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Renita Danabalan

PhD Ecology

Mosquitoes of southern England and northern Wales: Identification, Ecology and Host selection.

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Abstract

As early as 1901, ecological and epidemiological studies were conducted to understand malaria transmission in the UK. Unfortunately, following the eradication of malaria after WWII, ecological studies on local mosquito species has been intermittent, leading to a significant gap in knowledge of the current habitat preference, distribution and vector capabilities of the 33 recorded species. This lack of current information makes the assessment of possible transmission of enzoonotic diseases such as Chikungunya and West Nile virus in UK difficult. Thus the overall purpose of this thesis was to facilitate the identification of potential vector species through the documentation and characterisation of the ecology of adult and larval stages, and the host selection of British mosquitoes, in southern England and northern Wales.

A total of 13 out the 33 documented species are assessed in this study. Of which members of the Maculipennis and Pipiens Group comprised the bulk of the adult and immature collections respectively. The development of the ITS2 PCR-RFLP assay in this study allowed the identification of the three members of the Maculipennis Group, which revealed the widespread occurrence of the recently documented *An. daciae* in almost all localities sampled. While previously published assays discriminating the Pipiens Complex, did not yield congruent results questioning the prior identification methods and the validity of the taxonomic status of its members. In addition, host-specific primers designed herein to determine host selection in local mosquitoes revealed an indiscriminate host selection by *An. atroparvus*, *An. daciae*, *An. messeae* and *Cx. pipiens* thus indicating their potential role as vectors in the UK.

Chapter 1

General Introduction

1. General Introduction

1.1 History of Mosquito Systematics

For the first time, following Ross's (1897) publication of the role of mosquitoes in the transmission of malaria, serious efforts were taken to actively describe as many mosquito species as possible and to identify taxonomic groupings. In 1910, F. V. Theobald published his 'Monograph of the Culicidae' proposing the first classification of mosquitoes. This and other early taxonomic studies on the European fauna by F.W. Edwards, resulted in an initial classification of the family Culicidae, which comprised three subfamilies (Edwards, 1932): Culicinae (the "true mosquitoes") and Dixidae and Chaoboridae (both "midges") (Figure 1.1). Despite much scientific debate on whether the midges should be included as subfamilies of the family Culicidae, the taxonomic framework of Edwards (1932) was upheld until Knight & Stone (1977) formally removed the Dixidae and Chaoboridae from within the family Culicidae. This key change recognised Anophelinae, Culicinae and Toxorhynchitinae as subfamilies within Culicidae (Stone, 1956) and the reorganisation of the subfamily Culicinae into 10 recognised tribes (Belkin, 1962) resulted in the working framework of mosquito classification used today (Munstermann & Conn, 1997) (Figure 1.2).

The biomedical importance of many mosquitoes and the potential application that phylogenetics could provide in terms of answering questions on vector capacity, diversification of mosquitoes and epidemiology (Sallum *et al*., 2000; Krzywinski *et al*., 2001a, b) has resulted in a shift from descriptive taxonomy to natural classification (Zavortink, 1990; Reinert *et al*., 2004, Shepard *et al*., 2006). Ross (1951, in Harbach & Kitching, 1998) made the first attempt to create 'intuitive' phylogenetic trees based on morphology and species distribution. Despite the biomedical importance of mosquitoes, these hypotheses remained largely unchallenged for fifty years (Harbach & Kitching, 1998). According to Zavortink (1990), natural classification allows for species to be studied in greater detail thus accurately identifying genetic relationships at the generic and sub-generic level. Harbach & Kitching (1998) attempted natural classification based on morphological

