicken-baited traps was at Elmley (18/22 total). The mean bird-biting rate as calculated per chicken-baited trap per farm at Elmley was 0.89 bites/night in the 'high' position trap and 1.11 bites/night in the 'low' position trap. These values fit with the only previous UK study employing chicken-baited traps (placed at 1-1.5 m above the ground) in which 20 mosquitoes (12 *Cx. pipiens* and 8 *Cs. morsitans*) were collected over 19 catch nights, producing an overall mean biting rate of 1.05 bites/night (Service 1969c). The same study collected no mosquitoes by direct capture from a tethered chicken. It is possible that bird-biting rates are very low in comparison to the biting rates on mammals, particularly on livestock farm sites where large mammalian hosts such as cattle are always available. If indeed avian biting rates are very low, then this would impact on the chances that an imported arbovirus would be successfully maintained in a wild bird enzootic cycle within these farm settings.

A total of 10 mosquitoes were collected in the 'high' chicken-baited trap and 12 in the 'low' position trap; this difference was not found to be statistically significant. The low mosquito numbers collected in this study make it impossible to draw valid conclusions about differences in the vertical distribution of the species collected. However, the two species considered to feed on mammals as well as birds, *Cx. modestus* and *Cq. richiardii*, were found only in the low trap whilst *Cx. pipiens* s.l. were collected at both trap heights. These results are consistent with a previous study showing that *Oc. cantans*, a species that mainly feeds on mammals, was collected only in unbaited suction traps placed close (~30 cm) to the ground, whilst 90% of *Cx. pipiens* s.l. were collected in traps placed 5 – 6 m from the ground (Service 1971c). In the study in the Camargue, the same species profiles were collected in bird-baited traps placed at ~1m and at

~8m, with the overall abundance of mosquitoes being highest in the traps at ~1m at one site (six species) and in the traps at ~8m (only *Cx. pipiens* s.l.) at the other (Balenghien et al. 2006). Similar to the results of this study, very low numbers (3 specimens) of *Cq. richiardii* were collected in their bird-baited traps.

The low numbers of mosquitoes collected using the chicken-baited traps provides little information on seasonal trends in the avian-biting populations present on the studied farms, and does not allow for detailed comparison between farm sites. Overall, mosquitoes were collected in July and August from the 'high' position trap, and between June and September in the 'low' position trap. The collated results (Figure 4.3) show that the presence of mosquitoes in the chicken-baited traps reflects the presence of the same species in the HLC and MMP trap collections. Both *Cq. richiardii* and *Cx. modestus* were collected by HLC and MMP only from Elmley, reflecting the results of the chicken-baited trap collections. No mosquitoes were collected at Church Farm in any trap type, similar to the results in Chapter 3. This may be as a result of the host preferences of mosquitoes at Church Farm being for hosts other than chickens or humans, or may result from there being a generally low mosquito abundance at this site, although larvae and adult mosquito populations, including *Cx. pipiens s.l./torrentium*, were present during the pilot study (Chapter 2).

Chapter 5 – Molecular species identification, host preference and detection of myxoma virus in the *Anopheles maculipennis* complex (Diptera: Culicidae) in southern England, UK

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For the purpose of this thesis, references for this chapter are included together with the references from all chapters at the end of the document, and in the format appropriate to this thesis.

The author (VAB) declares that he was the sole collector of the fieldwork data, performed the blood meal and contributed to the species identification PCRs, analysed the data and prepared the manuscript. The study was conceived by VAB, NJ and ARF; the PCR methodology to identify myxoma virus conducted by NJ and DGW; LMH-T, SWJP and CW contributed to the setup of blood meal PCRs; LMH-T contributed to the mosquito species identification PCRs and performed the editing of sequences for mosquito species identification. All authors read and approved the final manuscript.

Molecular species identification, host preference and detection of myxoma virus in the Anopheles maculipennis complex (Diptera: Culicidae) in southern England, UK

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5.1 Abstract

Background: Determining the host feeding patterns of mosquitoes by identifying the origin of their blood meals is an important part of understanding the role of vector species in current and future disease transmission cycles. Collecting large numbers of blood-fed mosquitoes from the field is difficult, therefore it is important to maximise the information obtained from each specimen. This study aimed to use mosquito genome sequence to identify the species within *Anopheles maculipennis* sensu lato (*An. maculipennis* s.l.), identify the vertebrate hosts of field-caught blood-fed *An. maculipennis* s.l., and to test for the presence of myxoma virus (Poxviridae, genus Leporipoxvirus) in specimens found to have fed on the European rabbit (*Oryctolagus cuniculus*).

Methods: Blood-fed *An. maculipennis* s.l. were collected from resting sites at Elmley Nature Reserve, Kent, between June and September 2013. Hosts that *An. maculipennis* s.l. had fed on were determined by a PCR-sequencing approach based on the partial amplification of the mitochondrial *cytochrome c oxidase subunit I gene*. Mosquitoes were then identified to species by sequencing a region of the internal transcribed spacer-2. DNA extracts from all mosquitoes identified as having fed on rabbits were subsequently screened using PCR for the presence of myxoma virus.

Results: A total of 94 blood-fed *Anopheles maculipennis* s.l. were collected, of which 43 (46 %) provided positive blood meal identification results. Thirty-six of these specimens were identified as *Anopheles atroparvus*, which had fed on rabbit (n = 33, 92 %) and cattle (n = 3, 8 %). Seven mosquitoes were identified as *Anopheles messeae*, which had fed on cattle (n = 6, 86 %) and dog

(n = 1, 14 %). Of the 33 An. atroparvus that contained rabbit blood, nine (27 %) were positive for myxoma virus.

Conclusions: Results demonstrate that a single DNA extract from a blood-fed mosquito can be successfully used for molecular identification of members of the *An. maculipennis* complex, blood meal identification, and for the targeted detection of a myxoma virus. This study shows that *An. atroparvus* has a strong feeding preference for both healthy and myxoma-infected rabbits, providing evidence that this species may play a significant role in the transmission of myxomatosis among wild rabbit populations in the United Kingdom (UK).

5.2 Background

The identification of blood meal origin in haematophagous arthropods such as mosquitoes provides important information concerning vector-host interactions and associated disease transmission dynamics (Mukabana et al. 2002). Molecular techniques for blood meal identification and the increasing volume of openly accessible databases of host species identification data such as GenBank (Benson et al. 2005) and The Barcode of Life Database (Ratnasingham & Hebert 2007) have facilitated this area of research. Systematic characterisation of bird and mammalian host genetics in particular has increased the specificity of studies carried out driven by the use of polymerase chain reaction techniques. These techniques have largely replaced serological methods for blood meal identification such as the precipitin test and enzyme-linked immunosorbent assays (ELISA) (Kent 2009). The high copy number and conserved nature of mitochondrial genes such as *cytochrome b* and *cytochrome c oxidase I* (*COI*) have made them popular targets for identification applied to host-feeding preference studies (Muñoz et al. 2012). Ribosomal genes such as the internal transcribed spacer-2 (*ITS-2*) are also commonly used in species identification (Prakash et al. 2006). For example, both *COI* and *ITS-2* markers helped to identify a third member of the *An. maculipennis* complex, *An. daciae*, Linton, Nicolescu & Harbach 2004, from the previously recognised species *An. atroparvus* van Thiel 1927 and *An. messeae* Falleroni 1926 (Linton et al. 2005). Identifying

mosquitoes to species level is important in blood-feeding studies as sibling species may exhibit marked differences in feeding preferences which are likely to influence their role in patterns of disease transmission (reviewed in Takken & Verhulst, (2013)).

Myxomatosis is a widespread disease of rabbits resulting from infection with the myxoma virus (Poxviridae, genus Leporipoxvirus). The virus causes mild disease in its South American native host species, rabbits of the genus *Sylvilagus* including the South American tapeti (*Sylvilagus braziliensis*), but in the European rabbit (*Oryctolagus cuniculus*) infection results in severe disease (Kerr & Best 1998; Aragão 1943). Two years after its introduction into the UK in 1953 (Armour & Thompson 1955), myxomatosis was responsible for the death of up to 99% of the British rabbit population (Hudson et al. 1955) and although some resistance in natural populations has since emerged (Ross & Sanders 1984), the disease continues to cause deaths in wild and domestic rabbits. The primary vector of the myxoma virus in Britain is the rabbit flea, *Spilopsyllus cuniculi* (Lockley 1954). Flea mouthparts become contaminated with myxoma virus when biting an infected rabbit, often directly through a lesion, and the virus can subsequently be mechanically transmitted to another host (Fenner & Woodroofe 1953). Other biting insects have also been implicated in transmission, most notably several species of mosquito including members of the Australian species complex *An. annulipes* (Foley et al. 2007). In Australia, mosquitoes were the principle vector of myxomatosis until the introduction of *S. cuniculi* in 1969 in order to promote transmission of the disease for rabbit population control purposes (Sobey & Conolly 1971). However, the role of mosquitoes in transmission of myxoma virus in the UK is less clear. Although an early study following the initial outbreak of myxomatosis in domestic rabbits implicated *An. atroparvus*, the authors found no evidence that healthy rabbits were fed upon in the wild, therefore did not consider the species important for transmission cycles in wild rabbit populations (Muirhead-Thomson 1956a). Subsequent studies using direct capture from rabbits and rabbit-baited traps did provide evidence that healthy rabbits were bitten by mosquitoes, but at a relatively low frequency, particularly when alternative large mammals (such as livestock species) were in close proximity (Service 1971b;

Service 1971a; Service 1969a). The myxoma virus was successfully identified from specimens of 17 out of the 34 British mosquito species in a limited number of studies (see Table 5.1), experimental transmission of myxomatosis was demonstrated using *An. atroparvus* (Andrewes et al. 1956) and exposure of healthy rabbits in the laboratory to the biting of field-caught mosquitoes resulted in infection (Muirhead-Thomson 1956a; Service 1971a; Muirhead-Thomson 1956b). Yet the apparent low biting and feeding rates of mosquitoes on healthy wild rabbits appeared to be a limitation to the involvement of mosquitoes in natural transmission cycles. In the UK mosquitoes might become infected through opportunistic feeding on infected rabbits with reduced host defensive behaviour but were unlikely to subsequently bite a healthy rabbit and transmit the virus.

A major limitation to mosquito blood-feeding studies lies with the difficulty in collecting large numbers of blood-fed individuals with blood meals that are sufficiently undigested to allow successful DNA amplification (Kent 2009). This study aimed to maximise the data obtained from each blood-fed specimen by assessing whether a single DNA extract could be used for multiple purposes: firstly to identify the blood meal origin in members of the *An. maculipennis* s.l using a cocktail of 'universal' barcoding primers and secondly to identify individual species of the *An. maculipennis* s.l. present in the study area to assess whether different species exhibited different feeding preferences. *An. maculipennis* s.l. was abundant at the site during the collecting visits and the dominant anopheline species captured. During a visit to Elmley, Kent, in the summer of 2013, it was also observed that rabbits were frequently present within 25 metres of areas where human biting activity of mosquitoes had been reported by farm workers. On a subsequent visit, wild rabbits with obvious facial lesions indicative of myxomatosis were observed. Therefore, a further aim of the study was to test whether blood meal samples that originated specifically from rabbits also contained evidence of myxoma virus infection.

Mosquito species	Evidence of natural rabbit feeding	Identification of rabbit feeding through analysis of blood meals	Wild-caught mosquitoes positive for myxoma virus
Ae. cinereus	Yes (1, 2)[BC], [19][DC]	Yes (3, 4)	No
Ae. rusticus	Yes (3)[BC]	No	No
An. atroparvus[a]	No	No	Yes (6)
An. claviger	Yes (3)[DC, BC]	Yes (3, 4)	Yes (3)
An. plumbeus	Yes [19, 43][DC, BC]	Yes (1, 4)	Yes (3)
Cq. richiardii	Yes; (3, 5)[BC]	Yes (1, 3, 4)	No
Cx. pipiens s.l.	Yes (5)[BC]	Yes (3, 4)	Yes (3)
Cx. torrentium	Yes (5)[BC]	Yes (4)	No
Cs. annulata	Yes [19, 43][DC, BC]	Yes (1, 4)	No
Cs. litorea	Yes	Yes (4)	No
Cs. morsitans	Yes (5)[BC], (3)[DC]	Yes (1, 3)	No
Oc. annulipes	Yes (2)[BC]	Yes (2)	Yes (2)
Oc. cantans	Yes (2, 3)[BC]	Yes (2, 4)	Yes (2, 3)
Oc. detritus	Yes (5)[DC], BC, (3)[DC]	No	No
Oc. dorsalis	No	Yes (3, 4)	No
Oc. punctor	Yes (3, 4)[DC], (5)[BC]	Yes (3)	No
Oc. geniculatus	Yes; (3)[DC, BC]	Yes (3)	No

Table 5.1: Reported rabbit-feeding behaviour of British mosquitoes and their association with the myxoma virus prior to this study. Evidence of natural feeding provided by direct collections (DC) from rabbits, or rabbit-baited trap collections (BC). [a]*Anopheles atroparvus* was identified in these studies based on morphological and behavioural characteristics. *Culex pipiens* s.l. in these studies was identified morphologically and thus could include *Cx. pipiens f. pipiens* or *Cx. pipiens f. molestus*. Numbered references in brackets: (1) (Service 1969a), (2) (Muirhead-Thomson 1956b), (3) (Service 1971a), (4) (Service 1971b), (5) (Service 1969c), (6) (Muirhead-Thomson 1956a).

5.3 Methods

Collection of blood-fed mosquitoes *An. maculipennis* s.l. were collected over 15 visits from Elmley National Nature Reserve, Isle of Sheppey (51.377445, 0.784068), Kent, UK between June and September 2013. Elmley is a freshwater coastal marsh used to graze approximately 700 head of cattle. The collection site was within 200 meters of grazing cattle. The site is popular with birdwatchers all year-round owing to the abundance of local and seasonal migrant bird species that breed in the area. Mosquitoes were primarily collected using a mouth aspirator (John W Hock, Gainsville, Florida, USA) from inside the publically accessible toilet facilities where they were observed to be resting on walls and close to exposed sections of the wood enclosing the pipework (Figure 5.1). Additional attempts to collect blood-feds from a similar area were made using four CDC resting traps (Panella et al. 2011) placed in close proximity (~25 m) to the toilet block and run overnight (~14 hours) for nine of the 15 nights. Finally, any anophelines landing on and attempting to feed on the collector were captured where possible. Collected mosquitoes were placed into a cooler containing dry ice and transported to the laboratory. Blood-fed specimens were separated from non-blood-fed specimens on the same day as collection and stored at −20°C until processing at the Animal and Plant Health Agency (APHA).

DNA extraction from mosquito abdomens

Mosquitoes were identified based on morphological features as *An. maculipennis* s.l. following the key of Snow (Snow 1990). Abdomens of engorged mosquitoes were separated from the rest of the body on a chilled plate using forceps, and placed into individual 1.5 mL eppendorf tubes containing 200 µl phosphate buffered saline (PBS). The abdomens were pressed against the wall of the tube using the forceps to release the blood meal. The remaining head and thorax of each mosquito was stored at −20°C for morphological reference. Forceps were cleaned between specimens using a three stage wash to avoid crosscontamination. The first wash consisted of 5% decon, the second of 100% ethanol and the third of sterile water, at which point all excess liquid was removed with task wipes (Kimtech Science, Roswell, Georgia,

USA). Each sample was incubated with 20 µl proteinase K and 200 µl buffer AL for 30 minutes at 56°C in a water bath. DNA extraction was carried out using the DNeasy Blood and Tissue Kit (QIAgen, Manchester, UK), following the manufacturer's spin column-protocol. All DNA extractions were stored at 4°C until processing.

Figure 5.1: Photograph showing the primary collection area of resting mosquitoes in the toilet block at Elmley. Blood-fed *Anopheles maculipennis* s.l. mosquitoes were found resting directly on the walls and on or under the exposed wooden covering to the pipework.

Identification of blood meal host

Vertebrate host species in the blood meal were identified using a vertebrate specific, M13-tailed, triple primer cocktail (VF1_t1 + VF1d_t1 + VF1i_t1/VR1_t1 + VR1d_t1 + VD1i_t1) targeting a 685 base pair (bp) sequence of the *COI* gene (Messing 1983; Ivanova et al. 2007). This primer combination was expected to amplify all vertebrate species. Reaction contents were

obtained from Sigma-Aldrich (Sigma-Aldrich, Dorset, UK) unless otherwise stated. The final PCR reaction mix of 50 µl consisted of: 31.075 µl H_2O, 5 µl GeneAmp 10X PCR buffer I (Applied Biosystems, Life Technologies Ltd, Paisley, UK), 1 µl dNTPs (at 0.2 mM/µl), 1 µl of each primer (at 10pmol/µl),0.25 µl AmpliTaq Gold DNA Polymerase (10 units/µl) (Applied Biosystems, Life Technologies Ltd, Paisley, UK), 0.675 µl dimethyl sulfoxide (DMSO), 1 µl tetramethylammonium chloride (TMAC) and 5 µl extracted DNA. The thermal profile consisted of an initial denaturation step at 94 °C for 10 minutes followed by 40 cycles of: 94°C for 30 seconds, 53°C for 30 seconds, 72°C for one minute, followed by a final elongation step of 72°C for 10 minutes. PCR products were separated on a 1.5% agarose gel and samples producing a positive result were sequenced. Sequencing was performed using M13 primers (Ivanova et al. 2007) at 1pmol/µl. Amplification products were sequenced in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies Ltd, Paisley, UK). All sequences were edited using Lasergene version 12.1 (DNASTAR, Inc, Madison, Wisconsin, USA) and assigned to a particular vertebrate species when agreement was ≥98% to sequences of known species in GenBank (Martínez-de la Puente et al. 2013).

Species identification within Anopheles maculipennis s.l.

Species level identification was obtained by amplification of a 435 bp region of *ITS-2* using the primers 5.8SF and 28SR of (Collins & Paskewitz 1996). PCR products were obtained using an optimised real-time PCR assay in a Mx3000P real-time PCR system (Stratagene, Agilent Technologies, Cheshire, UK) in the following reaction mix, final volume 40 µl: 2 µl of DNA template, 14 µl H_2O, 20 µl SYBRGreen JumpStart Taq ReadyMix (Sigma-Aldrich, Dorset, UK) and 2 µl of each primer (each at 10 pmol/µl). The thermal profile consisted of an initial denaturation step at 94°C for 10 minutes followed by 35 cycles of: 94°C for 30 seconds, 53°C for 30 seconds, 72°C for one minute, followed by a final elongation step of 72°C for 10 minutes. PCR products were visualized on a 1.5% agarose gel, and samples showing bands of the correct size were sequenced in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing

Kit (Applied Biosystems). All sequences were edited in Lasergene version 12.1 (DNASTAR) and assigned to a particular mosquito species when agreement was ≥98% to sequences of known species in GenBank. *ITS-2* sequences for each of the species within *An. maculipennis* s.l. found in the UK are available.

Detection of myxoma virus

Myxoma virus genome was detected using two previously published methods. Samples were initially screened using Low-GC PCR primers that amplified a 220 bp sequence of the myxoma virus genome (Li et al. 2010). Samples giving a positive result were also amplified using a primer pair (M135Rfor/M135Rrev) (Belsham et al. 2010) that produced a 650 bp amplicon. Amplified products were excised from a 2% agarose gel and purified using a gel extraction kit (QIAgen, Manchester, UK). The resulting amplicon was then sequenced using flanking primers and ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies Ltd, Paisley, UK) following the manufacturer's instructions. The presence of myxoma virus was confirmed by BLAST (NCBI) search of the sequence.

5.4 Results

In total, 94 blood-fed specimens belonging to the *An. maculipennis* s.l. were collected from the Elmley site over 15 collection days. The toilet block yielded the majority (n = 92), with only one blood-fed *An. maculipennis* s.l. collected in the CDC resting traps and one that alighted on the collector. Of the total blood-fed samples extracted, 43 (46%) produced a 685 bp band when amplified with *COI* primers as illustrated in Figure 5.2. *An. maculipennis* s.l. at Elmley fed on cow (*Bos taurus*), European rabbit (*Oryctolagus cuniculus*) and dog (*Canis lupus familiaris*) (Table 5.2).

The molecular identification of the species from the same DNA samples using the *ITS-2* region revealed that 36 specimens were *An. atroparvus* (98–100% identity following BLAST search) and seven specimens were *An. messeae* (99–100% identity following BLAST search). For

the latter sequences, all shared greater than 99% identity with published *An. messeae ITS-2* sequences (GenBank accession number AY238412) and we are confident that no blood-fed samples of *An. daciae* were detected at the site. When the blood-feeding hosts were analyzed by species, *An. atroparvus* fed mainly on rabbits (n = 33, 92%) and cattle (n = 3, 8%). In contrast, *An. messeae* was found to have fed on cattle (n = 6, 86%) and dog (n = 1, 14%) (Table 5.2).

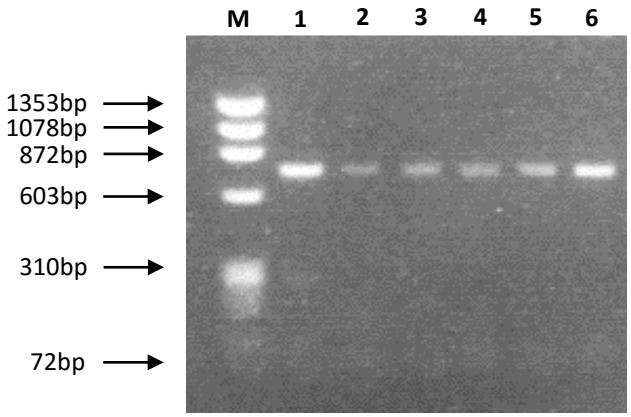

Figure 5.2: Gel image showing *COI* amplification products. The samples are PhiX174 DNA marker (M), negative control (1), mosquito blood meal samples (2 - 4) and a positive control of DNA (5, 6). The positive control was DNA extracted directly from horse blood.

Mosquito species	Cow	Rabbit	Dog	Total
Anopheles atroparvus	3	33	0	36
Anopheles messeae	6	0	1	7
Total of blood-fed	9	33	1	43

Table 5.2: Hosts selected by mosquito species at Elmley, Kent, between June and September 2013.

Rabbits at the collection site had been observed showing facial lesions suggestive of myxomatosis (Figure 5.3). All 33 specimens of An. atroparvus that were found to have fed on rabbits were tested for the presence of the myxoma virus. In total, amplicons were obtained

from nine blood meal samples (27%) (Figure 5.4). DNA sequencing confirmed that the amplicon was derived from the myxoma virus showing 100% sequence identity to previously characterised virus genomes from England (GenBank accession number KC660084 (Kerr et al. 2013).

Figure 5.3: Rabbit with swelling and lesions around the eyes indicative of myxomatosis infection (photographed by VAB at Elmley, Kent in July 2014).

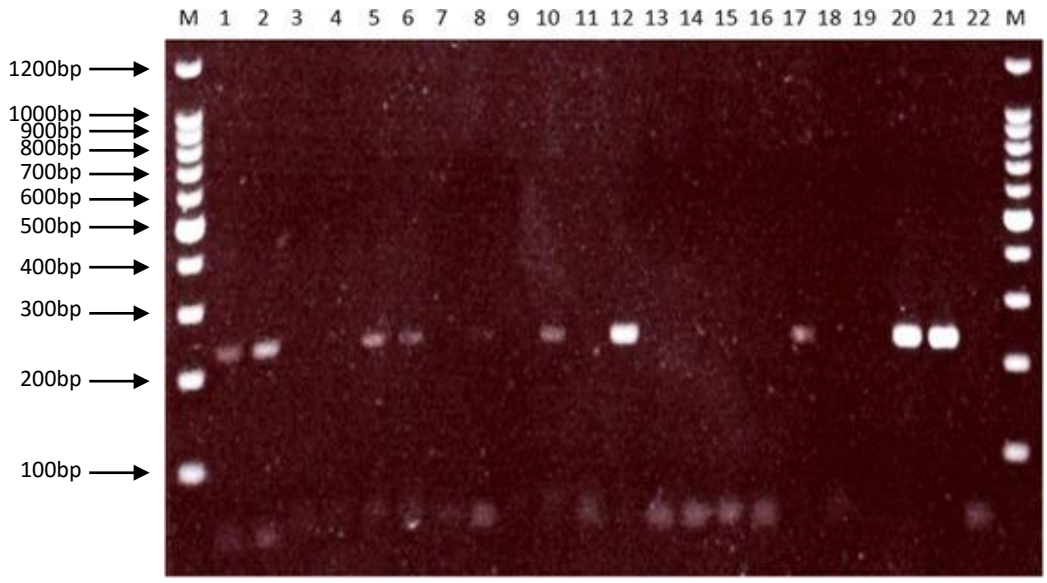

Figure 5.4: Gel image showing myxoma virus amplification products in mosquito blood-meal samples. The lane order is: 100 bp ladder (M), blood-fed *Anopheles atroparvus* DNA extracts BF1 (1), BF13 (2), BF14 (3), BF19 (4), BF20 (5), BF31 (6), BF47 (7), BF33 (8), BF39 (9), BF9 (10), BF85 (11), BF93 (12), BF99 (13), BF106 (14), BF108 (15), BF110 (16), BF111 (17), BF18 (18), 113 (19), myxoma virus positive controls (20, 21), negative control (22).

5.5 Discussion

This study demonstrates that a single DNA extract from the abdomen of a blood-fed mosquito can be successfully used for three purposes in a sequential workflow: (1) for the identification of the vertebrate origin of a blood meal by sequencing of a 685 bp region of the *COI* gene, followed by (2) the molecular identification of members of *An. maculipennis* s.l. by sequencing of a 435 bp region of *ITS-2*, then (3) the detection of a pathogen, in this case the myxoma virus. This approach provided definitive evidence that *An. atroparvus* feeds extensively on both healthy and myxoma-infected rabbits, thus indicating that this mosquito could play a more significant role in the transmission of myxomatosis in wild rabbit populations in the UK than was previously suspected.

Early studies on the feeding behaviour of *An. atroparvus* (Andrewes et al. 1956; Muirhead-Thomson 1956a) were limited to morphological identification of specimens. Despite suggestions as far back as the 1930s of a third member of the *An. maculipennis* s.l. in the UK (Edwards 1936), it was not until recent sequencing of *ITS-2* was performed that a third member, *An. daciae*, was discovered (Linton et al. 2005). Applying current molecular techniques to delineate mosquito species complexes is important as morphologically indistinguishable sibling species may exhibit different feeding behaviours that will affect their capacity to act as disease vectors. Owing to its recent discovery in the UK the feeding preferences of *An. daciae* have not been extensively studied, however one study indicated it may differ from *An. atroparvus* and *An. messeae* by feeding on birds as well mammals (Danabalan et al. 2014). In the present study no *An. daciae* were found but *An. atroparvus* and *An. messeae* were successfully identified by amplification of *ITS-2* (Collins & Paskewitz 1996). The presence of both species in the same collection site is to be expected as Elmley sits at the interface between the saline coastal habitats generally favoured by *An. atroparvus* and fresh-water breeding habitats in the grazing marshes more suited to *An. messeae* (see Sinka et al., (2010) for a review). Both these species were found to feed only on mammals, corresponding with previous studies (Danabalan et al. 2014).

However, 92% of *An. atroparvus* blood meals were taken from rabbits, whereas *An. messeae* was found to have fed only on cattle (86%) and dog (14%). It is worthy of note that although the 14% of *An. messeae* comprises only a single mosquito, only one dog is present on site (this belongs to the site owners; other dogs are not permitted) and thus the host abundance of this species is considerably lower than that of rabbits around the collection site. Nonetheless it is evident that further blood-fed collections from the site are required to draw conclusions on whether these results reflect a true difference in feeding preferences between the two mosquito species. Prior to this study, there have been no published reports of rabbit blood being detected in field-caught specimens of *An. atroparvus* in the UK (Table 5.1). The molecular approach used in this study facilitated the finding of this result as sequencing of the 'universal' barcoding region of the *COI* gene precludes the need to pre-select hosts on which mosquito feeding is considered most likely, a necessary step in the preparation of species-specific sera for serological assays such as the precipitin test or when designing primers for a multiplex assay. The previous study collecting *An. maculipennis* s.l. from a similar area of Kent utilised a multiplex assay design that did not include rabbit and therefore was not able to inform on rabbit-feeding behaviour (Danabalan et al. 2014).

The apparent strong feeding preference of *An. atroparvus* for rabbits is somewhat surprising considering the low numbers captured previously in a study employing direct capture of mosquitoes, rabbit-baited traps and precipitin testing in the UK (Service 1971a). There, only two individuals of the *An. maculipennis* s.l. were collected, both of which were negative for rabbit blood by precipitin testing and neither mosquito were identified to species. A recent host preference study in Spain provided evidence that *An. atroparvus* populations did feed on rabbits in the wild, albeit at a frequency of less than 2% (2/115 blood meals) (Martínez-de la Puente et al. 2013). This contrasts with observations of strong feeding preferences for rabbits by *An. atroparvus* elsewhere in Europe (Cambournac 1994), raising the question of whether different populations of the same species may exhibit different feeding preferences driven mainly by the local availability of hosts rather than an intrinsic preference (Chaves et al. 2010). Collecting larger

numbers of blood-fed *An. maculipennis* s.l. from several sites across the UK taking into account local rabbit abundance may provide evidence to this effect. Elmley Nature Reserve is a grazing marsh with cattle present close to the area of collection and throughout the collection period. It would appear that rabbits are being preferentially selected for feeding by *An. atroparvus* despite the presence of larger mammalian hosts, although host-baited choice experiments in the field or laboratory would provide stronger evidence for this behaviour (reviewed in Kent, (2009)).

It is important to maximise the data that can be obtained from a single blood-fed specimen as collecting large numbers of blood-fed mosquitoes in the UK by currently available methods is challenging (Burkot et al. 2013). Furthermore, the likelihood of successfully identifying bloodmeal host decreases rapidly with time as digestion within the insect takes place (reviewed in Kent, (2009)). The primer cocktail used in this study (Ivanova et al. 2007) successfully identified the blood meal host in 46% of the captured blood-fed mosquitoes. The time between feeding and collection was not known for mosquitoes in this study but assessing mosquitoes in future studies using the Sella score to rate level of digestion would be beneficial to assess the efficacy of this approach according to the degree of digestion (Detinova 1962).

The concomitant identification of both the mosquito and its blood meal host allowed for the targeted selection of specimens of epidemiological relevance for subsequent pathogen screening, saving both time and resources. In this instance we have screened samples for a mechanically transmitted pathogen. Prior to this study, there was little evidence from fieldwork studies that mosquitoes played an important role in the transmission of myxomatosis among wild rabbit populations in the UK. Evidence for occasional involvement in transmission to and within domestic rabbit populations has been reported (Muirhead-Thomson 1956a) supported by laboratory transmission data (Andrewes et al. 1956). This study found that 27% of blood meals derived from rabbit were positive for myxoma virus. According to evidence that arthropod transmission is mechanical and therefore requires the presence of a lesion through which

mouthpart contamination will occur (Fenner & Woodroofe 1953), this finding demonstrates that wild, myxomatosis-infected rabbits are being fed upon by *An. atroparvus* at Elmley. However, it is the observation that over two-thirds of rabbit-derived bloodmeals were negative for myxoma virus that is most important and differs from previous studies in the UK as this suggests that *An. atroparvus* is not simply opportunistically feeding on diseased rabbits, but also readily feeds on healthy rabbits in wild populations. We therefore conclude that mosquitoes could contribute to the transmission of the virus. Mosquito collections in this study were conducted between June and September, the period broadly considered to be the peak vector season in the UK (Service 1969a; Brugman et al. 2013), however, the number of mosquitoes collected make interpreting seasonal influences uncertain. If the peak incidence of myxomatosis in the rabbit population were to be closely associated with the peak period of *An. atroparvus* activity, then this would provide evidence of seasonal shifts in the relative importance of different vector groups in the area.

The myxoma virus possesses a DNA genome and was therefore present following the extraction procedure. However, using a DNA- or RNA-specific extraction protocol provides an additional limitation to the data that can be obtained from each specimen. Therefore, we advocate the use of a co-extraction procedure such as that of Griffiths et al. (2000) that would preserve both DNA and RNA, greatly widening the information that could be obtained from a single mosquito specimen. Further applications of such an approach could include screening for human viral pathogens present in mosquitoes that had fed on humans for the purposes of xenosurveillance (Grubaugh et al. 2015).

5.6 Conclusion

This study shows that a single DNA extraction can be successfully used to identify blood meal host, delineate to species level members of the *An. maculipennis* s.l. and to subsequently detect the presence of an animal pathogen, myxoma virus. This tripartite approach revealed that healthy and myxomatosis-infected rabbits are a major blood-feeding host at Elmley, Kent,

and therefore provides further evidence that *An. atroparvus* may play an important role in the transmission of this disease in wild rabbit populations.

Chapter 6 – Mosquito host selection and feeding preferences

6.1 Introduction

Identifying the vertebrate hosts of blood-feeding mosquitoes is an important component of understanding pathogen transmission and determining the role of different species in inflicting biting nuisance. A barrier to this process is the fact that blood-fed mosquitoes are among the most difficult physiological states to collect, due primarily to inhibition of host seeking, limiting attraction to semiochemical-baited traps (Silver 2007; Burkot et al. 2013). In the UK, a single CDC light trap operated two-three times per week between May and October 1965 and 1966 collected no blood-fed mosquitoes (Service 1969c). Similarly, in a more recent comparison of carbon-dioxide (CO_2)-baited CDC light traps with an early version of the Mosquito Magnet trap design, none of the 5414 mosquitoes collected were blood-fed (Hutchinson et al. 2007). A similar trend has been shown for mainland European mosquito assemblages. Only 781/33 033 (2.4%) of the mosquitoes collected using a combination of unbaited, CO_2 plus BG lure-baited and CO_2 plus 1-octen-3-ol-baited BG-sentinel traps and CO_2-baited CDC light traps were blood-fed in a wetland area of Spain (Roiz, Roussel, et al. 2012). In Italy, of 5063 mosquitoes collected using BG-Sentinel traps baited with BG lure, only 91 (1.8%) individuals were blood-fed (Roiz, Vazquez, et al. 2012).

Studies of resting blood-fed mosquito populations in the UK have largely relied on direct collection of individuals from indoor and outdoor resting sites using aspirators and sweep-nets. This has the advantage of providing additional and highly specific data concerning preferred resting habitats, but yields only limited numbers of mosquitoes for a substantial allocation of collection time. During studies conducted previously in the UK, a wide variety of habitats have been sampled such as bunkers (Danabalan et al. 2014), the eaves of buildings (Service 1994) and vegetation (Service 1969a; Service 1971b). The use of resting boxes to collect blood fed mosquitoes is an alternative approach to direct sampling that relies on providing a simulation of resting habitats. Boxes used are usually containers of various sizes, materials and colour, unified

by a reliance on the (generally passive) recruitment of engorged mosquitoes into the box interior from where they can be captured (see Silver (2007) for review). Mosquitoes are usually aspirated from inside boxes following collection, but other methods including draw-string bags placed within the box are sometimes used (Edman et al. 1968) and additional designs have used fans analogous to those in a light trap (Panella et al. 2011) or sticky sheets placed inside the boxes (Pombi et al. 2014).

Standardisation between designs of resting trap has been limited, with size, shape, habitat selection and frequency of emptying all varying between users. However, resting boxes are frequently coloured red on the inside to increase visibility of mosquitoes on surfaces, with darker colours used on the outside (Reisen & Pfuntner 1987; Edman et al. 1968). Different colours have not however been consistently found to be a significant factor in influencing collections (Morris 1981). Another source of potential variation was highlighted using resting box collections of the species *Culiseta melanura* Coquillett 1902, *Cs. morsitans*, *Anopheles punctipennis* Say 1823 and *An. quadrimaculatus* in the USA (Morris 1981). Within this study, morning collections from west-facing boxes were found to maximise mosquito collections, illustrating a potentially important variable that is rarely recorded in studies. Although resting box collections are frequently biased towards species that commonly rest inside artificial structures (such as those of the genera *Anopheles*, *Culiseta* and *Culex*), other genera including *Coquillettidia* and *Aedes* are also often collected. To date, save one anecdotal report of resting box use in London Zoo (Quintavalle Pastorino et al. 2015), systematic resting box collections of blood-fed mosquitoes have not been conducted in the UK.

As highlighted in Chapter 1, UK blood meal studies have to date lacked some resolution in identifying the full range of blood meal hosts available to mosquitoes in the field. This may have led to an overestimation of the amount of feeding determined to occur on selected hosts. Existing studies have been essential in understanding fundamental feeding patterns of many mosquito species, particularly from the perspective of broad 'mammal vs. bird' preferences.

However, current understanding of mosquito-pathogen-host transmission indicates that disproportionate feeding on certain pathogen-susceptible species in a given ecosystem can influence epidemiology. As an example, feeding by *Culex pipens* s.l. on American robins (*Turdus migratorius* Linnaeus 1766) between May-June in the USA is a key determinant of West Nile virus transmission intensity (Kilpatrick et al. 2006). This is driven by a significant preference of *Cx. pipiens* s.l. for this host species, constituting 51% of blood meals, despite American robins representing only 4.5% relative abundance among birds in the ecosystem. The reduction in the numbers of American robins as a result of its late-summer migration additionally results in an increase in mosquito feeding on humans, and therefore zoonotic West Nile virus transmission at the end of the summer (Kilpatrick et al. 2006).

Following collection of blood-fed mosquitoes, vertebrate blood meal origin is identified using one of a variety of techniques (see Kent (2009); Washino & Tempelis (1983) for reviews). The specific assay used for this purpose has evolved over time from precipitin testing, through enzyme-linked immunosorbent assays (ELISA) to more recent use of polymerase chain reaction (PCR) based techniques. This progression has enabled more consistent and specific identification of hosts, not only due to the increased robustness of the techniques developed but also due to substantial open access databases of sequence DNA now available for comparison. Two approaches are most commonly adopted: firstly, molecular markers within the DNA of the host's blood can be amplified using generic primers for vertebrates and then sequenced to compare to existing databases; secondly, multiplex primers can be designed from sequence data to differentiate blood from specific species or species groups of hosts. The former technique has the advantage of a high degree of specificity in identification, but is reliant on the quality and size of sequence databases for the marker concerned. The latter technique is more cost-effective and rapid in most scenarios, but is heavily reliant on accurately defining available hosts in the habitat of interest prior to use.

To date, the molecular marker most commonly sequenced for host identification is the *cytochrome c oxidase 1* (*COI*) gene (Kent 2009). To cope with variation in DNA sequence across vertebrates in this region, cocktails of primers containing degeneracies are usually used in studies, together with low annealing temperatures, as discussed by Ivanova et al. (2007). The *COI* marker gene is used widely for routine vertebrate identification studies, resulting in a diverse repository of several million taxonomically-linked sequences stored in the Barcode of Life database (Ratnasingham & Hebert 2007) and Genbank (Benson et al. 2005). While other markers are sometimes used (particularly a region of the vertebrate *cytochrome b* gene), these alternatives lack the substantial underlying dataset.

Multiplex PCR approaches are particularly useful where the potential host species are known *a priori*, making this technique a rapid and inexpensive alternative to sequencing. In the case of the *COI* region, a wide array of specific primer sets have been designed and tested successfully for this purpose (e.g. Garros et al. (2011); Ngo & Kramer (2003); Kent & Norris (2005)). Usually species-specific primers are paired with a single universal primer, allowing samples to be tested for several different host species in a single reaction. This requires testing for cross-reactivity between species and also optimisation of reaction conditions to enable broadly similar sensitivity to be achieved across the primers in use. A significant limitation in this approach is that the number of suitable polymorphic sites on the molecular marker chosen for siting species-specific primers tends to be limited and hence several assays are sometimes required to distinguish between larger numbers of potential hosts. In addition, unless the diversity of hosts within a particular habitat is extremely restricted (e.g. when directly collecting insects from host habitation), these assays are not suitable for screening blood meals in the majority of natural environments.

In addition to sequencing and multiplex PCR, a wide range of other, less frequently used, techniques have also been applied to identifying blood meals in arthropod vector species. Real-time (q)PCR assays (van den Hurk et al. 2007) permit high-throughput processing as no gel

electrophoresis or sequencing step is required, but are limited by the availability of suitable fluorescent probes and require more expensive equipment and reagents. It is also doubtful in most cases whether the increased speed of sample processing is a requirement for studies that are not usually on the front line of diagnostics. Both microsatellite analysis (Darbro et al. 2007; Torr et al. 2001) and restriction fragment length polymorphism (RFLP) PCR (Oshaghi et al. 2006) enable the separation of species (and in some cases individual hosts), but require the initial setup of a library to identify hosts and hence are more suited to specific behavioural experiments where the likely hosts have been identified. Similarly, microsphere assays using the Luminex system (Thiemann et al. 2012) provide a highly specific and high-throughput method of identification, but still require potential hosts to be predetermined. More recently, a combination of proteomic and mass-spectographic methodologies (Önder et al. 2014), yet to be extensively tested on field specimens, has also been described, but this again requires the initial setup of an extensive spectral library from reference samples.

To date, only one study using PCR to identify blood meals in mosquitoes within the UK has been carried out, which used a species-specific multiplex PCR assay based on the *cytochrome b* molecular marker region to identify the hosts of *An. maculipennis* s.l. (Danabalan et al. 2014). This enabled separation of the major available host species thought to be present across three sites in the UK (bird, human, deer, goat, horse, cow and dog), although no attempt was made to survey sites for these populations. Although this methodology permitted accurate identification of feeding on these hosts, the use of targeted multiplexed primers also introduced selection bias to the results, in comparison to the sequencing of molecular markers. It is therefore probable that many of the 99/237 blood-fed individuals for which no blood meal result was obtained could have resulted from feeding on non-target hosts (Danabalan et al. 2014). This highlights the value of using sequencing to establish feeding preference, combined with field surveys to assess host availability. While a powerful approach, this combination is rarely carried out, in part due to the logistics of surveying across diverse vertebrate groups and relating these populations to mosquito ecology.

The aim of this study was to create a complete picture of host selection by mosquitoes at a single farm site. Elmley was chosen as this site supported the greatest abundance of mosquitoes in prior sampling visits and, additionally, collections of blood-feds specimens in Chapter 5 indicated that with more intensive collection effort, even greater numbers of blood-fed specimens could be collected here. By identifying mosquito blood meals using a PCR-sequencing approach, information on the range of hosts selected by the mosquitoes could be obtained without introducing selection bias. Blood-fed collections from the field are often biased as a result of collecting mosquitoes that have fed on the most abundant host in an area (Takken & Verhulst 2013). By estimating the number of different hosts in the area by way of bird and mammal surveys and collecting mosquitoes using passive methods targeting the resting mosquito population (resting boxes and artificial structures), the aim was to obtain information on host preferences weighted by the relative abundance of hosts in the collection area, using the feeding index of Kay et al. (1979).

Study aim

To identify the blood-feeding patterns of mosquitoes at Elmley.

Objectives

1. To evaluate the influence of sampling location and meteorological variables on the collection of blood-fed mosquitoes using a novel resting box design.
2. To determine the vertebrate blood-feeding hosts of farm-associated mosquitoes at Elmley using a sequencing-based approach and species identification assays.
3. To draw preliminary conclusions regarding the host feeding preferences of mosquitoes at Elmley by comparison of hosts selected with vertebrate host abundance data.
4. To correlate successful blood meal identification with stage of oogenesis/digestion using the Sella scale.

6.2 Materials and Methods

6.2.1 Farm selection

The study was conducted at Elmley as results from the pilot study (Chapter 2) and the avian- and human- biting rate studies (Chapters 3 and 4) indicated that mosquito abundance was highest at this site. Additionally, opportunistic collections of blood-fed specimens in 2013 looking into myxoma virus transmission (Chapter 5 and Brugman et al. (2015)) found that the greatest numbers of blood-fed specimens were to be found at this farm.

6.2.2 Design and construction of resting trap

The resting boxes were constructed from flat-pack 'no-nail' 5 mm plywood boxes with exterior dimensions W: 500 mm x H: 500 mm x D: 500 mm (Davpack, Derby, UK) (Figure 6.1). The boxes were treated with a primer coating of paint and then a coat of matt red paint on the inside and matt black paint on the outside (B&Q, Guildford, UK). At least two hours was left between primer coats and paint coats to allow drying, with a final 48 hour drying period before deployment in the field. A 5 mm Perspex sheet with dimensions W: 600 mm x H: 600 mm (Display Pro, Norfolk, UK) with a 120 mm diameter hole in the centre was used when collecting from the resting boxes. Netting removed from a BugDorm insect cage (BugDorm, Taichung, Taiwan) was affixed using tape to prevent mosquito escape during aspiration.

6.2.3 Location of resting boxes

Five resting boxes were placed at four sites on the farm (see farm map in Chapter 2) and identified with a letter (A-D) and number (1-5) e.g. A1. Resting boxes at the same site were placed a minimum of 1.5 m apart to avoid interference between traps (Morris 1981). The open face of each resting box was faced west, except for site B where the boxes were faced into the vegetation (approximately east). Location A was in the centre of a small area of woodland strip called 'the orchard' consisting of fruit trees, Elder (*Sambucus nigra* L.) and Willow (*Salix* sp.) trees and bramble (*Rubus* sp.). Location B was approximately 10 m away from location A and situated at the border between the woodland area and the overflow carpark. At the time of

study, a large mound of gravel (used to cover the paths throughout Elmley) was present directly behind the traps at location B, which was utilised as a warren by rabbits. Location C was close to the machinery barn in a narrow strip of willow trees bordering a small freshwater pond. Location D was situated in a more exposed area away from trees and next to two wide freshwater ditches. A Google Photosphere is available for each trapping location; see .jpg files on enclosed CD-ROM which can be uploaded and viewed at http://photosphereviewer.net/.

6.2.4 Additional collection sites

In addition to collections made from resting boxes, direct collections were made from the following structures which might serve as resting sites (Figure 6.2):

- the public toilets at Elmley, previously identified as containing considerable numbers of resting mosquitoes in Chapter 5/Brugman et al. (2015)
- two chicken coops close to one of the houses on site
- a cattle barn (henceforth, “barn”), unused during the summer collection period
- a red cattle feeder
- a permanent, stone-and wood roofed structure (henceforth, “roofed structure”) exposed on one side and situated close to the car park and public toilets.