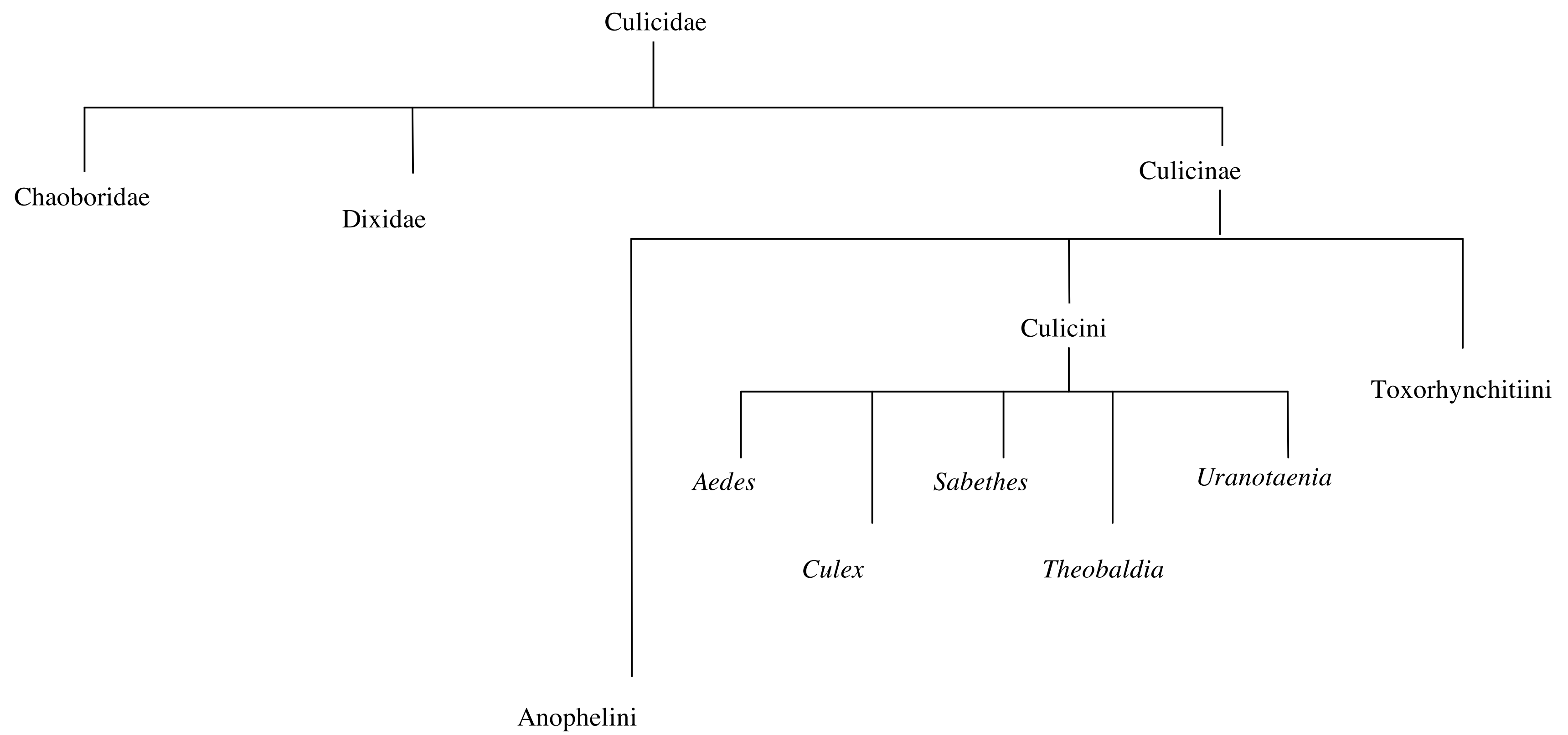

Figure 1.1 Classification of the Family Culicidae after Edwards (1932)