Figure 6.1: the resting box. Clockwise from top left: (1) fully assembled resting box next to its folded constituent parts, (2) resting box *in situ* at Elmley, with Perspex collection sheet covering entrance (3) 20 folded resting traps folded flat in the back of the fieldwork vehicle, (4) photo illustrating disassembly of the resting box using a flat head screwdriver.

6.2.5 Mosquito collection schedule

Resting boxes were set up at the beginning of June 2014 and left in position until collections ended in October 2014. Each box was visited for two consecutive days each week constituting a total of 36 collection visits. Each trapping day consisted of a collection in the morning (08:30-09:30), around noon (11:30-12:30) and in the afternoon (14:30-15:30). A

Mosquito Magnet Pro trap baited with 1-octen-3-ol (Midgetech), situated in a fixed location ~100 m from the nearest resting boxes was run overnight (~14 hours) on the first of the two nights each week, as a control to assess background mosquito populations. Mosquitoes were collected from resting boxes using a manual aspirator (John W Hock) and blown into cardboard pillboxes (Watkins and Doncaster) with insect mesh upper. Collections from the barn were made once per collection day by manual aspiration and collection using the 'Improved prokopack aspirator' (John W Hock). Collections from the chicken coops, barn and roofed structure commenced at the end of June when farm workers suggested these as collection sites. Pillboxes containing mosquitoes were transported to TPI in a cooler containing dry ice where they were stored at -20°C until processing.

6.2.6 Identification of mosquitoes

Mosquitoes collected in traps were identified based on morphological features following published keys (Snow 1990; Cranston et al. 1987). Additionally, mosquitoes morphologically identified as *Cx. pipiens* s.l./*torrentium* were identified by molecular methods to species level using the techniques detailed in sections 2.5.1 and 2.5.2. For reasons of cost it was not possible to identify all specimens of *An. maculipennis* s.l. to species level and hence a subsampling approach was used. All *An. maculipennis* s.l. identified as having fed on wild birds were identified to species level, while all those having fed on mammals, up to a limit of 10 individuals per host species, plus a randomly selected subset (~15 specimens) of those identified as having fed on chickens, cattle and rabbits, were also processed. Species-level identification of *Anopheles maculipennis* s.l. was conducted using the techniques detailed in section 2.5.3.

Figure 6.2: Photographs of artificial resting sites for mosquito collections. Clockwise from top left: (1) resting *Anopheles maculipennis* s.l. on the inside of one of the chicken coops, (2) the disused cattle barn, (3) the roofed structure, (4) the red cattle feeder.

6.2.7 Processing of blood-fed specimens and blood meal analysis

Female mosquitoes identified as containing a blood meal were manually separated from unfed specimens. The degree of blood meal digestion was classified as I-VI using the scale of Sella, following Detinova, (1962). All mosquitoes (except for those specimens of *An. maculipennis* s.l. omitted in the subsampling approach decribed above) identified as being stages II-VI were processed to identify the vertebrate blood meal origin according to the techniques detailed in section 2.5.4. Samples that did not produce a result in the PCR reaction (i.e. gel image) or sequencing were not repeated.

6.2.8 Vertebrate host surveys

Bird survey

A bird survey was conducted on the 22nd August 2014 in order to estimate the relative abundance of avian species present in the immediate vicinity (~2 km) of the trapping area, as approximated from the central woodland strip. A point transect was employed (following recommendations in Gregory, (2004)) consisting of nine points appropriately 200 m apart following the main track (Figure 6.3). Counts were made from inside a car and recording at each point lasted for 10 minutes after an initial settling time of one minute (to allow for local disturbance caused by arrival at the point). The survey was started at sunrise (06:20 on the day). It was considered that the maximum distance for observation was a 200 m radius around each transect point. The combined total numbers of birds for each recorded species were used as an estimate of abundance in the trapping area.

Rabbit and hare survey

A rabbit (*O. cuniculus*) and hare (*Lepus europaeus* Pallas 1778) survey was conducted at sunset on the 9th of September 2014 and at sunrise on the 10th of September in order to estimate the abundance of these hosts in the vicinity of the trapping area. Two continuous walking point transects were conducted (following recommendations in Toms, Siriwardena, & Greenwood, (1999)) (Figure 6.3), one towards the barn and the second towards the central woodland strip, with records made for rabbits/hares within eyeline of each transect point. The number of rabbits and hares were recorded within a maximum range of 200 m. Results from the evening and morning surveys were averaged and this figure used as the estimate for abundance.

Abundance of other vertebrate hosts

The number of mammalian species present at Elmley is heavily restricted by the use of an 'anti-predator' fence which runs across land and water, beginning shortly after the entrance to the site. There is additionally an annual winter hedgehog relocation plan (hedgehogs are

humanely trapped and released on the Kent mainland). These measures are enforced to minimise predation of breeding birds on site and thus predatory mammals such as foxes and badgers are not present on site. Details on the numbers of cattle, chickens and breeding barn owls on site were obtained from the farm owners. Data on average visitor numbers were also obtained from the owners however this was not considered an accurate measure of human exposure to mosquito biting, as most visitors were bird watchers who quickly moved several miles from the central woodland area after parking. Therefore, the number of farm occupants on site was used as an estimate of human numbers within the 2 km trapping area.

Host preferences

The preference for mosquito feeding on pairs of different hosts was calculated using the feeding index of Kay et al. (1979), using the formula $FI = \frac{Ne/Ne^1}{Ef/Ef^1}$, where Ne = number of feeds on host 1, Ne^1 = number of feeds on host 2, Ef = expected number of feeds on host 1, Ne^1 = expected number of feeds on host 2. To account for differences in host size, the expected relative number of feeds (Ef) was weighted by a factor dependent on an approximation of the relative size of the hosts being compared, as performed in Renshaw et al. (1994).

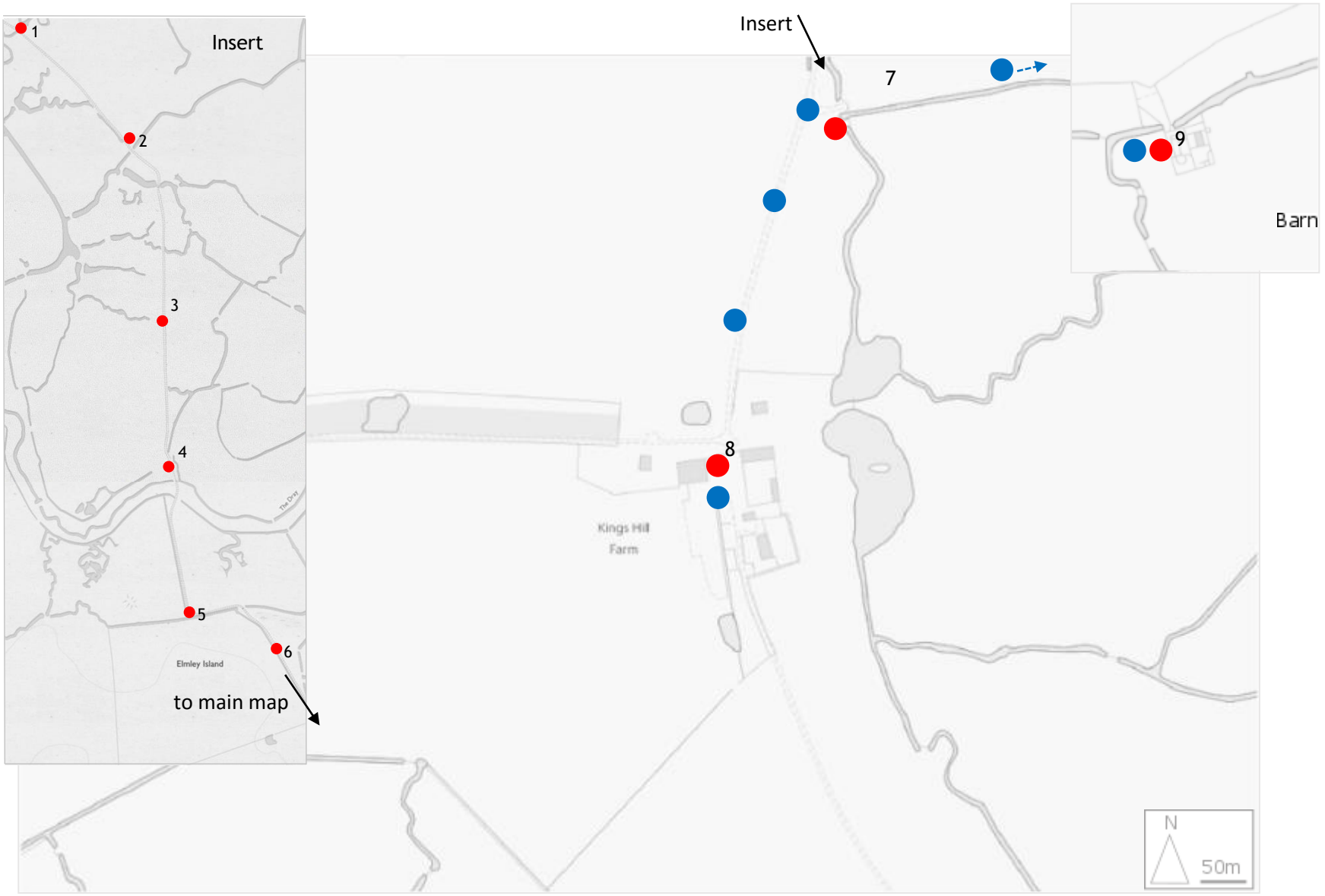

Figure 6.3: Bird (red dots, 1-9) and rabbit/hare survey transects (blue dotted lines) at Elmley. The bird surveys were conducted from inside a car, the rabbit/hare survey by foot. The insert on the left indicates the bird survey route from the entrance of the Elmley grounds.

6.2.8 Collection of meteorological data

Meteorological data was collected at hourly intervals using an automatic weather station (AWS) data logger model CR800 (Campbell Scientific, Loughborough, UK), placed in a fixed location (as in Chapter 3) throughout the trapping period. Variables collected were air temperature (°C), relative humidity (%), wind speed (m/s) and rainfall (mm). The hourly data points are a mean value calculated automatically from values recorded every minute. Data was stored on the on-board CR800 data logger and was downloaded onto a laptop at the end of the study and stored as an Excel file.

6.2.9 Analysis of results

Assessment of the effects of different variables on the number of blood-fed mosquitoes (Sella Stages II-VI) collected in the resting boxes in the morning collection period was made by fitting a generalized linear mixed model (GLMM) to the data using the 'glmmadmb' function in the 'glmmADMB' package in R version 3.2.0 (R Core Team 2015). The response (number of blood-fed mosquitoes collected) was a count variable, therefore the initial model consisted of a Poisson GLMM with a log link function, fitted by maximum likelihood with the Laplace approximation. The covariates in the model were *temperature*, *relative humidity* and *wind speed*, which were averaged over the 12 hours preceding the morning collection period (20:00 – 08:00), the period during which feeding and flight activity leading to entry into the boxes would be expected. Resting box locations at each location were combined for analysis (A – D) and included as a fixed factor. *Rainfall* was also included as a fixed factor (presence/absence) in the model. Collection *date* was included as a random factor. The Poisson model indicated that the data were overdispersed (residual deviances > degrees of freedom) and therefore a negative binomial GLMM was fitted to the data. The goodness-of-fit of the models to the data was assessed by comparison of Akaike information criterion (AIC) values using function 'AIC' in the 'stats' package in R, with lower values indicating a better model fit. Multiple comparisons to

assess the effect of individual factors on the response variable were made using the 'glht' function in the 'multcomp' package in R.

To assess the effect of blood meal digestion stage (Sella stages II – VI) on the chances of successfully obtaining a result for blood meal host, a binomial GLMM with logit link function was fit to the data using the 'glmer' function in the 'lme4' package in R. The response was a binary variable according to either obtaining a successful (1) or unsuccessful (0) result following sequencing, with blood meal digestion stage included as a fixed factor of five levels (each stage of digestion, II-VI). The results for all mosquito species were combined and *mosquito* included as a random factor in the model. The script for the resting box analysis is included in appendix A8 and for the blood meal identification success analysis in appendix A9.

6.3 Results

Summary

A total of 20 666 mosquitoes of ten species/species complexes were collected in the study, of which 2159 were blood-fed (Sella stages II-VI) (Table 6.1). Unfed females (stage I) (41.1%) and males (40.1%) accounted for the largest proportions of mosquitoes collected, with blood-fed and fully gravid (stage VII) females accounting for 10.4% and 8.4% respectively. Collections from the barn yielded the greatest number of mosquitoes (13 670), of which 1399 (10.2%) were blood-fed (Table 6.2). The resting boxes yielded a total of 5107 mosquitoes of which 485 (9.5%) were blood-fed. The MMP trap collected the lowest relative proportion of blood-fed mosquitoes (0.3% of its total).

Anopheles maculipennis s.l. was the most numerous species in collections, accounting for 15 653 individuals (Table 6.1). This was six times more than the number collected of the second most numerous species, *Cs. annulata* (2447) and nine times that of the third, *Cx. pipiens s.l./torrentium* (1726). These three species dominated collections from the resting boxes and other artificial resting sites, although resting boxes collected more *Cs. annulata* than *An. maculipennis* s.l. and *An. maculipennis* s.l. was the only species collected from the chicken coops (Table 6.2, Figure 6.4). The MMP collected a different species profile to the resting collections with *Cx. modestus*, *Cq. richiardii* and *Oc. flavescens* the most numerous species collected (Table 6.2, Figure 6.4).

Mosquito species	Males (%)	Sella stage of blood meal digestion							Totals
		I (%)	II (%)	III (%)	IV (%)	V (%)	VI (%)	VII (%)	
Anopheles claviger	0 (0)	2 (66.7)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**3**
Anopheles maculipennis s.l.	6294 (40.2)	6655 (42.5)	171 (1.1)	420 (2.7)	408 (2.6)	340 (2.2)	332 (2.1)	1033 (6.6)	**15 653**
Coquillettidia richiardii	45 (14.9)	216 (71.5)	1 (0.3)	0 (0)	3 (1.0)	3 (1.0)	3 (1.0)	31 (10.3)	**302**
Culex modestus	1 (0.3)	336 (97.4)	1 (0.3)	1 (0.3)	2 (0.6)	1 (0.3)	0 (0)	3 (0.9)	**345**
Culex pipiens s.l./torrentium	673 (39.0)	524 (30.4)	42 (2.4)	40 (2.3)	15 (0.9)	3 (0.2)	10 (0.6)	419 (24.3)	**1726**
Culex spp.	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**1**
Culiseta annulata	1267 (51.8)	601 (24.6)	32 (1.3)	113 (4.6)	80 (3.3)	68 (2.8)	53 (2.2)	233 (9.5)	**2447**
Culiseta morsitans	0 (0)	0 (0)	2 (66.7)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	**3**
Culiseta spp.	4 (16.0)	5 (20.0)	0 (0)	3 (12.0)	2 (8.0)	0 (0)	2 (8.0)	9 (36.0)	**25**
Ochlerotatus caspius/dorsalis	1 (10.0)	9 (90.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**10**
Ochlerotatus detritus	0 (0)	11 (61.1)	1 (5.6)	3 (16.7)	2 (11.1)	0 (0)	0 (0)	1 (5.6)	**18**
Ochlerotatus flavescens	0 (0)	130 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**130**
Damaged (not identifiable)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	**3**
Totals	**8285 (40.1)**	**8493 (41.1)**	**251 (1.2)**	**581 (2.8)**	**512 (2.5)**	**415 (2.0)**	**400 (1.9)**	**1729 (8.4)**	**20 666**

Table 6.1: mosquito species collected in the study in all trap locations and by all collection methods combined across 36 visits to Elmley in 2014.

Mosquito species	Resting boxes		Toilets		Barn		Chicken coops		Roof structure		Red feeder		MMP		Totals	
	total	*BF (%)*	*total*	*BF (%)*	*Total*	*BF (%)*	*total*	*BF (%)*	*total*	*BF (%)*	*total*	*BF (%)*	*total*	*BF (%)*	***total***	***BF (%)***
Anopheles claviger	0	-	0	-	1	1 (100)	0	-	0	-	0	-	2	0 (0)	**3**	**1 (33.3)**
Anopheles maculipennis s.l.	1809	95 (5.3)	776	166 (21.4)	12832	1329 (10.4)	131	73 (55.7)	21	1 (4.8)	59	7 (11.9)	25	0 (0)	**15653**	**1671 (10.7)**
Coquillettidia richiardii	106	9 (8.5)	2	0 (0)	1	0 (0)	0	-	0	-	0	-	193	1 (0.5)	**302**	**10 (3.3)**
Culex modestus	14	2 (14.3)	6	3 (50)	1	0 (0)	0	-	0	-	0	-	324	0 (0)	**345**	**5 (1.4)**
Culex pipiens s.l./torrentium	1038	85 (8.2)	72	13 (18.1)	567	11 (1.9)	0	-	35	1 (2.9)	1	0 (0)	13	0 (0)	**1726**	**110 (6.4)**
Culex spp.	1	0 (0)	0	-	0	-	0	-	0	-	0	-	0	-	**1**	**0 (0)**
Culiseta annulata	2104	279 (13.3)	33	7 (21.2)	267	57 (21.3)	0	-	2	2 (100)	1	0 (0)	40	1 (2.5)	**2447**	**346 (14.1)**
Culiseta morsitans	3	3 (100)	0	-	0	-	0	-	0	-	0	-	0	-	**3**	**3 (0)**
Culiseta spp.	25	7 (28.0)	0	-	0	-	0	-	0	-	0	-	0	-	**25**	**7 (28.0)**
Ochlerotatus caspius/dorsalis	2	0 (0)	1	0 (0)	0	-	0	-	0	-	0	-	7	0 (0)	**10**	**0 (0)**
Ochlerotatus detritus	5	5 (100)	0	-	1	1 (100)	0	-	1	0 (0)	0	-	11	0 (0)	**18**	**6 (33.3)**
Ochlerotatus flavescens	0	-	0	-	0	-	0	-	0	-	0	-	130	0 (0)	**130**	**0 (0)**
Totals	**5107**	**485 (9.5)**	**890**	**189 (21.2)**	**13670**	**1399 (10.2)**	**131**	**73 (55.7)**	**59**	**4 (6.8)**	**61**	**7 (11.5)**	**745**	**2 (0.3)**	**20663**	**2159 (10.4)**

Table 6.2: Summary of blood-fed mosquito species (% of total) collected in resting boxes, by aspiration from artificial structures and by Mosquito Magnet Pro + octenol (MMP). Damaged specimens are excluded from the table.

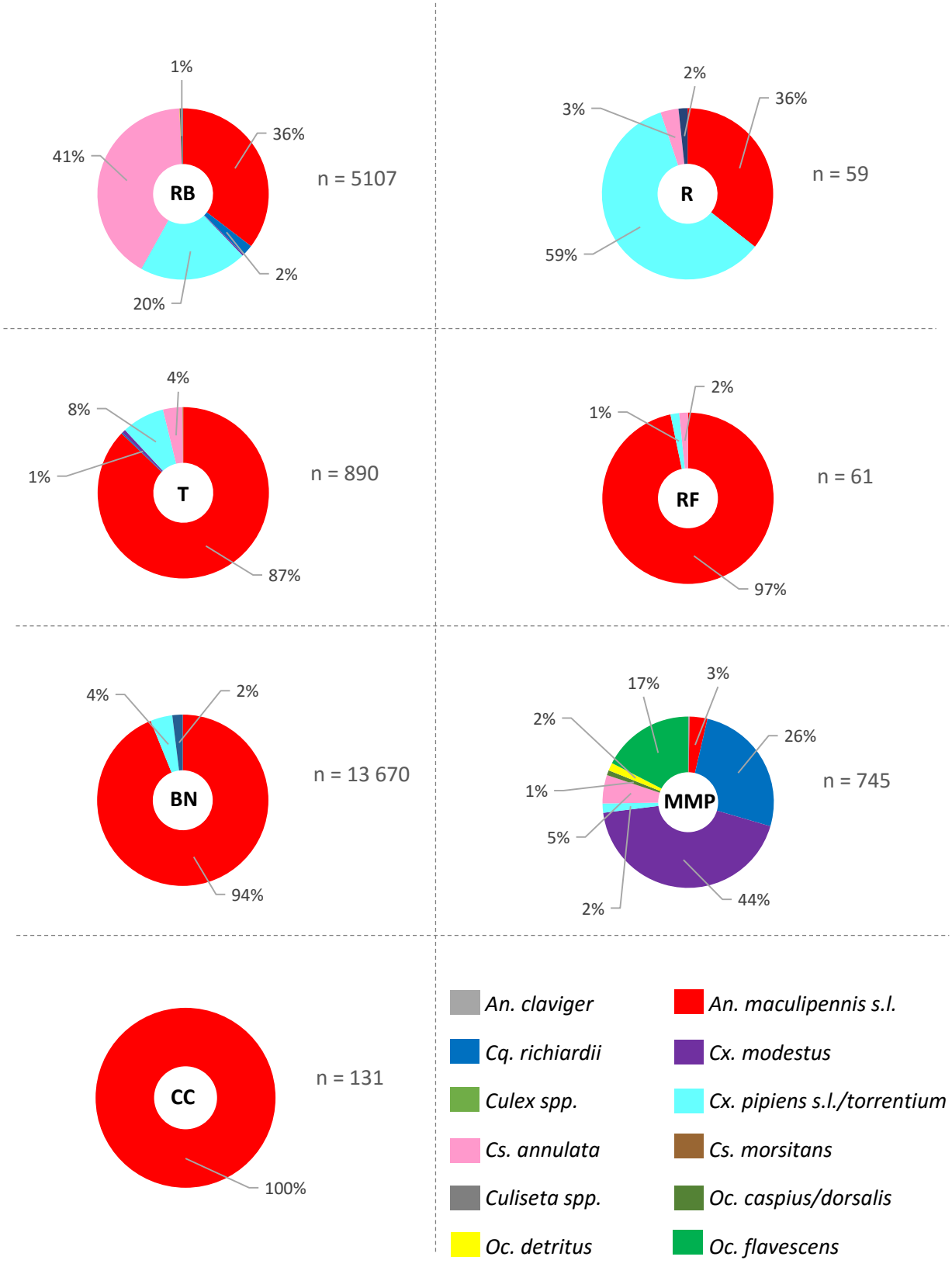

Figure 6.4: Pie charts showing the species assemblages collected from each resting site or trap type. RB = resting boxes, T = toilets, BN = barn, CC = chicken coops, R = roof structure, F = red feeder, MMP = Mosquito Magnet Pro. The total number represented by each chart is displayed to the right and a key bottom right of the page.

Resting box performance

Of the 5107 mosquitoes collected in the resting boxes, the morning collection yielded the greatest total number of mosquitoes of the three collection periods, 4089 (range 0 – 118), followed by the noon collection period, 724 (range 0 – 22), and the afternoon collection period, 294 (range 0 – 9) (Figure 6.5A). A similar trend was found for blood-fed female mosquitoes (stages II-VI), with the morning collection showing the greatest total numbers, 371 (range 0 – 47) then the noon collection period, 72 (range 0 – 10) and the afternoon collection period, 42 (range 0 – 6) (Figure 6.5B).

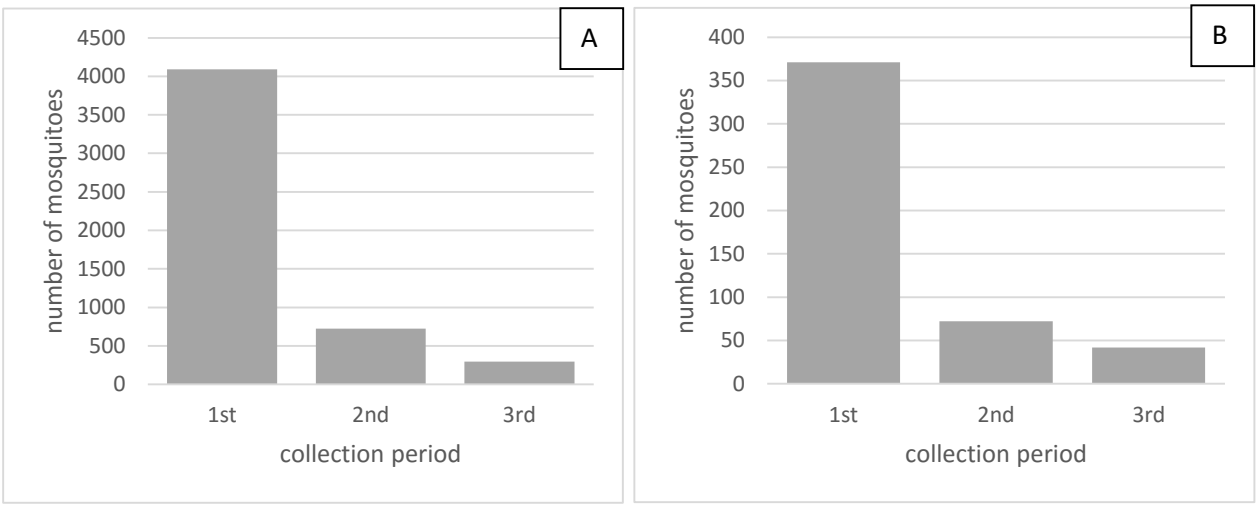

Figure 6.5: (A) Graph showing the total number of mosquitoes (all species) collected in each of the three collection periods and (B) the total number of blood-fed mosquitoes (all species) collected in each period.

The total number of mosquitoes collected from each resting box location varied. Combining the collections from the five resting boxes placed at each location, resting bxoes at site A collected the greatest total number of mosquitoes, 3488 (range 0 – 118), followed by site C, 1205 (range 0 – 57), site B, 248 (range 0 – 16) and site D, 166 (range 0 – 9) (Figure 6.6).

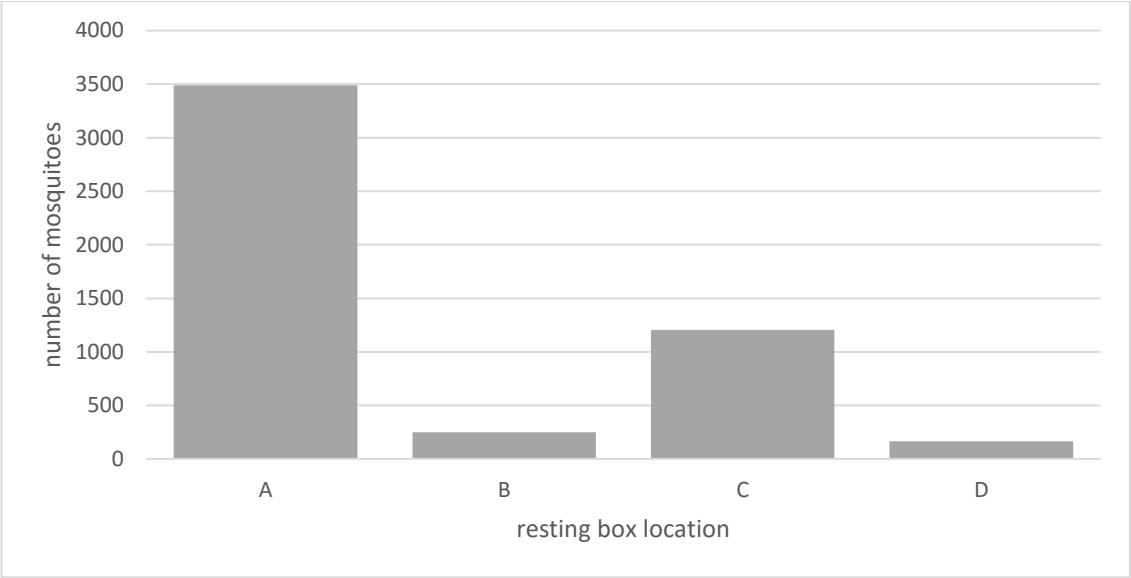

Figure 6.6: Graph showing the combined total number of mosquitoes collected from the resting boxes placed at each of the four locations on site in the study.

The relative abundance of mosquito species over the collection period (June-October) in the resting boxes showed some seasonal variation. Although *An. maculipennis* s.l. and *Cs. annulata* dominated the species profiles until late August, *Cx. pipiens s.l./torrentium* became more prominent in collections during this period, representing approximately a third to a half of the collected individuals until collections were completed (Figure 6.7). Other mosquito species represented a very minor proportion of individuals collected over the study with *Cq. richiardii* the only species present consistently in collections until late August, after which abundance fell. Three species sequentially dominated the MMP collections: (1) *Oc. flavescens*, at its peak in June and declining sharply into July, (2) *Cq. richiardii*, at its peak in July and declining into August, and (3) *Cx. modestus*, representing the majority of collections from August until mid-September. The species profiles collected in resting boxes did not closely resemble those collected in the MMP (Figure 6.7).

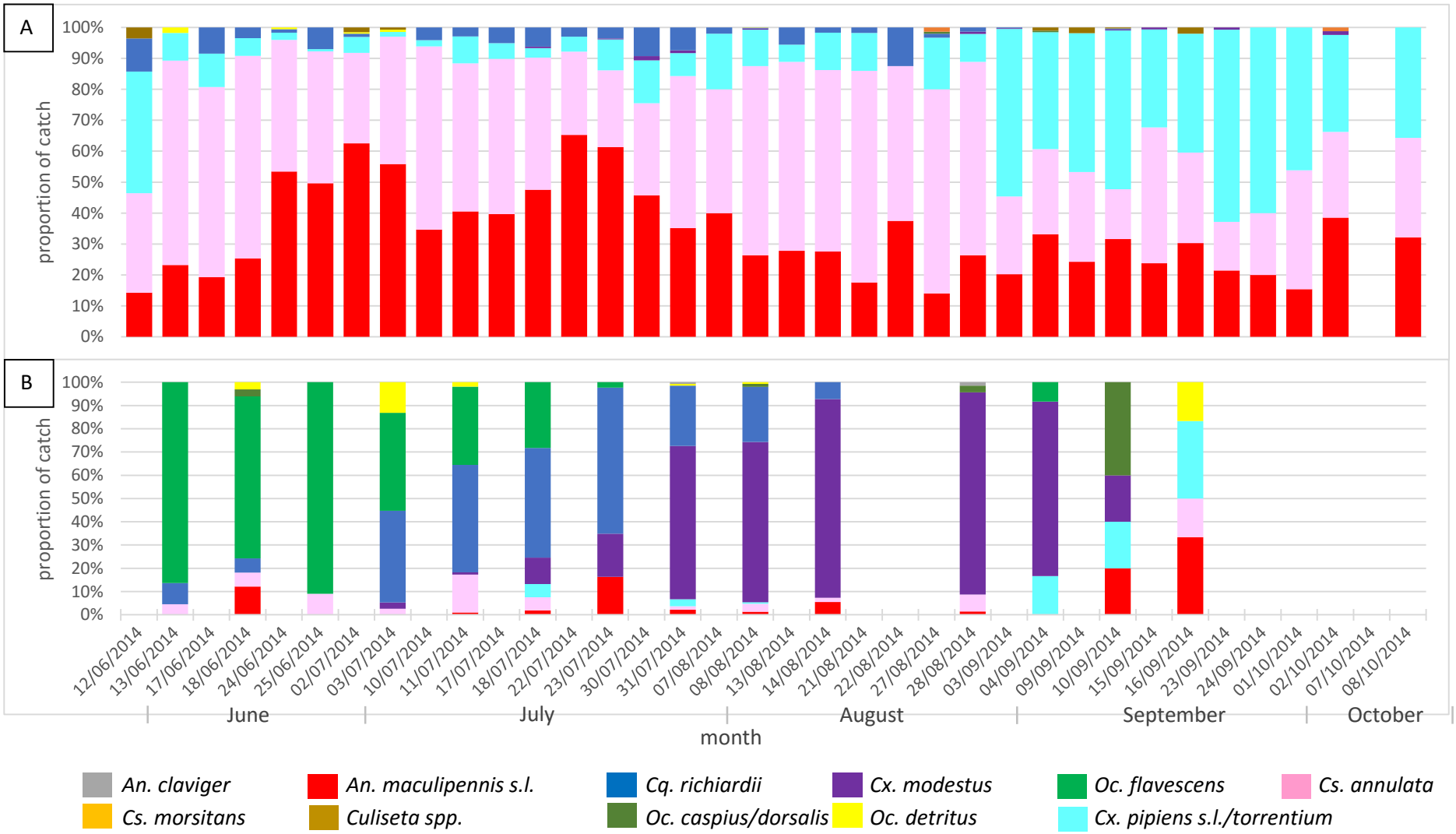

Figure 6.7: (A) Bar charts showing seasonal variation in the relative abundance of mosquito species collected between June – October in resting boxes and (B) in the Mosquito Magnet Pro (MMP). MMP collections were run overnight between two consecutive days of resting box collections.

Generalized linear mixed model results

Blood-fed mosquitoes in resting traps

Four GLMMs were fit: the first using data for the combined total of all blood-fed mosquito species collected in the resting boxes and then subsequently using data for each of the three most abundant species in the collections: *An. maculipennis* s.l., *Cs. annulata* and *Cx. pipiens s.l./torrentium*. All models included two fixed effects (resting box location (A – D) and rainfall (mm)), one random variable (date) and three continuous covariates (*temperature*, *relative humidity* and *wind speed*). The initial Poisson model for all mosquito species was 19 AIC units higher than the negative binomial model (1024 > 1005) indicating the latter was a better fit to the data (Table 6.3) (Burnham & Anderson 2002).

The covariates *wind speed* and *temperature* significantly influenced the number of blood-fed mosquitoes collected (Table 6.3). Over the night preceding the morning collection period, an increase of 1 m/s average wind speed would be expected to lead to an estimated 51% fewer blood-feds in the resting boxes ($P \leq 0.01$) and a 1°C increase in temperature would lead to an expected 127% more mosquitoes collected ($P \leq 0.001$). Multiple comparisons between resting box locations (A – D) showed a significant difference ($P \leq 0.001$) in the number of blood-fed mosquitoes collected from resting boxes in each of the four locations, with the exception of locations B and D which did not differ significantly (Table 6.4).

Coefficients	Estimate (95% CI)	Predicted % difference	Std. Error
(Intercept)	-3.149 (-8.31; 2.01)	-	2.63170
location B	-2.849 (-3.39; -2.31) ***	-5.79	0.27429
location C	-1.431 (-1.75; -1.11) ***	-23.92	0.16409
location D	-3.415 (-4.11; -2.73) ***	-3.29	0.35207
rainfall	-0.379 (-1.07; 0.31)	-68.48	0.35050
wind speed	-0.679 (-1.10; -0.25) **	-50.73	0.21707
temperature	0.243 (0.13; 0.36) ***	127.47	0.05945
relative humidity	0.006 (-0.05; 0.06)	100.61	0.02752

Table 6.3: Regression coefficients, plus Wald 95% confidence intervals and standard errors, for the final negative binomial GLMM, for all blood-fed mosquito species. Predicted % difference is the (exponent x 100) of the value in the estimate column and gives the estimated change in blood-fed numbers collected depending on the box location compared to resting box location A as a baseline, or for a one-unit increase in meteorological variables. *** $P \leq 0.001$, $P \leq$ ** 0.01.

Linear hypotheses: Resting box comparison	Estimate	Predicted % difference	Std. Error
B - A == 0	-2.849 ***	-5.79	0.2743
C - A == 0	-1.431 ***	-23.92	0.1641
D - A == 0	-3.415 ***	-3.29	0.3521
C - B == 0	1.418 ***	412.93	0.2869
D - B == 0	-0.566	-56.76	0.5174
D - C == 0	-1.985 ***	-13.74	0.4799

Table 6.4: Multiple Tukey's comparisons between the numbers of blood-feds collected in each resting box location for all species. Predicted % difference is the (exponent x 100) of the value in the estimate column and gives the estimated change of the catch between two resting boxes. *** $P \leq 0.001$.

Anopheles maculipennis s.l.

A Poisson GLMM (Table 6.5) was the best fit to the data, with an AIC of 421. This was lower than the value for the negative binomial alternative (423), although a difference of 2 AIC units is considered insufficient to entirely rule out the model (Burnham & Anderson 2002). As with the GLMM fit to all blood-fed mosquitoes, resting box location influenced the number of mosquitoes collected, although the only significant difference was between resting box location A and the other locations ($P \leq 0.001$, Table 6.6). Of the meteorological variables included in the model, only temperature was found to be a significant predictor of the catch of this species, with a 1°C increase in temperature predicted to lead to a 137% increase in the number of blood-feds collected ($P \leq 0.001$).

Coefficients:	**Estimates (95% CI)**	**Predicted % difference**	**Std. Error**
(Intercept)	-5.666 (-13.87; 2.54)	-	4.184600
location B	-1.629 (-2.31; -0.95) ***	-19.61	0.345840
location C	-1.447 (-2.08; -0.82) ***	-23.53	0.320840
location D	-2.322 (-3.24; -1.40) ***	-9.80	0.468620
rainfall	-0.720 (-1.86; 0.42)	-48.70	0.579990
wind speed	-0.614 (-1.33; 0.10)	-54.09	0.362640
temperature	0.316 (0.13; 0.50) ***	137.13	0.095798
relative humidity	0.0003 (-0.08; 0.089)	100.03	0.043471

Table 6.5: Regression coefficients, with Wald 95% confidence intervals and standard errors, for the final Poisson GLMM for *An. maculipennis* s.l. Predicted % difference is the exponent (x 100) of the value in the estimate column and gives the estimated change in blood-fed numbers collected depending on the box location compared to resting box location A as a baseline, or for a one-unit increase in meteorological variables. *** $P \leq 0.001$.

Linear hypotheses: Resting box comparison	Estimate	Predicted % difference	Std. Error
B - A == 0	-1.629 ***	-19.61	0.3458
C - A == 0	-1.447 ***	-23.53	0.3208
D - A == 0	-2.322 ***	-9.80	0.4686
C - B == 0	0.182	120.00	0.4562
D - B == 0	-0.693	-50.00	0.6730
D - C == 0	-0.876	-41.67	0.6915

Table 6.6: Multiple Tukey's comparisons of the differences in the catch of *An. maculipennis* s.l. between resting box locations. Predicted % difference is the exponent (x 100) of the value in the estimate column and gives the estimated change of the catch between two resting boxes. *** $P \leq 0.001$.

Culiseta annulata

A negative binomial model (Table 6.7) was the best fit to the data for *Cs. annulata* (AIC 697 < AIC 720 for Poisson GLMM). Resting box location once again influenced the number of mosquitoes collected (Table 6.8). Significantly more blood-fed mosquitoes were collected at location A ($P \leq 0.001$) than from all other locations; more blood-fed mosquitoes were collected at location C than B ($P \leq 0.001$) and D ($P \leq 0.01$) (Table 6.8). No significant difference in the abundance of blood-fed mosquitoes collected was found between locations B and D. Of the recorded meteorological variables, only wind speed and temperature were significant predictors of the catch of this species. An increase of 1 m/s in wind speed would be expected to lead to a 52% decrease in the number of blood-fed *Cs. annulata* collected ($P \leq 0.05$), whilst a 1°C increase in temperature would be predicted to lead to a 130% increase ($P \leq 0.001$).

Coefficients:	Estimate (95% CI)	Predicted % difference	Std. Error
(Intercept)	-4.026 (-9.96; 1.91)	-	3.02930
location B	-5.120 (-7.11; -3.13) ***	-0.60	1.01290
location C	-1.386 (-1.81; -0.96) ***	-25.01	0.21543
location D	-3.717 (-4.74; -2.69) ***	-2.43	0.52450
rainfall	-0.330 (-1.13; 0.47)	-71.92	0.40727
wind speed	-0.657 (-1.16; -0.15) *	-51.82	0.25667
temperature	0.265 (0.12; 0.40) ***	130.28	0.07147
relative humidity	0.006 (-0.06; 0.07)	100.56	0.03197

Table 6.7: Regression coefficients, plus Wald 95% confidence intervals and standard errors, for the negative binomial GLMM selected for *Cs. annulata*. Predicted % difference is the exponent x (100) of the value in the estimate column and gives the estimated change in blood-fed numbers collected depending on the box location compared to resting box location A as a baseline, or for a one-unit increase in meteorological variables. *** $P \leq 0.001$, * $P \leq 0.05$.

Linear hypotheses: Resting box comparison	Estimate	Predicted % difference	Std. Error
B - A == 0	-5.130 ***	-0.59	1.0030
C - A == 0	-1.346 ***	-26.04	0.1693
D - A == 0	-3.744 ***	-2.37	0.5059
C - B == 0	3.784 ***	4400.05	0.9974
D - B == 0	1.386	400.00	1.1928
D - C == 0	-2.398 **	9.09	0.6518

Table 6.8: Multiple Tukey's comparisons of *Cs. annulata* in resting box locations. Predicted % difference is the exponent (x 100) of the value in the estimate column and gives the estimated change of the catch between two resting boxes. *** $P \leq 0.001$, *** $P \leq 0.01$.

Culex pipiens s.l.

A Poisson GLMM (Table 6.9) was the best fit to the data, with an AIC of 362. This was lower than the value for the negative binomial alternative (364), although a difference of 2 AIC units is considered insufficient to rule out the alternative model (Burnham and Anderson 2002). Resting box location had less of a detected effect on the number of blood-fed *Cx. pipiens* s.l. collected than for other species, with significant differences in abundance found only between locations A and B and between A and C ($P \leq 0.001$, Table 6.10). None of the recorded meteorological variables were significant predictors of the number of blood-fed *Cx. pipiens* s.l. collected.

Coefficients:	**Estimate (95% CI)**	**Predicted % difference**	**Std. Error**
(Intercept)	-4.767 (-13.28; 3.74)	-	4.3446
location B	-2.197 (-3.12; -1.27) ***	-11.11	0.4714
location C	-1.242 (-1.86; -0.62) ***	-28.89	0.3149
location D	-11.562 (-106.25; 83.13)	-0.001	48.3120
rainfall	-0.187 (-1.29; 0.92)	-82.98	0.5664
wind speed	-0.611 (-1.29; 0.07)	-54.30	0.3452
temperature	0.101 (-0.08; 0.28)	110.58	0.0933
relative humidity	0.025 (-0.06; 0.11)	102.48	0.0456

Table 6.9: Regression coefficients, plus Wald 95% confidence intervals and standard errors, for the Poisson GLMM selected for *Cx. pipiens* s.l. Predicted % difference is the exponent (x 100) of the value in the estimate column and gives the estimated change in blood-fed numbers collected depending on the box location compared to resting box location A as a baseline, or for a one-unit increase in meteorological variables *** $P \leq 0.001$.

Linear hypotheses: Resting box comparison	Estimate	Predicted % difference	Std. Error
B – A == 0	-2.197 ***	-11.11	0.4714
C – A == 0	-1.242 ***	-28.89	0.3149
D – A == 0	-11.562	-0.001	48.3120
C – B == 0	0.956	260.05	0.1570
D – B == 0	-9.365	-0.009	48.7833
D – C == 0	-10.320	-0.003	48.6268

Table 6.10: Multiple Tukey's comparisons of differences between catch of *Cx. pipiens* s.l. in resting box locations. Predicted % difference is the exponent (x 100) of the value in the estimate column and gives the estimated change of the catch between two resting boxes. *** $P \leq 0.001$.

Molecular identification of mosquitoes

Only those mosquitoes containing blood (Sella stages II-VI) were identified to species level using molecular methods. All tested *Cx. pipiens s.l./torrentium* were identified as being *Cx. pipiens f. pipiens*. A total of 149 *An. maculipennis* s.l. were randomly selected from the total number collected for species-level identification of which 83 were identified as *An. atroparvus* and 66 as *An. daciae/messeae*. The sequence could not be used to discriminate *An. daciae/messeae* (identical BLAST results) and these species are thus presented as a species complex.

Vertebrate blood meal host results summary

Of the 2159 mosquitoes identified as being blood-fed, 1330 were selected for blood meal analysis to identify the blood meal host. As *An. maculipennis* s.l. dominated the collections, those specimens collected from the resting boxes and other artificial structures were tested together with a randomised subset (~10%) from each collection date in the barn (as described in section 6.2.6). Randomisation was performed by assigning each sample cell a random number using the 'rand' function in Excel, arranging cells in ascending order and selecting the first 10%

of specimens. Every specimen of all other mosquito species were processed to identify blood meal origin.

In total, 964 mosquitoes were successfully analysed for blood meal host (Table 6.11), of which the majority (709) were *An. maculipennis* s.l., *Cs. annulata* (204) and *Cx. pipiens f. pipiens* (100). The remaining species were represented by fewer than ten specimens each. Nineteen hosts, including five mammals and fourteen birds, were identified as blood meal hosts. Two of the bird species, the barn swallow (*Hirundo rustica* L.), fed on by *Cx. pipiens f. pipiens* and *Cx. modestus*, and yellow wagtail (*Motacilla flava* (L.)), fed on only by *Cx. pipiens f. pipiens* are summer migrants to the UK; the remaining 12 birds are resident species.

Nine hosts were fed upon by *An. maculipennis* s.l., predominantly cow (*Bos taurus*), 344/708 (49%) and rabbit (*O. cuniculus*), 228/708 (32%). Feeding was also confirmed on hare (*L. europaeus*) and brown rat (*Rattus norvegicus* Berkenhout 1769) and three species of bird, primarily chickens (*Gallus gallus*) 60/708 (8%). Cattle were also the main blood-feeding host for *Cs. annulata*, 187/204 (92%), with no feeding detected on birds. In contrast, *Cx. pipiens f. pipiens* was found to have fed exclusively on birds, with blood meals taken from 13 different avian hosts. Three host species accounted for the majority of blood meals detected in *Cx. pipiens f. pipiens*: wood pigeon (*Columba palumbus* L.), (43%); blackbird (*Turdus merula* L.), (20%) and house sparrow (*Passer domesticus* L.), 12/97 (12%). *Culex modestus* was also found to have fed only on avian species with barn swallow (75%) and mute swan (*Cygnus olor* Gmelin 1789) (25%) accounting for the four samples successfully identified. *Anopheles atroparvus* was found to have fed on seven vertebrate hosts including two bird species (chicken and stock dove *Columba oenas* L.) and five mammalian species (brown rat, hare, cow, rabbit and sheep *Ovis aries* L.). *Anopheles daciae*/*messeae* fed on five species including two birds (chicken and barn owl (*Tyto alba guttata* Scopoli, 1769) and three mammalian species (cow, hare and sheep) (Table 6.12). Table 6.13 provides common and Latin names for all species identified in this study.