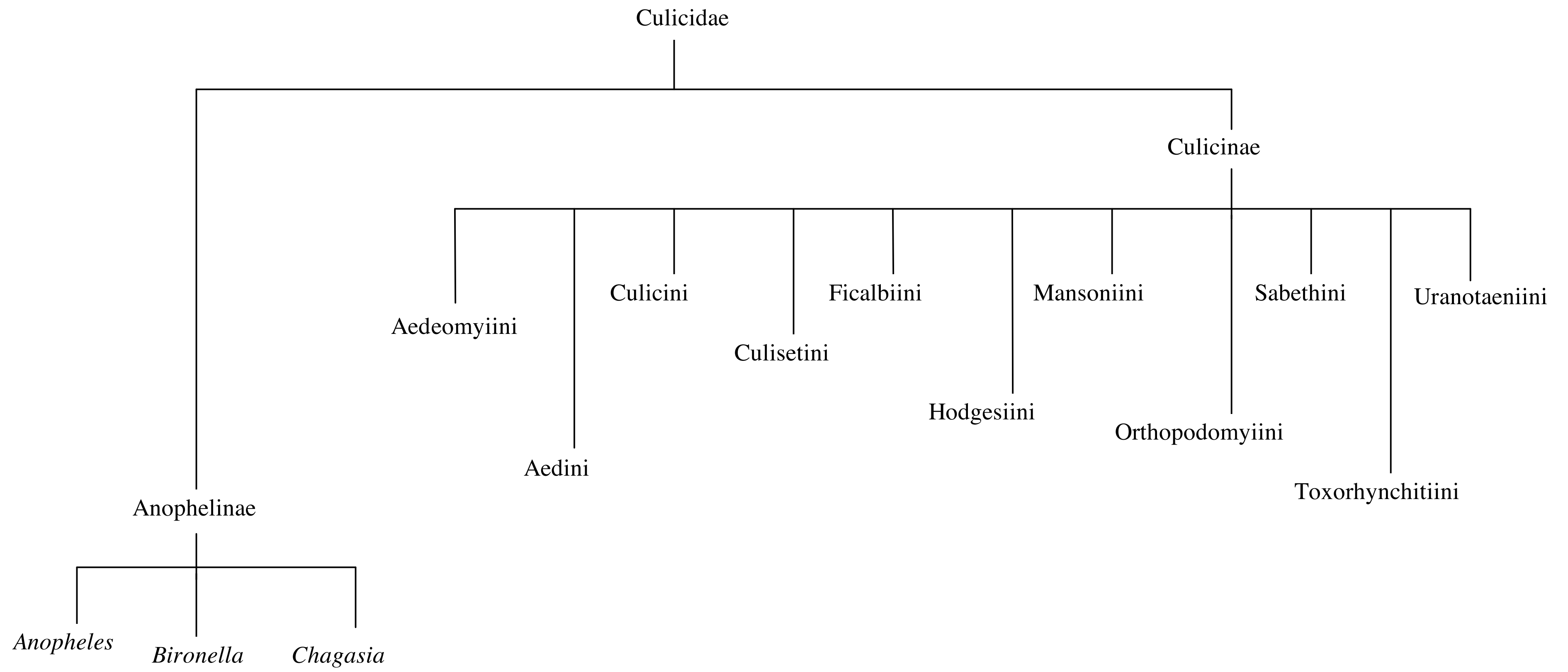

Figure 1.2 Organogram of Family Culicidae after Belkin (1962) and Harbach & Kitching (1998).

characters of larvae, pupae and adults. They found relationships within tribes of subfamily Culicinae to be poorly resolved. This could be explained by hybridisation of characters occurring between 2 or more taxa (Belkin, 1962; Zavortink, 1990), thus implying that mosquitoes within tribes are derived from the same ancestor (Harbach & Kitching, 1998).

One explanation for the lack of effort in mosquito systematics could simply be that formal species descriptions and identification keys have taken precedence due to their biomedical importance in disease transmission (Besansky & Fahey, 1997; Krzywinski *et al*., 2001a, b). However, the relatively recent advent of sophisticated cladistics methods to analyse morphological characters and the application of molecular techniques to mosquito systematics have catalysed the current heightened levels of activity in Culicid systematics (Harbach & Kitching, 1998), leading to several high-level phylogenies, particularly those dealing with the Tribe Aedini (Reinert, 2000; Reinert *et al.,* 2004; Reinert & Harbach, 2005; Reinert *et al.,* 2006; Reinert *et al.,* 2008; see section 1.1.2).

1.1.1 Internal Systematics of the Subfamily Anophelinae

According to the morphological study of Harbach & Kitching (1998), the subfamily Anophelinae comprises three genera: *Anopheles*, *Bironella* and *Chagasia*. Given the minor reported morphological differences between mosquitoes of genera *Bironella* and *Anopheles* (Belkin, 1962; Tenorio, 1977), Sallum *et al*. (2000) investigated the relationship of the genus *Bironella* within the subfamily Anophelinae. Based on morphology, Sallum *et al*. (2000) found that species in genera *Anopheles* and *Bironella* comprised three major lineages: Lineage 1 (proposed as the oldest) - Neotropical subgenera *Nyssorhynchus* and *Kerteszia*; Lineage 2 – the monophyletic subgenus *Cellia* (most closely related to lineage 3); and Lineage 3 which was paraphyletic, consisting of the subgenera *Anopheles*, *Lophopodomyia*, *Stethomyia* and the purportedly separate genus *Bironella*. That *Bironella* occurred as a subgenus within genus *Anopheles* (Sallum *et al*., 2000) contradicted the earlier findings of Harbach & Kitching (1998).

Morphological studies (Harbach & Kitching, 2005) and molecular studies (Krzywinski *et al*., 2001a, b) agree with the proposed monophyly of the subgenera *Nyssorhynchus*, *Kerteszia* and *Cellia*. However, their findings disagree with the placement of *Bironella* as a subgenus within *Anopheles* by Sallum *et al.* (2000). Using molecular data, *Bironella* was

placed basal to *Anopheles*, suggesting that it diverged much earlier and provided evidence for its separate generic status (Krzywinski *et al*., 2001b). In 2002, Sallum *et al*. re-examined the phylogeny of *Anopheles* and *Bironella* using both morphological and molecular characters. Their results still showed *Bironella* to be a distinct group within the genus *Anopheles*. As such the relationship of *Bironella* with respect to the genus *Anopheles* remains unresolved (Sallum *et al*., 2002).

Currently, the genus *Anopheles* includes 459 formally named and more than 50 provisionally designated extant species divided between seven subgenera: *Anopheles*, *Baimaia*, *Cellia*, *Kerteszia*, *Lophopodomyia*, *Nyssorhynchus* and *Stethomyia* (Harbach & Howard, 2007; updated on Mosquito Taxonomic Inventory website, 2008).

1.1.2 Internal Systematics of the Subfamily Culicinae

The subfamily Culicinae is the largest subfamily in Culicidae, comprising the tribes Culicini and Aedini (Harbach & Kitching, 1998). The tribe Aedini is the largest in subfamily Culicinae consisting of 1,235 recognised species to date (Reinert *et al*., 2006). According to Belkin (1962), the tribe Aedini appeared not to be a monophyletic group, but more a heterogeneous mixture of species that were superficially similar, making the construction of a natural classification challenging. One approach was to divide the species of the tribe into smaller groups by creating more genera, thus accurately determining phylogenetic relationships (Zavortink, 1990). Reinert (2000) provided morphological evidence based on characters of the male and female genitalia, 4th larval instar and one pupal character to elevate mosquitoes of the subgenus *Aedes* (*Ochlerotatus*), to generic status as *Ochlerotatus*. Of the thirteen British species in the genus *Aedes* at that time, only two remained in the genus *Aedes*, with the other eleven being transferred to the genus *Ochlerotatus* following the work of Reinert (2000) (Table 1.1). Although the results of this study were widely challenged at the time by fellow entomologists (AMCA, 2002), the elevation of *Ochlerotatus* has since been further supported by additional morphological studies (Reinert *et al*., 2004) and by molecular data (Spanakos *et al.*, 2006).

Following the papers of Harbach & Kitching (1998) and Reinert (2000), the authors collaborated to re-evaluate the entire internal classification of tribe Aedini using 172 morphological characters of both the adult and larval stages, representing the recognised 12

genera and 56 subgenera (Reinert *et al*., 2004). In some cases, the resulting phylogenies were shown to vary depending on the subsets of data used, character codings and weightings applied. However, some 55 groups were consistently identified as monophyletic regardless of data and weighting used and these robust subgeneric groupings were elevated to full generic status, creating a further 43 new genera. Shepard *et al*. (2006) maintained that whilst the characters used by Reinert *et al*. (2004) were diagnostic for species identification, they lacked sufficient depth to be employed in resolving evolutionary relationships. This reorganisation of the tribe Aedini caused so much controversy, especially amongst US mosquito workers who suggested that the evidence was not strong enough for such a radical shake to the taxonomic stability, that an internet based forum was established for the sole purpose of discussing these changes (Walter Reed Biosystematics Unit, 2008). Major concerns included the lack of inclusion of readily available molecular data to confirm these elevations, and the taxonomic instability that such a large number of changes would cause, thus leading to confusion over taxon names particularly amongst non-scientific mosquito-personnel. The most controversial of these is the name change of the Asian tiger mosquito, from *Aedes (Stegomyia) albopictus* to the almost unrecognisable *Stegomyia albopicta* (Reinert & Harbach, 2005).