Mosquito species	Vertebrate blood meal hosts																			
	Mammals					Birds													Sequencing failed	Totals
	Brown rat	Cow	European hare	European rabbit	Sheep	Blackbird	Barn swallow	Chicken	Dark-breasted barn owl	Eurasian skylark	European starling	Grey heron	House sparrow	Long-eared owl	Meadow pipit	Mute swan	Stock dove	Wood pigeon	Yellow wagtail	
An. claviger	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
%	-	*100*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1																				
An. maculipennis s.l.	2	344	2	228	6	0	0	60	1	0	0	0	0	0	0	0	10	0	0	55
%	*<1*	*49*	*<1*	*32*	*1*	-	-	*8*	*<1*	-	-	-	-	-	-	-	*1*	-	-	*8*
708																				
Cq. richiardii	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
%	-	*100*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3																				
Cx. modestus	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	1	0	0	0	0
%	-	-	-	-	-	-	*75*	-	-	-	-	-	-	-	-	*25*	-	-	-	-
4																				
Cx. pipiens f. pipiens	0	0	0	0	0	19	9	1	3	2	1	3	12	1	2	0	1	42	1	3
%	-	-	-	-	-	*19*	*9*	*1*	*3*	*2*	*1*	*3*	*12*	*1*	*2*	-	*1*	*42*	*1*	*3*
100																				

Table 6.11: Vertebrate blood meal hosts of mosquito species collected at Elmley by all collection methods.

Mosquito species	Vertebrate blood meal hosts																				Totals
	Mammals					Birds														Sequencing failed	
	Brown rat	Cow	European hare	European rabbit	Sheep	Blackbird	Barn swallow	Chicken	Dark-breasted barn owl	Eurasian skylark	European starling	Grey heron	House sparrow	Long-eared owl	Meadow pipit	Mute swan	Stock dove	Wood pigeon	Yellow wagtail		
Cs. annulata	0	187	0	4	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	**204**
%	-	*92*	-	*2*	*3*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*4*	
Cs. morsitans	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	**3**
%	-	-	-	-	-	*67*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Oc. detritus	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**
%	-	*100*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Culiseta spp.	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	**4**
%	-	*25*	-	-	-	*25*	-	-	-	-	-	-	*25*	-	-	-	-	*25*	-	-	
unknown (damaged)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	**1**
Total	2	538	2	232	11	22	12	61	4	2	1	3	14	1	2	1	11	44	1	66	**1030**

Table 6.11 (continued). Vertebrate blood meal hosts of mosquito species collected at Elmley by all collection methods.

Row Labels	barn owl	brown rat	chicken	cow	hare	rabbit	sheep	stock dove	Total
An. atroparvus	0	2	16	30	1	22	2	10	**83**
An. daciae/messeae	1	0	23	37	1	0	4	0	**66**
Total	**1**	**2**	**39**	**67**	**2**	**22**	**6**	**10**	**149**

Table 6.12: Comparison of vertebrate blood-feeding hosts selected by a subset of *Anopheles maculipennis* s.l. identified by molecular methods as *An. atroparvus* or *An. daciae/messeae.*

Common name	**Family**	***Genus species* (*subspecies*)**
Brown rat	*Muridae*	*Rattus norvegicus*
Cow	*Bovidae*	*Bos taurus*
European hare	*Leporidae*	*Lepus europaeus*
European rabbit	*Leporidae*	*Oryctolagus cuniculus*
Sheep	*Bovidae*	*Ovis aries*
Blackbird	*Turdidae*	*Turdus merula*
Barn swallow	*Hirundinidae*	*Hirundo rustica*
Chicken	*Phasianidae*	*Gallus gallus*
Dark-breasted barn owl	*Tytonidae*	*Tyto alba gutatta*
Eurasian skylark	*Alaudidae*	*Alauda arvensis*
European starling	*Sturnidae*	*Sturnus vulgaris*
Grey heron	*Ardeidae*	*Ardea cinerea*
House sparrow	*Passeridae*	*Passer domesticus*
Long-eared owl	*Strigidae*	*Asio otus*
Meadow pipit	*Motacillidae*	*Anthus pratensis*
Mute swan	*Anatidae*	*Cygnus olor*
Stock dove	*Columbidae*	*Columba oenas*
Wood pigeon	*Columbidae*	*Columba palumbus*
Yellow wagtail	*Motacillidae*	*Motacilla flava*

Table 6.13: Common and Latin names for vertebrate blood meal hosts identified in this study.

Blood meal hosts by trap and location

Figure 6.8 presents photographs of some of the vertebrate hosts identified in various trap locations during the study. Comparing the hosts of *An. maculipennis* s.l. (Figure 6.9) and *Cx. pipiens f. pipiens* (Figure 6.10) to the locations/traps in which they were collected demonstrates evidence of location-specific feeding activity. The vast majority (98%) of *An. maculipennis* s.l. collected from the chicken coops had fed on chickens. Blood meals originating from chickens, however, formed a very small proportion of the total identifications in *An. maculipennis* s.l. in the other artificial sites (toilets, resting boxes A and C) and were not found in traps situated further away from the chicken coop, indicating opportunistic feeding. Similarly, both *An. maculipennis* s.l. and *Cx. pipiens f. pipiens* collected from the barn had fed on stock dove, where nests of this species were recorded, and blood meals from this host were not detected from any other collection area. *Culex pipiens f. pipiens* blood-fed on chickens were only collected from the toilets (chickens were observed in here, Figure 6.8) whilst feeds on barn swallow were only detected from the barn and toilets where nestling barn swallows were observed during the collection periods. Feeds from small passerine birds like blackbirds and house sparrows were detected only from resting boxes and toilets, in close proximity to the central woodland strip where they were observed during the bird survey.

Figure 6.8: Clockwise from top left: (1) chickens in toilet block, (2) barn swallow nest in toilet block, (3) nesting box utilised by dove/pigeons or barn owls in the barn, (4) resting *An. maculipennis* s.l. inside chicken coop, (5) nestling dove/pigeon in barn, (6) barn owl on

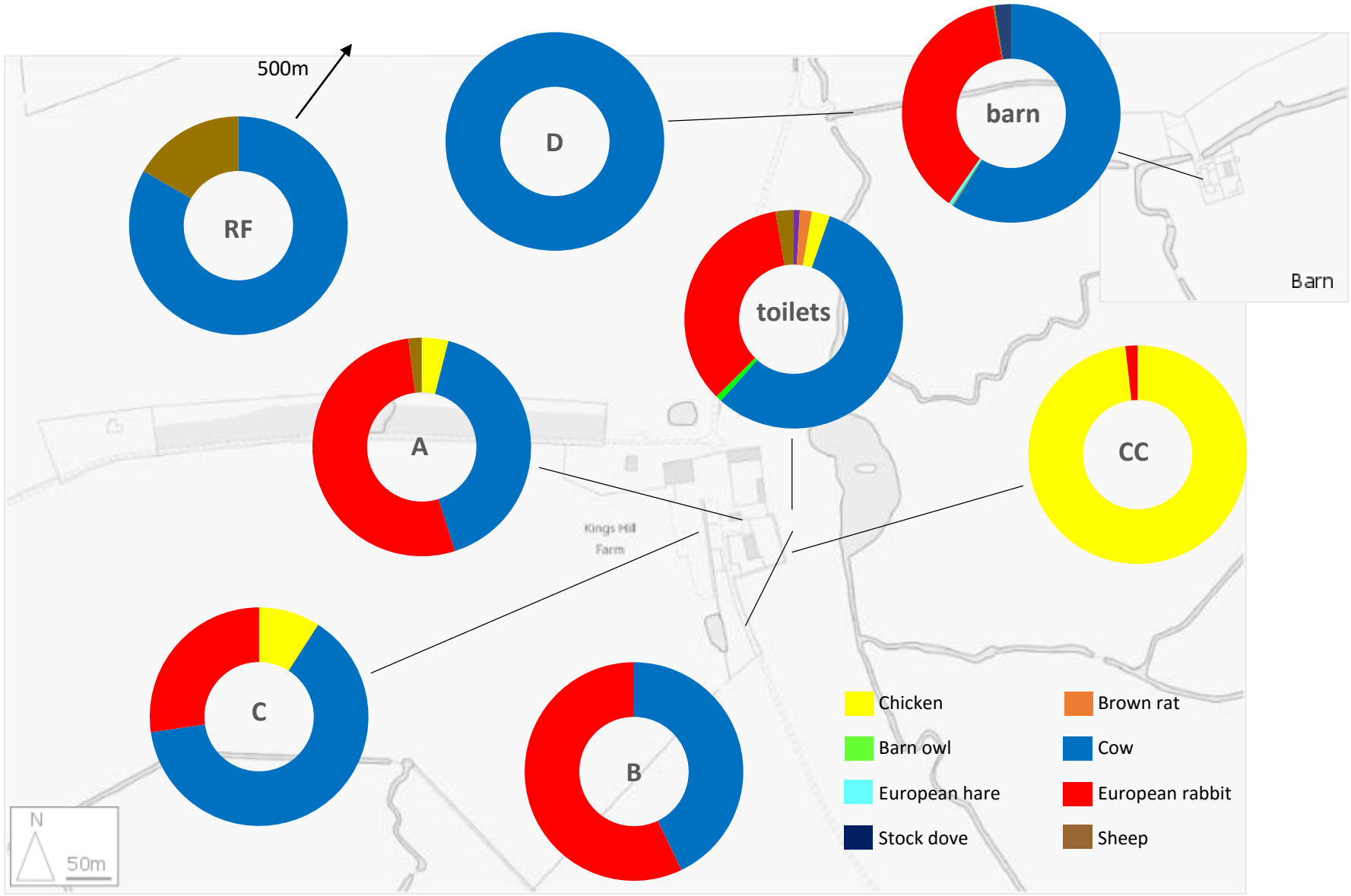

Figure 6.9: Vertebrate blood meal origin of *Anopheles maculipennis* s.l. according to site of collection: resting boxes A, B, C D; red feeder (RF); chicken coops (CC), toilets and barn. Key to colours is at bottom right.

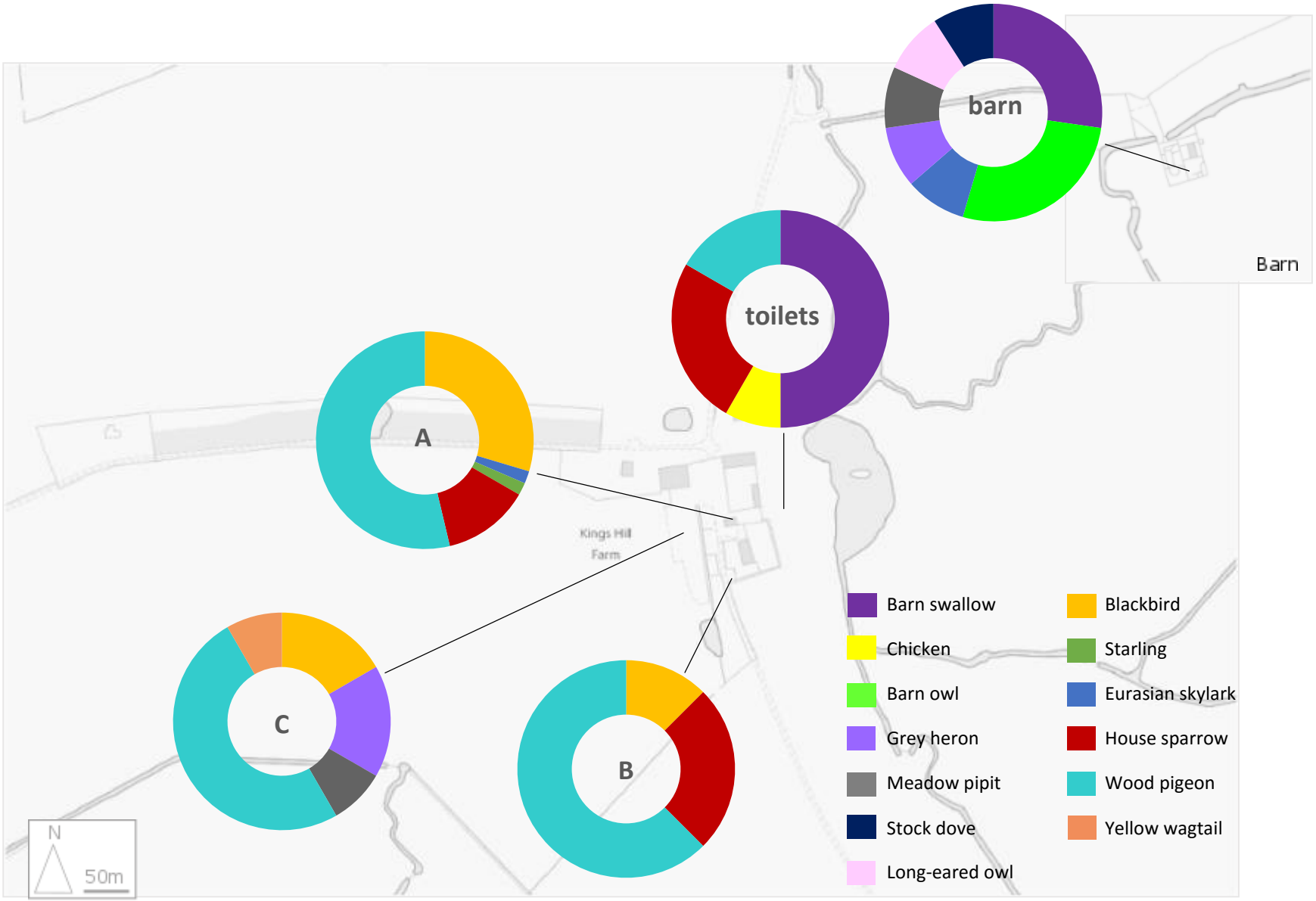

Figure 6.10: Vertebrate blood meal origin of *Culex pipiens f. pipiens* according to site of collection: resting boxes A, B and C; chicken coops (CC), toilets and barn. Key to colours is at bottom right.

Host abundance and feeding index results

Host abundance data on a total of 36 different vertebrate hosts was obtained, comprising five mammal and 31 wild bird species (Table 6.14). Rabbits were found to be in greatest number close to the barn and close to the central woodland strip, the latter of which had a visible warren present. The feeding index (FI) was calculated for four mosquito species, *An. atroparvus, An. messeae/daciae*, *Cs. annulata* and *Cx. pipiens f. pipiens*; the numbers of other species collected were not considered sufficient for analysis. For the purposes of calculating feeding indices (Kay et al. 1979), total bird numbers recorded over all survey points and the average number of rabbits and hares observed over the evening and morning collections was used to represent host abundances. Host abundance data was weighted using an approximation of the relative size of hosts. A cow was considered to be 20X the size of a rabbit, hare, chicken and 'pigeon' (including both the wood pigeon and stock dove owing to difficulty distinguishing these in the survey). Among the birds, species were considered to be in four size categories relative to the smallest category, containing barn swallow, house sparrow and yellow wagtail; (2X size) blackbird, European starling (*Sturnus vulgaris* L.), (3X) chicken, dark-breasted barn owl, pigeon and (4X) grey heron (*Ardea cinerea* L.). As no information on feeding success of mosquitoes on different hosts was available this optional corrective value (Kay et al. 1979) was omitted from the calculation.

Species common name	Latin name	Number observed
Barn swallow	*Hirundo rustica*	62
Blackbird	*Turdus merula*	4
Buzzard	*Falco tinnunculus*	1
Chicken	*Gallus gallus*	20
Coot	*Fulica atra*	9
Cormorant	*Phalacrocorax carbo*	1
Cow	*Bos taurus*	30
Crow	*Corvus spp.*	4
Dark breasted barn owl	*Tyto alba guttata*	6
Goldfinch	*Carduelis carduelis*	44
Great crested grebe	*Podiceps cristatus*	2
Grey heron	*Ardea cinerea*	4
Hare	*Lepus europaeus*	4
Herring gull	*Larus argentatus*	2
House sparrow	*Passer domesticus*	40
Human	*Homo sapiens*	5
Kestrel	*Falco tinnunculus*	2
Linnet	*Carduelis cannabina*	1
Little egret	*Egretta garzetta*	2
Little grebe	*Tachybaptus ruficollis*	2
Mallard	*Anas platyrhynchos*	16
Marsh harrier	*Circus aeruginosus*	8
Meadow pipit	*Anthus pratensis*	2
Moorhen	*Gallinula chloropus*	1
Mute swan	*Cygnus olor*	8
Peregrine falcon	*Falco peregrinus*	1
Pheasant	*Phasianus colchicus*	9
Pied wagtail	*Motacilla alba*	5
Pigeon/dove	*Columba spp.*	6
Rabbit	*Oryctolagus cuniculus*	28
Robin	*Erithacus rubecula*	1
Sandpiper	*Tringa spp.*	2
Sparrowhawk	*Accipiter nisus*	1
Starling	*Sturnus vulgaris*	227
Wheatear	*Oenanthe oenanthe*	2
Yellow wagtail	*Motacilla flava*	4

Table 6.14: Host abundance data collected by survey at Elmley, 2014.

Culiseta annulata showed a distinct feeding preference for cows over rabbits ($FI_{cow:rabbit}$ = 2.3). Despite being the most numerous blood-feeding host for *An. atroparvus*, cattle were considered to be less attractive for feeding than the other four species, with a FI of more than one for all comparable species (Table 6.15). Stock doves were considered to be more attractive for feeding of *An. atroparvus*. Chickens were the preferred host for *An. daciae/messeae*, with cattle once again considered unfavourable hosts for feeding (Table 6.16). Bird abundance estimates were only available for nine of the thirteen bird species on which *Cx. pipiens f. pipiens* was found to feed and thus feeding indices were only calculated for these (Table 6.17). Blackbirds were considered to be consistently the most favoured blood-feeding host in comparison to all the other bird species with FIs >1. Chickens were only the preferred host when compared to the European starling.

	Cow	rabbit	hare	chicken	stock dove
Cow	X	0.06	0.20	0.06	0.05
Rabbit	10.43	X	3.14	0.98	0.79
Hare	4.62	0.32	X	0.31	0.25
Chicken	17.67	1.02	3.20	X	0.80
Stock dove	19.41	1.30	4.00	1.25	X

Table 6.15: Host feeding indices for *Anopheles atroparvus*, given to 2 decimal places. Values >1 indicate a preference for a host, values <1 indicate avoidance of host.

	cow	hare	chicken	barn owl
Cow	X	0.24	0.05	0.37
Hare	3.86	X	0.22	1.49
Chicken	20.72	4.6	X	6.91
Barn owl	2.70	0.67	0.13	X

Table 6.16: Host feeding indices for *Anopheles daciae/messeae*, given to 2 decimal places. Values >1 indicate a preference for a host, values <1 indicate avoidance of host.

	Grey heron	DB Barn owl	Chicken	Pigeon	Blackbird	European starling	Barn swallow	House sparrow	Yellow wagtail
Grey heron	X	0.75	7.5	0.03	0.04	42.9	0.85	0.42	0.50
DB Barn owl	1.33	X	10	0.12	0.05	56.6	0.86	0.42	0.50
Chicken	0.13	0.10	X	0.01	0.01	5.68	0.09	0.04	0.05
Pigeon	34.4	8.58	86.0	X	0.45	488.6	7.41	3.58	4.30
Blackbird	25.0	19.2	190	2.2	X	1056	16.37	7.92	9.50
European starling	0.02	0.02	0.18	0.002	0.0002	X	0.02	0.01	0.01
Barn swallow	1.16	1.16	11.6	0.14	0.06	64.29	X	0.48	0.58
House sparrow	2.40	2.40	24.0	0.28	0.13	136.4	2.07	X	1.20
Yellow wagtail	1.94	1.98	20.0	0.23	0.12	113.7	1.72	0.83	X

Table 6.17: Host feeding indices for *Culex pipiens f. pipiens*. Values >1 indicate a preference for a host, values <1 indicate avoidance of host. DB Barn owl = dark-breasted Barn owl

Sella Stage and likelihood of blood meal identification

The identity of vertebrate blood meals in mosquitoes was successfully identified in 72% of individuals (Table 6.18), a rate of 77% at the amplification stage (i.e. observing a band of the correct size (~685 bp)) and a sequencing success rate of 93% (i.e. vertebrate host identified). Comparing *An. maculipennis* s.l., *Cs. annulata* and *Cx. pipiens* s.l., there was a general trend of a slightly decreasing likelihood of successful identification moving from Sella stage II to stage VI, although, with the exception of *Cx. pipiens* s.l., the greatest drop in success rate was between stages V and VI. *Culex pipiens* s.l. had the highest overall success rates of the three species with stages II-VI all over 94%. As compared to freshly blood-fed specimens (Sella stage II), only mosquitoes of stage V (odds ratio (OR) = 0.19, $P \leq 0.01$) and VI (OR = 0.04, $P \leq 0.001$) had a significantly reduced odds of obtaining a successful blood meal identification (Table 6.19). Success rates of sequencing were generally high (>84%) for all three species except at Sella stage VI where the rate dropped sharply. Sequencing failures most commonly consisted of detections of mosquito DNA, with results less frequently returning BLAST results pertaining to bacterial, fungal or rarely, a poor-quality vertebrate sequence.

Processing stages	**All species**	***An. maculipennis* s.l.**					***Culiseta annulata***					***Culex pipiens* s.l.**				
	all stages	*II*	*III*	*IV*	*V*	*VI*	*II*	*III*	*IV*	*V*	*VI*	*II*	*III*	*IV*	*V*	*VI*
Total mosquitoes tested	1341	340	1245	444	405	486	62	342	324	340	312	84	114	64	10	54
PCR positive	1034	306	1044	368	280	372	50	204	204	185	144	82	108	60	10	36
PCR success rate	**0.77**	**0.90**	**0.84**	**0.83**	**0.69**	**0.77**	**0.81**	**0.60**	**0.63**	**0.54**	**0.46**	**0.98**	**0.95**	**0.94**	**1.00**	**0.67**
Successful blood meal ID	964	302	1005	352	235	192	44	201	204	175	126	80	108	60	10	24
Sequencing success rate	**0.93**	**0.99**	**0.96**	**0.96**	**0.84**	**0.52**	**0.88**	**0.99**	**1.00**	**0.95**	**0.88**	**0.98**	**1.00**	**1.00**	**1.00**	**0.67**
Final success rate	**0.72**	**0.89**	**0.81**	**0.80**	**0.58**	**0.40**	**0.71**	**0.63**	**0.63**	**0.51**	**0.40**	**0.96**	**0.95**	**0.94**	**1.00**	**0.45**
%	**72**	**89**	**81**	**80**	**58**	**40**	**71**	**63**	**63**	**51**	**40**	**96**	**95**	**94**	**100**	**45**

Table 6.18: The probability of successful blood meal identification for mosquitoes classified according to stage of blood meal digestion (Sella Scale II-VI).

Coefficients:	**Estimate (95% CI)**	**Odds Ratio**	**Std. Error**
(Intercept)	3.975 (2.94; 5.01) ***	-	0.529633
stage 3	-0.095 (-1.07; 0.87)	0.91	0.494845
stage 4	0.002 (-1.28; 1.28)	1.00	0.654019
stage 5	-1.659 (-2.69; -0.62) **	0.19	0.528356
stage 6	-3.184 (-4.11; -2.26) ***	0.04	0.470784

Table 6.19: Binomial GLMM regression coefficients, with 95% Wald confidence intervals and standard error, for the likelihood of successfully obtaining a vertebrate host blood meal identification at increasing Sella stages of digestion. The odds ratios are the exponent of the values in the 'estimate' column and indicate the odds of successful identification in comparison to a mosquito with a blood meal at Sella stage II. *** $P \leq 0.001$, ** $P \leq 0.01$.

6.4 Discussion

The data presented in this chapter represent the most detailed investigation of blood-fed mosquitoes conducted at a single site, to date, in the UK. The novel resting box design based on a collapsible plywood packaging crate was demonstrated to collect significant numbers of mosquitoes, primarily of the species *Cs. annulata*, *An. maculipennis* s.l. (comprising both *An. atroparvus* and *An. daciae/messeae*) and *Cx. pipiens s.l.*, with blood-fed mosquitoes comprising 9.5% of the total catch. The PCR-sequencing approach subsequently applied to these mosquitoes successfully identified blood-feeding on 19 different vertebrate hosts, comprising five mammals and fourteen birds, and was able to detect vertebrate blood meal origin in mosquitoes until Sella Stage VI of digestion. The blood-feeding hosts of nine mosquito species were identified, including, for the first time, conclusive demonstration of the ornithophagic activity of *Cx. modestus* and *An. atroparvus* in the UK.

Four of the nine mosquito species collected, *An. claviger*, *Cq. richiardii*, *Cs. annulata* and *Oc. detritus*, were identified as feeding solely on mammals. Of these, only *Cs. annulata* was collected in sufficient number (204) to enable conclusions to be drawn about host preference. A previous study showed that 37.5% of *Cs. annulata* blood meals were taken from cattle but that in addition, 20% of blood meals were taken from birds (Service 1971b). In this study, *Cs. annulata* did not feed on birds despite the presence and utilisation by other mosquito species of many bird species in the area, taking 92% of its blood meals from cattle and the remainder from other mammals. The reasons underlying this difference are unclear, but could potentially relate to differences in available avian fauna between the two field sites.

With the exception of one study (Curtotti 2009), previous studies of blood-feeding in the UK concerning *Cx. pipiens* s.l. did not have the molecular tools to identify its two ecoforms. Here, all blood-fed *Cx. pipiens* s.l. were identified as being *Culex pipiens f. pipiens* and, in agreement with the available literature (Medlock et al. 2005; Snow 1990; Cranston et al. 1987), all blood meals were found to have been taken from birds. Mosquitoes fed on thirteen avian

host species including both resident and migratory species. Barn swallows were preferentially fed upon in comparison to chickens and European starlings, but as compared to other avian hosts were opportunistically fed upon or actively avoided (Table 6.17). This contrasts with a northern Italian study of *Cx. pipiens* s.l. where no blood meals were recorded from barn swallows despite large numbers being present in the study area (Rizzoli et al. 2015). The reasons for feeding on this species at Elmley perhaps relates to the presence of nestling barn swallows in the toilets and barn which would allow feeding to occur that otherwise would not be possible on active adults, a factor that would similarly have facilitated the feeding of *Cx. modestus* on this host.

In agreement with the one previous UK blood meal study employing molecular separation of *An. maculipennis* s.l., *An. daciae/messeae* was identified as feeding on both mammals and birds (Danabalan et al. 2014). The earlier study only definitively identified a captive, non-native species, Darwin's Rhea (*Rhea pennata* d'Orbigny 1834) as a blood-feeding host. In the current study, the number of avian species reported as hosts of *An. daciae/messeae* is expanded to include domestic chickens and dark-breasted barn owls. In contrast, *Anopheles atroparvus* has not previously been identified as feeding on birds in the UK, with neither Danabalan et al. (2014) nor the results of the blood meal analyses conducted at Elmley in 2013 (Chapter 5/Brugman et al. (2015)) detecting avian feeds. Evidence points to these species exhibiting opportunistic feeding preferences, with a recent study in Spain detecting a low frequency of feeding on chickens (2/115, 1.7%), but no other avian species (Martínez-de la Puente et al. 2013). In the current study, the birds being fed upon by *An. maculipennis* s.l. were all available 'indoors' either resting and/or nesting in the barn (stock doves, barn owls) or inside the chicken coops (Figure 6.8). During the course of collections from these sites, blood-fed specimens of *An. maculipennis* s.l. were seen to rest close to the chickens in their nesting box and to rise up, when disturbed by the aspirator, from around a dove or pigeon nest in the barn. The lack of developed feathers and minimal defensive behaviour of the nestlings may permit easier feeding by opportunistic feeders less adapted to bird feeding. Techniques such as

videoing mosquito landing in nests (Griffing et al. 2007) or utilising microsatellite analysis of blood meals (Ligon et al. 2009) would better inform on whether nestlings or adult birds were the source of blood meals.

The absence of humans from all blood-fed collections is perhaps surprising given that all the species, with the exception of *Cs. morsitans*, were collected by human landing catch at Elmley (Chapter 3). However, although a small number (~5) humans are present on site, their actual availability to mosquito feeding is likely to be fairly limited as much human activity takes place during the day when mosquito feeding activity is minimal. Additionally, bed nets are sometimes used by the farm workers which is likely to further prevent human feeding. Similarly, although there is one dog present on site, and feeding on a dog by *An. messeae* was detected in chapter 5, no evidence of dog-feeding was found in this study. Taken together with the presence of blood meals in mosquitoes collected from resting locations in close proximity to certain hosts, this indicates that the mosquito species recorded on site exhibit a level of opportunistic feeding behaviour, but that in general, feeding is most frequently restricted to broad host groups such as mammals (*Cs. annulata*, *An. maculipennis* s.l.) or birds (*Cx. pipiens f. pipiens*). Comparing this to similar studies elsewhere, precipitin analysis of blood-fed mosquitoes collected using cloth exit traps fitted to chicken huts in Nigeria revealed none to have fed on the chickens, with feeding instead detected from cattle, sheep and goats (Service 1964). This suggests that the mosquitoes were simply using the chicken huts as a resting site rather than also supplying blood-feeding hosts, in contrast to what appears to be the case in the present study.

This study attempted to provide estimates of the bird and mammalian species present within the limited sampling area, in order to compare mosquito host selection in relation to host availability and thus draw conclusions on host preference. This is the first time this approach has been attempted in a blood meal analysis study of its kind in the United Kingdom and enabled inferences regarding mosquito host preference to be made. However, due to limitations in the availability of time and, most importantly, of personnel experienced in bird surveys and

identification, the surveys provide only a fairly crude 'snapshot' estimate of most of the vertebrate hosts present on site on the survey date. However, the presence of the 'anti-predator fence' and the active relocation of hedgehogs from Sheppey means that most ground-based mammalian predators can be considered to be absent from the site. This therefore restricts mammalian hosts to those for which numbers were available or for which estimates were obtained in this study: humans, cattle, sheep (seasonally moved into the area), rabbits and hares. Smaller mammals including rats, mice and other rodents were not however included in the survey, limiting conclusions that can be made about feeding preferences for these species. Future, more accurate attempts to survey the population would require more intensive, repeated sampling effort, with a minimum of two – four visits for bird point transect surveys, as recommended by Gregory et al. (2004), and three twilight surveys for rabbit populations as advised by Trout & Tittensor (1989). Additionally, multiple survey visits would enable comparison of temporal changes in mosquito feeding preferences to be made in accordance with seasonal variation in host densities, including changes in the migrant bird population and any disease-related (e.g. myxomatosis in rabbits, chapter 5) alterations in population. Furthermore, better estimates could have been obtained by using a laser rangefinder to estimate the distance of birds and mammals from the observer. This distance sampling approach would take into account the fact that the number of birds seen or heard decreases with distance from the observer and thus considers the varying detectability of hosts (see Buckland (2006) for further discussion). The availability of computer software such as 'Distance'[4] enables the appropriate calculations to be made to provide an improved estimate of vertebrate host density (Thomas et al. 2010). Using the rangefinder would also be a more accurate method to establish the maximum range over which animals could be seen by the collectors, rather than the simple estimate of 200 m that was used in this study.

[4] Latest versions for multiple platforms available at http://distancesampling.org/

Considering the rabbit and hare survey specifically, one major limitation of the walking transect approach was the ability of the animals to spot the observer from quite a distance away and rapidly disperse out of eye line, for example into burrows or behind vegetation. This likely led to an underestimate of the true numbers present within the study area. Further underestimation is likely to have occurred as a result of conducting twilight counts, estimated to reveal only 40-60% of the rabbit population during the summer (Toms et al. 1999). An alternative approach to that used would be spotlighting, relying on the reflected eye shine at night to obtain individual counts as used for hares, but not generally used for rabbits (Toms et al. 1999), or for more detailed information small mammals could be trapped in mark-release capture experiments (see Powell & Proulx (2003) for a detailed overview and discussion of the relative merits of different trapping methodologies).

In addition, visual counts of bird populations are by themselves not necessarily sufficient to capture the full vertebrate species complement of a given habitat. Whilst much of the transect area at Elmley was open marsh with little impediment to the view from the survey vehicle, the final transect point was close to the central woodland strip. In this area birds would have been difficult to see when roosting or resting in the trees. To address this issue, visual counts could be complemented with the monitoring of bird calls. These could be recorded and played back when no longer in the field, thus precluding the need for a bird identification expert to be present on the survey day. For even more comprehensive monitoring of bird populations, regular mist-netting, such as that conducted in several blood meal studies in the USA (e.g. Hamer et al. (2009), Molaei et al. (2006)), could be conducted. This would however require significant time and trained personnel, which were not available to this project.

This study also highlights the limitations of the feeding index calculations which, notwithstanding the limitations of the host survey techniques used, do not take into account fine-scale variation in host availability. In addition, the figures used to 'weight' the feeding index calculations for comparative host size (Kay et al. 1979) are only loose approximations. Although

differences in host size have been shown to influence the number of bites experienced by individual humans by *Anopheles gambiae* Giles 1902 s.l. (Port et al. 1980) and pigs by *Culex tritaeniorhynchus* Giles 1901 (Sota et al. 1991), and cattle with a larger surface area associated with slightly higher feeding rates by *Anopheles arabiensis* and *An. quadriannulatus* (Prior & Torr 2002) correcting for host mass may be a better means of weighting host preference calculations. The mass of hosts has been correlated with CO_2 production, shown to be important for influencing *Stomoxys* biting activity on cattle (Torr et al. 2006). Furthermore, no attempt was made to understand the seasonal variation in host feeding patterns that might have occurred during the collection period, with the simple exception of finding feeding on the two summer migrant bird species by *Cx. pipiens f. pipiens*.

The abundance of the different mosquito species collected in this study reflect the bias of the collection techniques towards those species willing to rest in artificial structures (endophily), particularly *An. maculipennis* s.l., *Cs. annulata* and *Cx. pipiens* s.l. For a Site of Special Scientific Interest (SSSI) like Elmley, the use of a passive collection method like resting boxes is advantageous as it avoids the collection of non-target species that would occur if using a backpack aspirator on potential resting habitat. Resting box collection also resulted in specimens of good quality with little damage, facilitating morphological identification. The simplicity of the design facilitated easy construction (only painting was required), portability, and would enable more boxes to be deployed in future if more blood-fed mosquito collections were required. The portable nature of this trap design is analogous to that of Pletsch (1970) who constructed a collapsible and stackable plywood resting box design by means of joining plywood sheets together using canvas strips. This current design however requires considerably less construction, thus saving time in fieldwork preparation. Prior to this study, resting boxes placed outdoors have not been used extensively in the UK, however boxes of varying design have been more widely used in the USA, collecting blood-fed mosquitoes mainly of the genera *Culex*, *Culiseta* and *Anopheles* (e.g. Morris (1981); Edman (1974); Sandhu et al. (2013); Howard et al. (2011)). The use of resting boxes outside of the USA has been more limited and in some cases

aimed at testing their ability to serve as an alternative to the human landing catch (HLC) for monitoring human-biting mosquito populations. Success in this regard has been mixed; for example, a recent study comparing resting boxes with window exit traps, Ifakara tent traps, CDC light traps and human landing catches found that resting boxes performed poorly in sampling malaria vector species *An. gambiae* s.l., *Anopheles funestus* Giles 1900 and *Anopheles ziemanni* Gruenberg 1902 in comparison with the HLC (Govella et al. 2011). However, cow odour-baited resting boxes showed promise in potentially replacing overnight HLCs for sampling *Anopheles arabiensis* Patton 1905 in northern Tanzania (Kweka et al. 2009). Resting boxes have seen less successful use in the African subcontinent for blood-fed collections; a recent study in Tanzania that collected mosquitoes from 10 villages, for four consecutive days at each over a three-year period, did manage to collect a total of 550 *An. gambiae* s.l. and 28 *An. funestus* in outdoor resting boxes (Mayagaya et al. 2015). Collected mosquitoes fed on all five of the target vertebrate hosts included in the PCR approach utilised: humans, cattle, goat, dog and chicken.

Resting box location had a significant influence on the mosquito collections even within the relatively small collection area. Resting boxes in wooded areas, location A and, to a lesser extent, location C, collected more mosquitoes than the others and even placing boxes just outside the wooded area (location B) resulted in much lower numbers collected. However, boxes at location B were faced into the vegetation, following the methodology of Morris (1981) in facing boxes towards areas where mosquitoes would be expected to emerge. The inconsistency of the box orientation in this regard makes it difficult to interpret whether orientation or location was the significant factor here influencing the numbers collected. The higher catches in wooded areas are likely due to mosquitoes actively seeking sheltered, vegetated areas to rest after feeding as the nature of the farm environment means such resting habitat is mainly restricted to ditches and edge habitats on field margins. In addition, it is likely that many of the blood-feeding hosts were clustered in or around the central woodland strip and therefore mosquitoes would need to move only a short distance from resting sites in the woodland to feed on the hosts.

Were it to become necessary, mosquito control techniques for *An. maculipennis* s.l. in an area such as Elmley could be designed to exploit the affinity of mosquitoes for resting in large structures like the barn and toilet block by selective insecticidal treatment of these areas. Such targeted methods would likely inflict considerable mosquito mortality whilst minimising, cost, time and undesirable effects on non-target species. Exploiting the endophilic behaviour of mosquitoes - such as that of *Anopheles gambiae* s.s. in sub-Saharan Africa - by means of indoor residual spraying of houses with insecticides such as DDT and pyrethroids has been an important part of integrated vector control strategies against malaria (see Pluess et al. (2010) for a systematic review of the topic).

The recruitment of mosquitoes (both blood-fed and unfed) into the resting boxes during the day, albeit in low numbers, indicates that some level of daytime movement of mosquitoes does occur. This movement may be as a result of disturbance caused by increasing light intensity or potentially the movement of a nearby host (Service 1971d). However, as the numbers of mosquitoes collected were low, it would be sufficient to target future studies simply to the morning collection. Collecting mosquitoes from resting boxes in the morning, generally in the period between 08:00 and 09:00, is the most commonly-used approach worldwide (e.g. in the USA (Sandhu et al. 2013), Tanzania (Mayagaya et al. 2015) and Australia (Kay 1983)). Both the wind speed and temperature experienced over the twelve hours preceding the morning collection significantly influenced the overall number of mosquitoes collected in the resting boxes. This is expected as these factors would be likely to impact on the ability of the mosquitoes to host-seek (see discussion in chapter 3). Daytime recruitment into the resting boxes is also likely to be affected by meteorological conditions, as shown previously (Morris 1981; Edman et al. 1968), although the limited catches of mosquitoes collected in the second and third visits did not permit detailed analysis of this.

The PCR-sequencing approach used in this study was successful in identifying host origin in 72% of blood-fed mosquitoes, with a decrease in success rates for all three of the most

numerous species (*An. maculipennis* s.l., *Cs. annulata* and *Cx. pipiens* s.l.) as Sella stage increased from stage II through to VI. This result is consistent with a previous study in Spain (Martínez-de la Puente et al. 2013) using the PCR assay of Alcaide et al. (2009). The major limitation with the methodology used in this study is that no mixed feeds can be detected, as the most abundant blood meal source will dominate the PCR reaction. In a previous UK study, only one of the seven targeted hosts, a dog, was detected only as part of a mixed blood meal of *An. maculipennis* s.l. (Danabalan et al. 2014).

From the perspective of mosquito-borne pathogen transmission, this study provides new insights into the potential involvement of UK mosquitoes and wild vertebrate hosts in pathogen transmission cycles. Barn swallows are long-distance migrants, over-wintering in southern Africa, and have been identified as having been exposed to WNV in Germany (Seidowski et al. 2010). The presence of this host at Elmley being fed upon by *Cx. pipiens f. pipiens* and *Cx. modestus* indicates that, from the mosquito-host contact perspective, Elmley could be a favourable site for enzootic virus transmission. Barn swallows and blackbirds have also been found to be highly susceptible to infection with USUTV in Europe (Weissenböck et al. 2002). The preference for feeding on blackbirds displayed by *Cx. pipiens f. pipiens*, reflecting the results of similar populations in Europe (Rizzoli et al. 2015), could facilitate transmission in the case of an introduction. In contrast, however, the lack of evidence of feeding on humans, despite clear evidence of exposure from human landing catches (Chapter 3), argues against zoonotic spread from these reservoirs.

This study additionally enhances the results of chapter 5 showing that higher proportions of blood meals are taken from rabbits than other mammals by *An. atroparvus* at Elmley, thereby implicating this species in the transmission of myxoma virus. In Chapter 5, both infected (27% of total rabbit feeds detected) and uninfected rabbits were being fed upon, indicating that feeding on rabbits is not simply a function of reduced host defensive behaviour caused by myxoma virus infection, but the result of a preference for this host. Here, the $FI_{rabbit:cow}$

was >1 indicating that rabbits are indeed preferentially fed upon by *An. atroparvus* over cattle at Elmley. Interestingly, no rabbit-feeding by *An. daciae/messeae* was detected in either study although this could be simply as a result of which samples happened to be selected for species identification.

Chapter 7 – General discussion

This thesis aimed to further the understanding of mosquito-vertebrate host interactions within livestock farm environments in the UK and the ecological factors influencing biting and feeding behaviour, both within and between different farms. Following an initial pilot study assessing the presence of mosquitoes on seven farms, standardised collections of adult mosquitoes were conducted on four farms between 2013 and 2014 using chicken-baited traps, human landing catches and resting collections, linked with molecular analysis of blood-fed females. These studies yielded a total of 22 693 adult mosquitoes of 7 genera and 18 species (Table 7.1) and together provide evidence that UK livestock farms support ornithophagic, mammalophagic and anthropophagic mosquito populations.

Human biting by farm-associated mosquitoes

The results indicate that farm-associated mosquito population assemblages are currently capable of causing a severe human-biting nuisance on certain farms, with at least 17 species collected by human landing catch (HLC). The highest biting pressure occurred at Elmley, Kent, with a maximum rate of 89 mosquitoes collected per 25 minutes (3.6/minute) on a single collector, over sunset. This biting rate is in line with the 200 bites per hour recorded in Sandwich, Kent, some 40 miles south of Elmley, in 1981 (Ramsdale & Snow 1995). Guided by preliminary HLCs (Chapter 2) indicating that the majority of human biting activity on the farms occurred close to sunset, four-hour, multi-volunteer collections were conducted, starting two hours before sunset. These demonstrated a close association between overall biting pressure and time of sunset for several species including *Coquillettidia richiardii* and *Culex modestus*, although the trends were the most defined only at Elmley. Notably, the biting rate of mosquitoes on humans varied considerably between the four farms studied, with, for example, no biting recorded on collectors at Church Farm, Oxfordshire, throughout the collections in Chapter 3, despite

preliminary human landing catches in the pilot study (Chapter 2) collecting two specimens of *Ochlerotatus punctor*.

Species	**Site(s) collected from**	**Chapter ref(s)**
Aedes cinereus/geminus [H]	Glendell Livery, White Lodge	2, 3
Anopheles claviger [H]	ADAS Arthur Rickwood, Church Farm, Elmley, Northney Farm	2, 3, 4, 6
Anopheles maculipennis s.l. [H]	ADAS Arthur Rickwood, Elmley, White Lodge	2, 3, 5, 6
Anopheles atroparvus [H]	Elmley	3, 4, 5, 6
Anopheles messeae/daciae [H, C] *	Elmley	
Anopheles plumbeus [H]	Coombelands Farm, Glendell Livery, Mudchute Farm, Northney Farm, White Lodge	2, 3, 4
Coquillettidia richiardii [H, C]	ADAS Arthur Rickwood, Coombelands Farm, Elmley, Northney Farm, White Lodge	2, 3, 4, 6
Culex modestus [H, C]	Elmley	2, 3, 4, 6
Culex pipiens s.l. [H, C]	All farms	2, 3, 4, 6
Culex pipiens f. pipiens [H, C]	Church Farm, Elmley, Northney Farm	3, 4, 6
Culiseta annulata [H]	All farms	2, 3, 4, 6
Culiseta morsitans	Elmley	4, 6
Culiseta subochrea	White Lodge	4
Dahliana geniculata [H]	White Lodge	3, 4
Ochlerotatus caspius/dorsalis [H]	ADAS Arthur Rickwood, Elmley, Northney Farm	2, 4, 6
Ochlerotatus cantans/annulipes [H]	Elmley, Glendell Livery, White Lodge	2, 3, 4
Ochlerotatus detritus [H]	ADAS Arthur Rickwood, Elmley, Northney Farm	2, 3, 4, 6
Ochlerotatus flavescens [H]	ADAS Arthur Rickwood, Coombelands Farm, Elmley	2, 3, 4, 6
Ochlerotatus punctor [H]	Glendell Livery, White Lodge	2, 3, 4
Ochlerotatus rusticus [H]	Elmley, White Lodge	3, 4

Table 7.1: Mosquito species collected during work comprising this thesis. * *An. daciae/messeae* could not be separated by molecular means in Chapter 6, although in Chapter 5 there was no evidence of *An. daciae* having been collected. Superscript 'H' indicates species collected by human landing catch and superscript 'C' indicates species collected using chicken-baited traps.

Bird biting by farm-associated mosquitoes

The studies in chapter 4 indicate that birds do serve as hosts for mosquito biting on UK farms, but that, notwithstanding the drawbacks of the chosen trap design (as discussed in Chapter 4), the overall biting rate per individual bird may be quite low in comparison to the biting rate experienced by humans on the farms surveyed. The low number of mosquitoes collected in the chicken-baited traps did not permit conclusions to be drawn regarding variation in ornithophilic mosquito species composition at different heights or even between sites. Nonetheless, the chicken-baited traps collected three species including *Culex modestus and Coquillettidia richiardii*, the first time these two species have been collected by a bird-baited trap in the UK. Together with the results of the trap efficiency study, which indicated that a greater proportion of mosquitoes entered and were retained by the trap when the chickens were in them, this justifies the use of chickens as a suitable host for bird-baited trapping studies in future. It would however be advisable to further modify the trap and obtain Home Office permission to allow the bait birds to be fed upon, in order to reduce trapping bias, maximise numbers retained by the trap following entry, and to gain an understanding of feeding rates on the bait following initial attraction.