The revision of the internal systematics of the tribe Aedini is still ongoing. Reinert *et al.* (2006) have recently reevaluated the classification and phylogeny of *Finlaya*. Based on the morphology of the egg, fourth instar larvae, pupae and adults and on the preferred habitats of the aquatic stages, another 11 subgenera were elevated to generic status; thus resulting in further name changes. The British species *Aedes (Finlaya) geniculatus* (Olivier, 1919), changed to *Ochlerotatus geniculatus* (Reinert *et al*., 2004), has been changed again to *Dahliana geniculata,* following the most recent review of *Finlaya* mosquitoes (Reinert *et al*., 2006). It seems clear that the taxonomic community must clarify these changes by adopting integrated molecular and morphological character assessments before a final resolution is agreed upon.

Based on the number of taxa in the family Culicidae, Zavortink (1990) proposed that the internal classification would comprise 225 genera; by this proposal, the tribe Aedini should comprise circa 87 genera (Reinert *et al.,* 2004). At the moment, 64 separate genera have been recognised in the tribe Aedini (Mosquito Taxonomic Inventory, 2008). The reassessment of this tribe Aedini is a four-phase project aimed to create a natural

classification of the tribe (Reinert *et al.,* 2006), as such we can probably expect further changes to the internal systematics of Aedini in the future.

Unlike Aedini, tribe Culicini was found to be a monophyletic group (Harbach & Kitching, 1998) comprising of 4 currently recognised genera: *Culex, Deinocerites, Galindomyia* and *Lutzia* (Belkin, 1962). Navarro & Liria (2000) determined the phylogenetic relationships of eighteen Neotropical Culicini species based on 30 characters of the larval mouthparts; their findings supported the proposed monophyly of the tribe by Harbach & Kitching (1998). However, their analysis indicated that the genus *Deinocerites* arises well within the genus *Culex*, contradicting the sister-group relationship proposed by Harbach & Kitching (1998). Further, they suggested the reduction of *Deinocerites* to a subgenus within *Culex* (Navarro & Liria, 2000). Given the number of taxa occurring within *Culex*, a number of morphological characteristics have been found to be polymorphic (Harbach & Kitching, 1998) accounting for the poor resolution encountered between subgenera based on morphological assessments alone (Navarro & Liria, 2000).

The genus *Culex* has a worldwide distribution and comprises more than 762 species in 26 subgenera (Mosquito Taxonomic Inventory, 2008) with *Culex pipiens* being the nominotypical species for this genus (Linnaeus, 1758, in Harbach *et al.,* 1985). Following the differentiation of *Culex torrentium* based on the structure of the male phallosome (Martini, 1925), the internal systematics of the genus *Culex* began to take shape. Sirinivanakarn (1976) initially proposed the subgenus *Culex* to be split into two groups: Pipiens Group and Sitiens Group. The addition of two Nearctic species allowed for the Pipiens Group to be further divided into 4 subgroups (Figure 1.3). Due to the morphological and genetic overlap between the species within the two subgroups, Dahl (1978) proposed that the Pipiens and Trifilatus subgroups be collapsed. However, based on distinct differences on the male genitalia between the Pipiens and Trifilatus subgroups, this proposition was overturned by Harbach (1988) and the division of subgenus *Culex* was reverted to the four groups previously listed.