Blood meal analysis, delineation of mosquito species and targeted pathogen detection

The results of chapter 5 successfully show that the amount of data obtained from a single blood-fed mosquito specimen can be effectively maximised by applying a sequential, targeted workflow to a single DNA extract from a blood-fed mosquito abdomen. In this approach, the mosquito (*Anopheles maculipennis* s.l.) was identified to species level (*An. atroparvus* or *An. messeae*) by PCR-sequencing of the *ITS-2* gene region, and the vertebrate blood meal origin was determined by PCR-sequencing of the *COI* gene. Those specimens that had blood-fed on rabbits were subsequently screened by PCR for myxoma virus, with subsequent sequencing to confirm positive samples. This approach will be very useful for ongoing work worldwide into the blood-feeding behaviour and pathogen-transmission potential

of mosquitoes, especially as it remains a challenge to capture large numbers of blood-fed females, particularly those species that do not display, or display limited, endophilic resting behaviour. The myxoma virus is a DNA virus and thus viral DNA was extracted alongside the insect and blood meal DNA. However, any RNA viruses are unlikely to be isolated using the chosen DNA extraction procedure (DNeasy blood and tissue kit) due to the sensitive nature of RNA. In this regard, it may be best to either extract RNA, which will likely still contain a DNA contaminant, or better still, use a co-extraction procedure such as that of Griffiths et al. (2000) which will enable the isolation of both nucleic acids.

Host-feeding patterns of farm-associated mosquitoes

The range of blood-feeding hosts for UK mosquitoes was greatly expanded by the work in Chapter 6 (see Table 7.2 for a complete list of updated host ranges), with definitive identification of UK mosquitoes having fed on nineteen vertebrate hosts. Arguably of greatest importance is the identification of the avian hosts of *Culex pipiens f. pipiens*, for which little data currently exist. This species is considered to be a potential vector of arboviruses such as West Nile virus (WNV) (Medlock et al. 2005; Medlock, Snow, et al. 2007), with implication in transmission in France and further afield (Fonseca et al. 2004; Farajollahi et al. 2011; Balenghien et al. 2008). In case of incursion, transmission is likely to be facilitated by the ubiquitous distribution of this species across the UK in both rural and urban areas of the UK (Townroe & Callaghan 2014). The present study demonstrated a very broad host range of *Cx. pipiens f. pipiens*, with blood meals taken from 13 bird species including two migratory species (Barn swallow and Yellow wagtail), plus others associated with arboviruses in Europe. These include, for example, the blackbird, associated with Usutu virus outbreaks in Austria (Weissenböck et al. 2002).

Vertebrate blood-feeding host	Mosquito species																					
	Aedes cinereus/geminus	*Anopheles maculipennis s.l.**	*Anopheles atroparvus*	*Anopheles claviger*	*Anopheles daciae*	*Anopheles messeae*	*Anopheles plumbeus*	*Coquillettidia richiardii*	*Culex modestus*	*Culex pipiens s.l.*	*Culex pipiens f. pipiens*	*Culex pipiens f. molestus*	*Culex torrentium*	*Culiseta annulata*	*Culiseta litorea*	*Culiseta morsitans*	*Dahliana geniculata*	*Ochlerotatus annulipes*	*Ochlerotatus cantans*	*Ochlerotatus caspius*	*Ochlerotatus detritus*	*Ochlerotatus dorsalis*	*Ochlerotatus flavescens*	*Ochlerotatus punctor*
Humans	✓	✓	✓		✓	✓	✓	✓	✓	✓		✓		✓	✓	✓	✓		✓		✓	✓		✓
Domestic mammals																								
Cow	✓		✓	✓	✓	✓	✓	✓		✓				✓	✓	✓	✓		✓		✓	✓	✓	✓
Sheep					✓	✓								✓					✓					✓
Goat			✓		✓	✓																		
Horse					✓														✓		✓	✓		
Pig														✓					✓		✓	✓		
Dog						✓						✓												
Domestic birds																								
Chicken		✓	✓																					
Wild mammals																								
Deer			✓		✓	✓																		
Rabbit	✓		✓	✓			✓	✓		✓			✓	✓	✓	✓			✓			✓		✓
Hare		✓	✓																					
Brown rat			✓																					
Wild birds																								
Blackbird											✓					✓								
Barn Swallow									✓		✓													
Barn owl		✓									✓													
Skylark											✓													
Starling											✓													
Grey heron											✓													
House sparrow											✓					✓								
Long-eared owl											✓													
Meadow pipit											✓													
Mute swan									✓															
Stock dove			✓								✓													
Wood pigeon											✓													
Rock pigeon											✓													
Yellow wagtail											✓													

Table 7.2: Updated blood-feeding host range of UK mosquitoes from blood meal studies conducted to date and in this thesis. * includes results where *An. daciae/messeae* produced identical BLAST results. Non-specific results omitted.

This study also demonstrated the successful use of a simple resting box design to collect blood-fed mosquitoes, the subset of the mosquito population that remains one of the hardest to survey. Of the more than 20 000 specimens collected in the 2014 study (Chapter 6), over 5000 mosquitoes were collected from the resting boxes, of which nearly 10% were blood-fed. Although the numbers collected were far less than from the barn, a similar proportion of the catch were blood-fed. However, an expected bias towards endophilic species was observed, with *An. maculipennis* s.l., *Culex pipiens* s.l. and *Culiseta annulata* constituting the majority of the blood-fed catch, with other species represented by a very low number of specimens, and thus a method of collecting a less biased subset of the blood-fed mosquito population remains to be found.

In an attempt to identify whether UK mosquitoes exhibited clear feeding preferences, blood meal analysis was combined with estimations of vertebrate host abundance (Chapter 6) to obtain values for the feeding index of Kay et al. (1979). Notwithstanding the limitations of this approach (detailed in chapter 6), the feeding indices do indicate that a level of preference is exhibited by mosquitoes at Elmley but that preference is most notably restricted to broad host groupings (bird, mammal). Within each grouping there may be more variability in host selection but this may be modulated in large part by the local availability of hosts, mirroring earlier work into the blood-feeding behaviour of *Oc. cantans* (Renshaw et al. 1994). It is interesting to note that no human-derived blood meals were detected in the specimens collected in Chapters 5 and 6, and yet, very high human biting rates were observed from this same farm, Elmley, during the work of Chapter 3. This is likely to partly result from the relatively low availability of human hosts in comparison to alternative vertebrate hosts at peak mosquito biting times and in close proximity to collection sites, influencing the likelihood of collecting human-fed specimens. Future work incorporating the collection of blood-fed mosquitoes from inside the houses on site with subsequent blood meal analysis may provide more information on the incidence of human biting at Elmley, as the farm workers still report on the need to use bed nets at certain times of the year to reduce indoor biting.

The digestion of blood meals and thus the rapid destruction of DNA sequences within mosquitoes and other haematophagous arthropods is a major limiting factor to blood meal studies. In this study, the blood meals of colony-fed *Cx. pipiens* s.l. were only identifiable until 24 hours post-feeding. Other studies have achieved positive results after longer periods of time, such as the 72 hours reported for *Simulium damnosum* Theobald 1903 s.l. fed with human blood (Boakye et al. 1999). In the present study, blood meals were successfully identified from field-caught specimens of *Cx. pipiens f. pipiens* from Elmley from digestion stages II-VI, indicating that identification could take place for longer than 24 hours. The field-caught *Cx. pipiens f. pipiens* were identified as having fed on avian blood, which, being nucleated, will contain greater concentrations of DNA than mammalian blood and may result in greater ease of identification. Avian blood was not used during the time course experiment (in part due to the expense of sourcing chicken blood as compared to mammalian blood), thereby illustrating the importance of using ecologically appropriate methods when testing laboratory protocols, i.e. blood from a host group relevant to current knowledge of feeding preferences.

Factors influencing trap catches

Of the meteorological variables examined, only wind speed was found to significantly influence the human biting rate experienced on the sites, with total biting pressure (all mosquito species) predicted to decrease by 58% with a 1 m/s increase in wind speed (Chapter 3). Together with temperature, wind speed was also an important factor influencing the mosquito catch of the resting boxes (Chapter 6). These results tie in with available literature that have tested the maximum flight speeds of mosquitoes elsewhere (Snow 1980; Gillies & Wilkes 1981), indicating that above certain wind speeds mosquitoes cease to be able to fly. However, the relationship between wind speed and the numbers of host-seeking mosquitoes captured at human bait may be more complicated than a simple speed-induced inhibition of flight. In field experiments using an electric fan to generate controlled wind speeds around CDC light traps baited with CO_2, Hoffmann & Miller (2003) demonstrated that an increase in the release rate of carbon dioxide

led to a predictable increase in mosquito catch (dominant species *Aedes vexans*, *Anopheles walkeri* Theobald 1901 and *Coquillettidia perturbans* Walker 1856) over wind speeds ranging between zero and 3.7 m/s. This indicated that the observed reduction in mosquito trap catches with increases in wind speed may actually result not simply from the maximum flight speed of the insects being exceeded, but from a dilution of host-derived attractants. This fits with controlled wind tunnel experiments with *Aedes aegypti* showing that the fine-scale structure of host odour plumes, influenced by wind-induced turbulence, influences the upwind host-seeking behaviour of mosquitoes depending on the odour (Geier et al. 1999).

In future field experiments it would also be useful to investigate the effect of localised variation in meteorological variables, particularly wind speed, in influencing mosquito biting activity. The heterogeneity of mosquito distribution across a single site has been demonstrated previously (Service 1971d) and within farm settings differences in vegetation structure, buildings and farm equipment will all likely influence the wind speeds experienced by a host situated in and around them. Furthermore, farm-specific seasonal changes in land use may occur which may affect the distribution of resting mosquitoes over a site. As an example, we can consider grass cutting. Grass grown for animal fodder and silage is cut, dried and baled in late spring/summer (Countryside 2015), a process dependent on meteorological conditions (dry and warm) which also favour mosquito activity. Farmers work long hours to cut the grass, exposing them to potential biting over mosquito activity peaks at sunrise and sunset, more than is usual at other times of the year. The cutting and baling of grass will, however, remove large areas of outdoor resting habitat, as well as likely causing considerable direct mortality to resting mosquito populations. Mosquitoes surviving the process may move to and increase in density in field margins or, in the case of smaller sites, migrate further to more favourable resting sites in adjacent areas, thereby reducing the local biting population. In other invertebrate groups including spiders and beetles, the grass cutting process has been demonstrated to cause direct mortality rates of between 25-60% over a given area, as well as inducing the emigration of survivors from the cut areas (Thorbek & Bilde 2004). Future studies directed at assessing the

effect of these processes on the vertebrate host-biting rates at the time of grass cutting, both within fields and at their edge, would be useful.

Larval mosquito habitats associated with the farms

The pilot study (Chapter 2) identified, on a simple presence/absence basis, larval mosquito habitats present on seven different livestock farms. This will have influenced the diversity of adult species assemblages found on the farm. However, some distinction is required between habitat provision on the farms themselves (i.e. within their boundaries) and those existing on adjacent land which, from a habitat management and control perspective, may not be accessible to the farm. The range of habitats found within farm boundaries differed considerably between farms (Chapter 2) and was influenced in large part by the land area covered by the farms. The Elmley site, for example, covers a very large area, sitting at the interface between freshwater habitats generally favoured by *An. messeae* and the saline habitats favoured by *An. atroparvus* (see Sinka et al., (2010) for a review). The White Lodge site, in contrast, occupies a small land area and thus many of the species collected from humans were most likely a result of recruitment of host-seeking adults from adjacent woodland habitats. This is reflected in the collection of woodland species such as *Oc. rusticus*, *Oc. punctor* and the dendrolimnic species *Da. geniculata*, not collected from the other farms. Importantly, all farms supported permanent and semi-permanent container habitats and drainage ditches. Taken together with information from available distributional datasets (e.g. on the NBN Gateway), this provides an element of predictability of which species will be most commonly found on farm sites across the UK. Those species able to exploit these container habitats and which are already found across large parts of the UK include (but are not limited to) *Cx. pipiens f. pipiens*, *Cs. annulata* and potentially *An. plumbeus* (Townroe & Callaghan 2014). The presence of these habitats also lay the foundations for recommendations for farm-wide control measures in the event of an exotic arbovirus outbreak. These could include alterations of water levels in ditches (either flushing or drainage, depending on the species) and where this is not possible, targeted

application of larvacides such as *Bti*, and the periodic emptying of container habitats to interrupt mosquito development.

Current pathogen transmission and potential future transmission

Many of the 18 mosquito species collected in work comprising this thesis have been historically associated, are currently associated, or may in future be associated with the transmission of important pathogens. Members of the *An. maculipennis* complex, most importantly *Anopheles atroparvus*, were responsible for the historical transmission of *Plasmodium vivax* in the UK (Hutchinson & Lindsay 2006a; Ramsdale & Snow 1995) and still contribute to the human biting mosquito population (Chapters 3 and 4). This serves as a reminder that maintaining and updating knowledge of local mosquito population dynamics, including their host feeding preferences and biting periodicities, is important to avoid future local pathogen transmission events occurring in such manner as the local Vivax malaria outbreak shortly after WWI (Ramsdale & Snow 1995; Shute & Maryon 1969). Whilst the risk of Vivax malaria reestablishment in the UK is considered to be low (Lindsay et al. 2010), the finding that *An. atroparvus* feeds on both healthy and myxoma virus-infected wild rabbits (Chapter 5) has now implicated this species in the transmission of this veterinary pathogen.

Arboviruses are considered to pose the greatest threat of emergence to the UK (Gould et al. 2006; Medlock, Snow, et al. 2007), with transmission likely to be facilitated or enhanced by invasive vector species such as *Aedes albopictus* entering the country and becoming established (Medlock et al. 2006). Emerging arboviruses have caused outbreaks of human disease in southern Europe – autochthonous Dengue fever occurred on Madeira island, Portugal in 2012 (Sousa et al. 2012), a Chikungunya virus outbreak occurred in Italy in 2007, and human infections with both lineages 1 and 2 West Nile virus (WNV) have occurred in Greece and Italy (see Hernández-Triana et al. (2014) for review). Further afield, arboviruses continue to show their potential for rapid emergence, even across continents; Zika virus for example, previously restricted to isolations in central Africa (e.g. Haddow et al. (1964); Weinbren & Williams (1958))

crossed the globe causing outbreaks in Micronesia (Duffy et al. 2009), French Polynesia (Musso et al. 2015) and more recently in Brazil and other south American countries (Zanluca et al. 2015; Musso et al. 2015).

Ten of the 14 UK mosquito species considered to be potential enzootic and/or bridge vectors for WNV in the UK (Brugman et al. 2013; Medlock et al. 2005) were collected by HLC across the studies in this thesis. The role of the widely-distributed species *Cx. pipiens f. pipiens* in potential arbovirus transmission is largely thought to be as an enzootic (bird-mosquito-bird) vector (Brugman et al. 2013). However, HLCs in both Chapters 3 and 4 collected this species. Although it cannot be definitely concluded that landing individuals of this species would have bitten and completed a blood meal, the human blood meals within other species collected alongside, using the same collection methods (Chapter 3), indicate that it is likely to have fed. Without knowing the relative abundance of those individuals flying but not landing on humans however, it is not possible to determine whether this landing/biting behaviour is more common than was previously believed (Snow 1990; Cranston et al. 1987). Nonetheless, the appearance of this species in collections from two sites (Elmley and White Lodge) indicates that the biting activity of the *Culex pipiens* complex as a whole in the UK warrants further attention.

The second species of particular interest in collections is *Cx. modestus*, thought to be fairly rare in the UK until the discovery of larval populations in 2010 (Golding et al. 2012), and thus the behaviour of which remains poorly understood. This species was collected in both chicken-baited traps and by HLC at Elmley, Kent, indicating that it exhibits similar host-feeding preferences to relatively nearby populations in the Camargue, France (Balenghien et al. 2006) and thus may also be able to serve as a vector for WNV (Balenghien et al. 2008) although the competence of UK specimens for virus transmission has yet to be confirmed. Notably, *Cx. modestus* was the second most abundant human-biting species in the HLC collections of Chapter 3, with only *Cq. richiardii* showing a higher mean biting rate. This contrasts to the reports of human-biting species collected from Sandwich, Kent in 1981 (Ramsdale & Snow 1995), where only *Ochlelrotatus detritus* was reported; in the present study *Oc. detritus* played only a

minor role constituting 0.37% of the total catch. The fact that *Cx. modestus* has gone unreported since the 1940s (Marshall 1945) despite the Thames/Medway region being a focal point for several mosquito control programs over the past century (Ramsdale & Snow 1995) and the subject of field studies as recently as 2003 (Hutchinson et al. 2007) does lend support to the argument that this species could have been recently re-introduced to the UK. However, since 2010, reports of this species in Mosquito Magnet collections as far apart as Dorset and Cambridgeshire, as well as in other parts of Kent (Medlock & Vaux 2014b; Medlock & Vaux 2012; Vaux et al. 2015) indicate that perhaps this species has been present, but unrecognised, for some time. One potential reason for this could be that this mosquito has been considered rare in the UK literature and thus has not been expected in collections, increasing the chances of it being overlooked. This is compounded by the fact that the pale-scale abdominal patterns of *Cx. modestus* and *Cx. pipiens* s.l./*Cx. torrentium* adults can be difficult to separate in older specimens and the fact that few studies have the resources for molecular confirmation of species. It is clear that there is a need for increased attention to be focused not only on the biology and distribution of *Cx. modestus*, but of mosquitoes as a whole across the UK. This will facilitate a better understanding of the potential for nuisance biting and pathogen transmission across the country, including providing data for risk models, but also will provide a baseline against which to assess any population changes which might occur.

Future work and emerging technologies

No specific mosquito control activities took place on the studied farms during the studies. However, some use of topical pour-on chemical treatments (no type or brand was specified) for sheep was reported by Church Farm for protection against flystrike. This economically important myiasis is most commonly caused by the blowfly *Lucilia sericata* Meigen 1826 in the UK and has been estimated to affect some 75% of UK holdings (Bisdorff et al. 2006). To the knowledge of the author, there are currently no UK studies to have investigated the effects of such insecticidal treatments on other biting arthropods. Given that UK mosquitoes

feed on sheep (Table 7.2), and other farm-associated arthropods including ticks and *Culicoides* are also known to feed on sheep, it would be of interest to conduct a study investigating the effects of such chemical treatment on broad host groups. The low mosquito abundance at Church Farm make this an unsuitable site for this type of study. However, a site like Elmley which supports a high abundance of mosquitoes, is known to support tick populations (*Haemaphysalis punctata*) (Tijsse-Klasen et al. 2013) and is likely to support *Culicoides* populations as well, would be a useful alternative.

There is considerable research benefit to incorporating novel and emerging technology into ecological studies of mosquito behaviour. In this study, the characterisation of trap locations was aided by the use of Google Photospheres™, available as an application on Android and some Apple smartphones. If fieldwork study sites across the UK and further afield made use of these 360° images and uploaded these to publically-accessible mapping websites (or, to take into account privacy concerns, password-protected mapping websites), the local habitats in which vector studies were conducted could be better understood by other research groups. This would also facilitate the standardisation of site characterisation approaches which at present range from the very detailed to only brief descriptions in published literature. Other technological advances which may be of use in mosquito studies include the use of remotely-controlled drones to photograph and characterise sites, or even to collect mosquito trap catches in remote locations, such as is the goal of a Microsoft-funded project, Project Premonition[5].

The relatively large number of blood-fed mosquitoes processed during the blood meal analysis studies in this thesis (chapters 5 and 6) was in part possible due to the decreasing cost of molecular techniques. However, each specimen needed to undergo a spin-column DNA extraction procedure which added cost and time to processing (about 1.5 – 2 hours from freezer to extracted DNA sample). Currently, direct-to-PCR kits are becoming readily available, which enable a blood sample to serve as a DNA template directly, precluding the need to perform DNA

[5] see http://blogs.microsoft.com/next/2015/06/10/project-premonition-mosquitoes-drones-cloud-computing/

extraction. These kits work by allowing the PCR reaction to take place in the presence of PCR inhibitors, such as haemoglobin, present in blood (see Wilson, (1997) for a detailed review of PCR inhibition) and which are usually reduced or removed during the DNA extraction process. This resistance to inhibition is conferred either by modification of the Taq polymerase enzyme to give it greater stability[6], or by inclusion of buffering mastermix reagents[7]. Furthermore, the present methodology was limited in that it could not detect mixed blood meals. The multiple sequence reads produced by next-generation sequencing approaches could be an alternative approach to detecting mixed blood meals in advanced stages of digestion.

Identifying patterns of blood feeding over time could be a further extension to the project. At the Elmley site, the availability of large mammals on site is consistent over the summer but the small mammal populations are likely to be far more variable due to natural variation in population abundance. Of interest is whether myxomatosis-induced rabbit mortality leads to changes in the blood-feeding behaviour of *An. atroparvus*. Theoretically, an increase in the number of myxomatosis-infected rabbits with reduced defensive behaviour could lead to an initial increase in feeding on these hosts. However, when rabbit populations decline as a result of the disease later in the summer, the reduced availability of these hosts could lead to increased feeding on alternative hosts. Shifts in feeding patterns over time are also of particular interest for the primarily ornithophagic *Cx. pipiens f. pipiens* in the context of arbovirus transmission. This species was collected in landing catches (Chapter 3), indicating (with some caution, discussed above) opportunistic anthropophagy. Increased feeding on humans could, in the event of enzootic circulation of an arbovirus like WNV, lead to increased human infections when preferred avian hosts decrease in number, such as has been reported for the American robin in the USA (Kilpatrick et al. 2006).

[6] e.g. ThermoFisher Scientific Phusion Blood Direct PCR kit
[7] e.g. Bioline MyTaq™ Blood-PCR kit

The use of blood meal analysis techniques can extend beyond understanding arthropod feeding patterns. One alternative use is the screening of blood-fed mosquitoes for human, non-mosquito-borne pathogens, in an approach referred to as xenosurveillance. This approach offers several benefits, primarily the avoidance of the need for invasive screening (such as blood-taking) from humans. Using this approach on collected *An. gambiae* s.s collected from living quarters in Liberia, Grubaugh et al., (2015) detected the presence of Epstein-Barr and Canine distemper viruses. This approach relies, firstly, on the ability of diagnostic tests to detect pathogens of interest, secondly, on mosquitoes taking blood meals from epidemiologically-relevant vertebrate hosts and finally, on being able to collect sufficient numbers of blood-fed mosquitoes, which still remains a recognised challenge. The absence of human blood meals from Elmley (and the small human population there), however, do not strongly justify testing the blood meals of mosquitoes collected there for human pathogens.

A second alternative use of blood meal analysis is in the tracking of rare or difficult-to-access mammalian species in the wild for conservation purposes, as hinted at in a recent study in Spain. Following the identification of a single field-caught specimen of *An. atroparvus* as having fed on an Iberian lynx (*Lynx pardinus* Temminck 1827), the blood meal was then tested using microsatellite markers. Three of the eleven successfully amplified, indicating that this approach could have utility in identifying individual animals, although it would require an original reference blood sample from the target animal and, again, the collection of sufficient numbers of blood-fed mosquitoes. From a similar perspective, blood meal analysis in Chapter 6 detected the presence of dark-breasted barn owls at Elmley, a subspecies from continental Europe which has not been previously reported as breeding in this part of Kent. This has prompted the site owners to undergo further investigations into their owl populations.