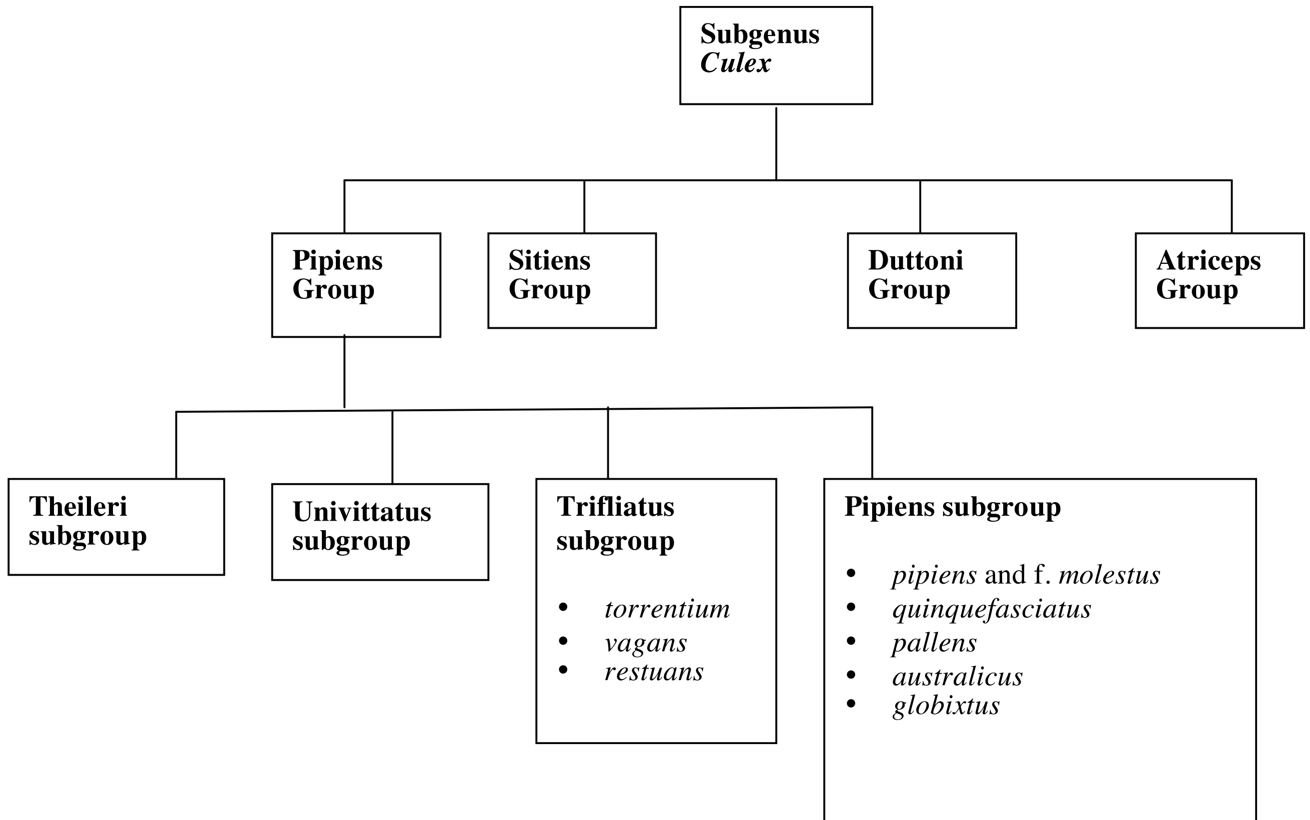

Figure 1.3 Organogram of the subgenus *Culex*, following the most recent classification of Harbach (1988).

1.2 British Mosquitoes

1.2.1 Species List and Feeding Preferences

Mosquitoes of the British Isles comprise thirty-four recognised species in eight genera: *Aedes* (3 sp.), *Anopheles* (6 sp.), *Culex* (4 sp. plus one form), *Dahliana* (1 sp.), *Culiseta* (7 sp.), *Coquillettidia* (1 sp.), *Ochlerotatus* (11 sp.) and *Orthopodomyia* (1 sp.) (Snow, 1990; Ramsdale & Snow, 2000, Linton *et al.,* 2002a; Linton *et al.,* 2005; Reinert *et al.,* 2006) (Table 1.1). Following the eradication of human malaria after WWII (Dobson, 1989) and the subsequent lack of importance of British mosquitoes as vectors, little is known about the current distribution, ecology and feeding behaviour of these mosquitoes. Data gathered from previously published reports on the preferred hosts of British mosquitoes (Table 1.1) show, 28 species feeding on human hosts, whilst only 13 are thought (but not confirmed) to be ornithophilic (Table 1.1). *Culiseta annulata* is opportunistic in its feeding behaviour, possibly feeding on mammals, man and birds. *Culex europaeus* is unusual in that it is the only British species to feed primarily on amphibians and reptiles. The host preference of *An. daciae* and *Cs. fumipennis* has not yet been determined. Determination of host preferences can provide valuable information on the potential vectorial capacity of these mosquitoes, thus enabling the identification and incrimination potential vectors of animal and human diseases as well as bridge vectors of zoonotic diseases, in the UK.