Conclusion

Understanding vector-host contact is essential to understanding the risk of nuisance biting and the risk of the establishment, or re-establishment, of pathogens in the UK. This thesis

provides key data on mosquito biting rates, host selection and preference and a targeted methodology for detection of blood meal, mosquito species and pathogens present at a site. These data, together with data assessing the effect of meteorological effects on mosquito catches, also provide an important starting platform for further mosquito behavioural and distributional studies which should ideally encompass a greater number of farms. Against a backdrop of continued arbovirus range expansion worldwide, as interest in the mosquitoes of the UK continues to grow, and as information on vector competence, a particular focus of current research, continues to emerge, it is important that essential ecological and behavioural mosquito data continues to be collected. This will ensure a balanced picture is maintained of the risk of nuisance biting on humans and animals, and the risk of pathogen emergence in the UK.

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Appendices

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(Byrne & Nichols 1999; Snow 2000; Lindsay et al. 2010; Hutchinson et al. 2005; Lindsay & Joyce 2000; Lindsay & Thomas 2001; Mpho et al. 2002; Buckley et al. 2003; Buckley et al. 2006; Higgs et al. 2004; Gould et al. 2006; Hutchinson 2004; Department of Health 2004; Medlock et al. 2005; Ramsdale & Gunn 2005; Ramsdale & Harbach 2003; Snow & Medlock 2006; Hutchinson & Lindsay 2006a; Hutchinson & Lindsay 2006b; Medlock & Vaux 2009; Medlock, Snow, et al. 2007; Medlock, Barrass, et al. 2007; Medlock et al. 2006; Hutchinson et al. 2007; Medlock & Snow 2008b; Snow & Medlock 2008; Phipps et al. 2008; Brugman et al. 2013; Brugman et al. 2015; Roy et al. 2009; Medlock & Jameson 2010; Medlock & Vaux 2010; Danabalan 2010; Danabalan et al. 2014; Danabalan et al. 2012; Vaux et al. 2011; Medlock 2015; Medlock & Leach 2015; Horton et al. 2013; Vaux et al. 2015; Medlock & Vaux 2011; Golding et al. 2012; Murphy et al. 2013; Medlock et al. 2012; Medlock & Vaux 2012; Brown et al. 2012; Engler et al. 2013; Hesson et al. 2014; Medlock & Vaux 2013; Golding 2013; Medlock & Vaux 2014a; Townroe & Callaghan 2014; Medlock & Vaux 2014b; Bessell et al. 2014; Vaux & Medlock 2015; Medlock & Vaux 2015a; Medlock & Vaux 2015b; Purse & Golding 2015; Townroe & Callaghan 2015; Mackenzie-Impoinvil et al. 2015; Manley et al. 2015; Quintavalle Pastorino et al. 2015; Snow & Snow 2004; Medlock & Snow 2008a; Curtotti 2009)

Appendix A1: Reference list for studies concerning UK mosquitoes 1998-2015 included in Figure 1.1.

ID	Description
1	Saltmarsh soils
2	Shallow very acid peaty soils over rock
3	Shallow lime-rich soils over chalk or limestone
4	Sand dune soils
5	Freely draining lime-rich loamy soils
6	Freely draining slightly acid loamy soils
7	Freely draining slightly acid but base-rich soils
8	Slightly acid loamy and clayey soils with impeded drainage
9	Lime-rich loamy and clayey soils with impeded drainage
10	Freely draining slightly acid sandy soils
11	Freely draining sandy Breckland soils
12	Freely draining floodplain soils
13	Freely draining acid loamy soils over rock
14	Freely draining very acid sandy and loamy soils
15	Naturally wet very acid sandy and loamy soils
16	Very acid loamy upland soils with a wet peaty surface
17	Slowly permeable seasonally wet acid loamy and clayey soils
18	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils
19	Slowly permeable wet very acid upland soils with a peaty surface
20	Loamy and clayey floodplain soils with naturally high groundwater
21	Loamy and clayey soils of coastal flats with naturally high groundwater
22	Loamy soils with naturally high groundwater
23	Loamy and sandy soils with naturally high groundwater and a peaty surface
24	Restored soils mostly from quarry and opencast spoil
25	Blanket bog peat soils
26	Raised bog peat soils
27	Fen peat soils

Appendix A2: Cranfield University's "Soilscapes" soil type categories and descriptions.

Source: http://www.landis.org.uk/soilscapes/soilguide.cfm.

Order		Group	
C010	Cropped land	C011	Field crops
		C012	Fallow land
		C013	Horticulture
		C014	Orchards
C020	Grass	C021	Improved grass
		C022	Unimproved grass
		C023	Recreational and amenity grass
C030	Woodland and shrub	C031	Conifer woodland
		C032	Mixed woodland
		C033	Broad-leaved woodland
		C034	Shrub
C040	Heathland and bog	C041	Heathland
		C042	Bracken
		C043	Bog
		C044	Montane
C050	Inland rock	C051	Inland rock
C060	Water and wetland	C061	Standing water
		C062	Running water
		C063	Freshwater marsh
C070	Coastal features	C071	Sea and coastal waters
		C072	Inter-tidal sand and mud
		C073	Salt marsh
		C074	Dunes
		C075	Coastal rock and cliffs
C080	Buildings and structures	C081	Building
		C082	Other built structure
C090	Permanent made surfaces	C091	Metalled roadway
		C092	Railway
		C093	Pathway
		C094	Other made surface
C100	General land surfaces	C101	Multiple surface
		C102	Bare surface

Appendix A3. National Land use database: land use and cover classification. Table following Harrison, (2006).

Anopheles atroparvus

GAGCTGGTCTTGTATCTCTGCTGCTATGGTTGGGGGTAACCATGAGATACACGCAGCAGCTGGTGCTTC
TCCGTTAGGTAACGCCTCACGATGACCGAACTGGGCCGAACACGCTACACAGCACGCTGATGGTGGTG
AGGTGTCAGCCCCATGGCCACTTTGCAAGTTGAAACCTGGGGTTGCTACACGCTACGACTTCGATGCAA
GAGAAAGGATGGCGTATCCAGACCCTTTCATCAACTCCACGTACGGTGAGGTACGCCGTTTGGCTTGG
GTTATGATCAAATATGGGCACTCAAAAATGTGTACATCGAGCTGTGTCGCACGATGCGCAATATGCGTT
CAACTTATCGGTGTTCATGTGTCCGCAGTTCACACATTGAACATAATTGTTGAACGCGTGGTGCTATCGT
GGGAGCGGTTTGATGTACACGTTTTTGAGTGCCCATATTTGATCATAACCCAAGCGAAACGGTGTAGCT
CACGAACGTGGAGTTGATGAATGGGTCTGGATACGCCTTCCTTTCTCTTGCATCGAAGTCTTAGGGTGT
AGCAACCCAGTTTTCAACTTGCAGAGTTGGCCATGGGGCTGACACTCACACCATCGGCATGCTGTGTAA
CGGGTGTCGTCCTAGTATCATCGTTGAGGGGCCTTTACTTAACGAAGAACACCAGCTCTGCGGGTATCT
AAGGCGTACCGCAGCGTAGCACAAAGATCGAGACAGATCTGACACAGAGCTCATGGATTCACATTTGT
GAGAACTACCCCTTAATTTTAGCCTTACA

Anopheles daciae/messeae

ATACGCTGAGTCCGCGGCTAGGAGCTGGTCTTGTATCTCTGCTGCTATGGTTGGGTTAGAACCACGAG
ATACGCGCAACAGTGTGTGCTTCCCCGTTAGGTGACGCCTCACGATGACCTTACTGGGCCGAACACGCT
ACACAGCACGCTGATGGTGGTGAGGTGTCAGCCCCATGGCCACTTTGCAAGTTGAAACCTGGGGTTGC
TACACGCTGCGCTTTCAATGCAAGAAATGGATGGTGTTCCAAACTCTTTCATCATCATGCACGTACACG
GAGGTACGTAGTTTGACTTGAATGGGTCAAATATGGGCACTCAAAAATGTGTACATCGAGCTGTGTCG
CACGATGCGCAATATGCGTTCAACTTATCGGTGTTCATGTGTCTGGAAGTTCACAAATTGAGATGATCA
GTTGAACGCATATGGCGCATCGTGCGACACAGGCTGGATGTACACATTTTTGAGGTGCCCATATCTGAC
CCATTCAAGTCGAACTACGTACCTCCCTGTACGTGCATGATGATAAAAGAGTTTGGGAACACCATCATA
TCTTGCATTGAAAGCGCAGCGTGTAGCAACCCGGGTTTCACTTGCAAAGTGGCATGGGGCTGACACTC
ACACCATCGCGTGACTGTGGTAGCGTGGTTTCGGCCAAGTAAGGTCATCGTGTGGGCGTTCCCTAAGG
GGGCAAGCCAACACTTCTGGCGCGTGATTTAGGCTTCTACCAGCAGTAGCACCATAGATACAGACAGA
CTCCGGAGCCCGCGGGGCCTCAAGAGGCCTCACTGTGGTGGTGAGAAATTACCCCTAAAAGTTTAAAG
CCTTAAAAA

Appendix A4: Example *Anopheles maculipennis* s.l. species identification sequences (*ITS-2*). *Anopheles daciae/messeae* sequences produce identical results in BLAST searches.

Cow *Bos taurus*

TGGGTGAAAAAATCAAATAGTGTTGATATAGAATAGGGTCTCCTCCTCCTGCCGGGTCGAAGAAGGTT
GTATTTAGGTTCCGGTCTGTTAATAGCATTGTGATGCCGGCTGCTAATACAGGGAGCGAGAGTAGTAG
TAGTACGGCGGTAATTATTACGGATCATACGAACAGAGGGGTTTGGTATTGTGACATTGCGGGGGGCT
TTATGTTGATAATTGTTGTAATGAAGTTGATGGCTCCTAAAATTGAGGAAACTCCTGCTAAGTGTAAAG
AGAAAATGGTTAGATCTACTGAAGCTCCTGCATGGGCTAGGTTGCCTGCTAAGGGAGGGTACACGGTT
CAGCCTGTTCCTGCCCCAGCTTCAACTATAGAGGATGCGAGGAGTAGTAGGAATGAGGGAGGGAGGA
GTCAGAAGCTTATATTATTTATTCGGGGAAATGCTATATCGGGAGCACCAATTATTAGGGGAACAAGTC
AGTTACCGAATCCTCCAATTATGATTGGTATTACTATGAAGAAGATTATTACAAATGCGTGTGCGGTTAC
AACTACGTTGTAGATTTGGTCGTCTCCGAGCAGAGTTCCGGGTTGGCCTAATTCAGCGCGAATTAGAAG
GCTTAGAGCTGTTCCTACTATACCGGCCCAAGCACCAAATAGTAGATAAAGGGTACCCATCTCCTTCTG
GTTGGTTGAGAACTGGCCGTCCGTTTTAGAAA

European rabbit *Oryctolagus cuniculus*

TGTCAGATAGATAGGTGTTGGTAGAGGATAGGGTCTCCTCCTCCTGCAGGATCAAAGAAGGTTGTATTT
AAGTTTCGGTCTGTTAAAAGCATTGTAATGCCAGCAGCTAGGACCGGTAAAGAGAGAAGAAGAAGTAC
GGCTGTGATTAGAACAGATCATACGAATAAGGGGGTTTGATATTGAGATATTGCAGGGGGTTTCATAT
TAATAATAGTTGTAATAAAGTTAATAGCCCCTAAAATAGATGATACTCCAGCTAAGTGAAGGGAGAAA
ATAGTAAGATCCACTGAGGCTCCAGCATGTGCAAGATTACCGGCTAGAGGTGGATAAACAGTTCAGCC
AGTCCCCGCCCCAGCTTCTACTATTGAGGAGGCTAGTAGAAGAAGGAATGAAGGGGGGAGAAGTCAG
AAGCTCATATTATTTATTCGGGGGAAGGCTATGTCAGGAGCCCCAATTATCAGGGGGACAAGCCAGTT
CCCGAAGCCTCCAATTATAATAGGTATGACTATAAAGAAGATTATTACAAAGGCATGTGCGGTGACGAT
TACATTATAGATTTGATCATCCCCGAGTAGAGTCCCTGGCTGACCTAATTCTGCTCGAATTAGCAGGCTA
AGGGCTGTTCCCACCATCCCAGCTCAAGCTCCAAATAGGAGATAAAGAGTGCCCATGTCCTTGTGGTTG
GTTGAGAACTGCGCCGTTTTTTCAAAA

Barn swallow *Hirundo rustica*

CAGGAAGATGGTACCCTATACTTAATCTTCGGCGCATGAGCCGGCATGGTAGGTACCTCCCTCAGTCTC
CTAATCCGAGCAGAATTAGGCCAACCTGGCGCCCTACTCGGAGACGACCAAATCTACAATGTGGTAGTT
ACAGCCCACGCTTTCGTAATAATCTTCTTCATAGTTATGCCAATTATGATCGGAGGATTCGGAAACTGAC
TAGTTCCCCTAATAATCGGCGCCCCCGACATAGCATTCCCACGAATGAACAACATAAGCTTCTGACTACT
TCCCCCATCATTCCTCCTCCTCCTAGCCTCATCCACGGTAGAAGCAGGAGTAGGTACTGGATGGACCGT
ATACCCGCCCCTAGCCGGAAACCTAGCACACGCAGGGGCCTCTGTAGACCTGGCCATTTTCTCCCTACA
TCTAGCAGGAATTTCCTCAATCCTAGGTGCAATCAACTTTATCACCACAGCAATCAACATAAAACCCCCA
GCTCTATCACAGTACCAAACACCACTATTCGTCTGATCAGTATTAATCACCGCAGTTCTTCTTCTCCTATC
ACTACCCGTACTAGCCGCTGGCATCACAATGCTACTTACAGACCGCAACCTAAACACTACCTTCTTCGAT
CCAGCTGGAGGAGGAGACCCAGTACTTTACCAACACCTATTCTGATTCTTCGGCCACCCAGAAGTCTAG
CAGAGAAGAGAAAAAGTACTTACGTTTTTTATCCCGTTTGACTAACAAACAAATGGTGCAAAAAGCAAG
AAAACAGAACTCTA

Appendix A5: Example blood meal identification sequences (*COI*).

```
# run script for Latin square randomisation
result <- matrix( "", 4, 4)
okay <- F
while( ! okay ){
result[1,] <- sample( c("collector 1","collector 2","collector 3","collector 4"), 4, replace=F)
result[2,] <- sample( c("collector 1"," collector 2"," collector 3"," collector 4"), 4, replace=F)
result[3,] <- sample( c("collector 1"," collector 2"," collector 3"," collector 4"), 4, replace=F)
result[4,] <- sample( c("collector 1"," collector 2"," collector 3"," collector 4"), 4, replace=F)
if( ( all( table( result[,1] ) == 1 ) ) &
( all( table( result[,2] ) == 1 ) ) &
( all( table( result[,3] ) == 1 ) ) &
( all( table( result[,4] ) == 1 ) ) ) okay <- T}
result
```

Appendix A6: R script for Latin square randomisation of collectors, provided by Dr Lara Harrup (TPI). # indicate lines not included in the code.

(A)

```
# fitting GLMM to human landing catch data using the glmmADMB package; follow installation instructions at http://glmmadmb.r-forge.r-project.org/

require(sandwich)

require(msm)

require(lme4)

require(MASS)

require(glmmADMB)

# load data

p <- read.csv("datafile.csv")

# identify factors in the data

p <- within(p, {

   farm <- factor(farm, levels = 1:3, labels = c("Northney", "Bisley", "Elmley"))

   volunteer <- factor(collector)

   site <- factor(site)

   rainfall <- factor(rainfall)})

# check structure of data

str(p)

# with the glmmadmb package, need to remove the NAs from the data before running the model; run na.omit function on the data

pmod <- na.omit(p)

# fit models using pmod and look at summary of models

# first fit a standard poisson model

summary(modelpois <- glmmadmb(mosq_catch ~ farm + (1 | volunteer) + (1 | site) + sunset + temp + windspeed + rainfall,

data=pmod,

zeroInflation = FALSE,

family = "poisson"))
```

Appendix A7 (A): R script for running GLMM analysis for human landing catch data assessing total biting pressure, chapter 3. # indicate lines not included in the code. Code is continued onto the following page.

```
# fit negative binomial model

summary(modelNB <- glmmadmb(mosq_catch ~ farm + (1 | volunteer) + (1 | site) + sunset + temp + windspeed + rainfall,

data=pmod,

zeroInflation = FALSE,

family = "nbinom"))

# compare AIC values

AIC(modelpois, modelNB)

# The lowest AIC values are for modelNB

# To test effect of single fixed factor or covariate (not random factors), sequentially refit modelNB excluding each factor and variable and compare the AIC values to the full model

AIC(modelNB, #modified model here) # etc. etc.

# if the AIC value is >2 units greater than that of the original model then the variable is a significant predictor of 'mosq_catch'. # If not, then can omit variable from the model

# run final, simplified model

summary(modelNB <- glmmadmb(mosq_catch ~ farm + (1 | volunteer) + (1 | site) + sunset + windspeed,

data=pmod,

zeroInflation = FALSE,

family = "nbinom"))
```

Appendix A7 (A) continued.

(B)

```
# fitting a GLMM to the data for Coquillettidia richiardii only

require(sandwich)

require(msm)

require(lme4)

require(MASS)

require(glmmADMB)

Cqrichdata <- read.csv("Cq rich data.csv")

# identify factors in the data

Cqrichdata <- within(Cqrichdata, {

  volunteer <- factor(volunteer)

  site <- factor(site)

  })

# check structure of data

str(Cqrichdata)

# with the glmmadmb package, need to remove the NAs from the data before running the model; run na.omit function on the data

Cqrichdata2 <- na.omit(Cqrichdata)

# fit models and look at summary of outputs

# first fit a poisson model

summary(modelpoisCqrich <- glmmadmb(mosq_catch ~ (1 | volunteer) + (1 | site) + sunset + temp + windspeed,

data=Cqrichdata2,

zeroInflation = FALSE,

family = "poisson"))

# fit a negative binomial model

summary(modelNBCqrich <- glmmadmb(mosq_catch ~ (1 | volunteer) + (1 | site) + sunset + temp + windspeed,

data=Cqrichdata2,

zeroInflation = FALSE,

family = "nbinom"))

# compare model fit using AIC

AIC(modelpoisCqrich, modelNBCqrich)
```

Appendix A7 (B): R script for analysis of human biting activity of *Coquillettidia richiardii* at Elmley, chapter 3. Continued on the following page.

```
# run neg bin model without temp

summary(modelNBCqrichnotemp <- glmmadmb(mosq_catch ~ (1 | volunteer) + (1 | site) + sunset + windspeed,

data=Cqrichdata2,

zeroInflation = FALSE,

family = "nbinom"))

# compare the full model and model without temperature using AIC

AIC(modelNBCqrich, modelNBCqrichnotemp)

# sequentially re-run model excluding each covariate (temperature, wind speed and sunset, comparing AIC values to the full model. More than a 2 unit increase in AIC means the covariate significantly influences the biting rate.

# obtain and run final, simplified model:

summary(modelNBqrichfinal <- glmmadmb(mosq_catch ~ (1 | volunteer) + (1 | site) + sunset + windspeed,

data=Cqrichdata2,

zeroInflation = FALSE,

family = "nbinom"))
```

Appendix A7 (B) continued.

(C)

```
# fitting a GLMM to the data for Culex modestus only

Cxmoddata <- read.csv("HLC Cx mod data.csv")

# identify factors in the data

Cxmoddata <- within(Cxmoddata, {

  volunteer <- factor(volunteer)

  site <- factor(site)

  })

# check structure of data

str(Cxmoddata)

# Remove the NAs from the data before running the model; run na.omit function on the data

Cxmoddata2 <- na.omit(Cxmoddata)

# fit models and look at summary of outputs

# first fit a poisson model

modelpoisCxmod <- glmmadmb(mosq_catch ~ (1 | volunteer) + (1 | site) + sunset + temp + windspeed,

data=Cxmoddata2,

zeroInflation = FALSE,

family = "poisson")

# fit a negative binomial model

modelNBCxmod <- glmmadmb(mosq_catch ~ (1 | volunteer) + (1 | site) + sunset + temp + windspeed,

data=Cxmoddata2,

zeroInflation = FALSE,

family = "nbinom")

# compare model fit using AIC

AIC(modelpoisCxmod, modelNBCxmod)

# sequentially re-run model excluding each covariate (temperature, wind speed and sunset, comparing AIC values to
the full model. More than a 2 unit increase in AIC means the covariate significantly influences the biting rate.

# obtain the final, simplified model and run

summary(modelNBCxmodfinal <- glmmadmb(mosq_catch ~ (1 | volunteer) + (1 | site) + sunset,

data=Cxmoddata2,

zeroInflation = FALSE,

family = "nbinom"))
```

Appendix A7 (C): R script for analysis of human biting activity of *Culex modestus* at Elmley, chapter 3.

```
# script for analysing resting box data for blood-fed mosquitoes
require(sandwich)
require(msm)
require(lme4)
require(MASS)
require(glmmADMB)
# load data
p <- read.csv("restingboxbfsmorning.csv")
# make collection time ("time"), month and rainfall into factors
p <- within(p, {
 month <- factor(month, levels = 1:5, labels = c("june", "july", "august", "september", "october"))
 rainfall <- factor(rainfall, levels = 0:1)
})
# initially try fitting poisson model
model1 <- glmmadmb(bloodfeds ~ box + (1 | date) + rainfallbin + windspeed + temp + rel.hum,
                   data = p,
                   zeroInflation = FALSE,
                   family = "poisson")
# fit negative binomial model
model2 <- glmmadmb(bloodfeds ~ box + (1 | date) + rainfallbin + windspeed + temp + rel.hum,
                   data = p,
                   zeroInflation = FALSE,
                   family = "nbinom")
# compare AIC values
AIC(model1, model2)
# Poisson model (model1) best fit
# conduct multiple comparisons analysis
summary(glht(model1, linfct=mcp(box="Tukey")))
```

Appendix A8: R script for running analysis on resting box data in chapter 5. Script is for all mosquito species combined; the same script applies for analysis of individual species. # indicate lines not included in the code.

```
# script for analysing success of blood meal ID for different Sella stages

# load packages

require(sandwich)

require(msm)

require(lme4)

require(MASS)

require(multcomp)

# load data

success <- read.csv("bm success data.csv")

# convert Sella stage into a factor, levels 2:6, seq_positive is dependent variable

success <- within(success, {

  stage <- factor(stage, levels = 2:6)

})

# run binomial GLMM with logit link function

modelsuccess <- glmer(seq_positive ~ stage + (1 | mosquito), data = success, family = binomial)

# conduct multiple comparisons

summary(glht(modelsuccess3, linfct=mcp(mosquito="Tukey")))
```

Appendix A9: R script for running analysis for blood meal identification success in chapter 5. Script is for all mosquito species combined; the same script applies for analysis of individual species. # indicate lines not included in the code.

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