SPECIES	HOST PREFERENCE
Aedes cinereus (Meigen, 1818)	cattle, mammals, man, birds
Aedes geminus Peus 1970	?
Aedes vexans (Meigen, 1830)	man
Anopheles algeriensis Theobald, 1903	man
Anopheles atroparvus Van Thiel, 1927	man
Anopheles claviger (Meigen, 1804)	mammals, man
Anopheles daciae Linton, Nicolescu & Harbach, 2004	?
Anopheles messeae Falleroni, 1926	man
Anopheles plumbeus Stephens, 1828	mammals, man, birds
Coquillettidia richiardii (Ficalbi, 1889)	birds, cattle, man
Culex europaeus Da Cunha Ramos, Ribeiro & Harrison (2003)	amphibians, reptiles, man
Culex modestus Ficalbi, 1889	man
Culex pipiens Linnaeus, 1758 (f. *pipiens*)	birds
f. *molestus* Forskål 1775	man
Culex torrentium Martini, 1925	birds
Culiseta annulata (Schrank, 1776)	man, mammals, birds
Culiseta alaskaensis (Ludlow, 1906)	man
Culiseta fumipennis (Stephens, 1825)	?
Culiseta litorea (Shute, 1928)	mainly birds, man
Culiseta longiareolata (Macquart, 1838)	birds
Culiseta morsitans (Theobald, 1901)	mainly birds, man
Culiseta subochrea (Edwards, 1921)	man
Dahliana geniculata (Olivier, 1919)	man
Ochlerotatus annulipes (Meigen, 1830)	mammals, man
Ochlerotatus cantans (Meigen, 1830)	mainly cattle, also man/birds
Ochlerotatus caspius (Pallas, 1771)	man
Ochlerotatus communis (De Geer, 1776)	man
Ochlerotatus detritus (Haliday, 1833)	mainly cattle, also man/birds
Ochlerotatus dorsalis (Meigen, 1830)	cattle, man
Ochlerotatus flavescens (Muller, 1764)	mammals, man
Ochlerotatus leucomelas (Meigen, 1804)	man
Ochlerotatus punctor (Kirby, 1837)	mainly cattle, also man/birds
Ochlerotatus rusticus (Rossi, 1790)	man
Ochlerotatus sticticus (Meigen, 1838)	man
Orthopodomyia pulcripalpis (Rondani, 1872)	birds

Table 1.1 List of British Culicid fauna, showing the 33 reported species and feeding preferences where known (Snow, 1990; Ramsdale & Snow, 1995; Medlock *et al.*, 2005; Linton *et al.*, 2005).

1.2.2 Distribution of British Mosquitoes

As part of an investigation into vectors of malaria in Britain, Nuttall *et al.* (1901) plotted the known distributions of *Anopheles* mosquitoes in England and Wales and Marshall (1938) provided a comprehensive distribution of the mosquitoes in the U.K. Since then a series of papers have reviewed the distribution of British mosquitoes by genus: *Anopheles* (Rees & Snow, 1990), *Culex* (Rees & Snow, 1992), *Coquillettidia*, *Culiseta* and *Orthopodomyia* (Rees & Snow, 1994), *Aedes* (Rees & Snow, 1995) and *Ochlerotatus* [Rees & Snow, 1996 (as a subgenus of *Aedes*)]. The data used to create these maps were obtained from Marshall (1938), Cranston *et al.* (1987) and the British Mosquito Recording Scheme; however up-to-date distribution data is needed, especially at species level for potential vectors and members of species complexes, for e.g. Maculipennis Complex.

1.2.2.1 Distribution of the subfamily Culicinae in the UK

Prior to publications by Rees & Snow (1990, 1992, 1994, 1995, 1996), no distribution maps for British mosquitoes of the genera *Aedes* (including *Ochlerotatus* and *Dahliana* species), *Culex*, *Orthopodomyia*, *Coquillettidia* and *Culiseta* had appeared in the literature. *Culex* species appear to be widespread, with distribution over the coastal areas of south England ranging from Norfolk through to Cornwall and Wales. However, older records for *Culex* do not differentiate between *Cx*. *pipiens* f. *pipiens*, *Cx. pipiens* f. *molestus* or *Cx. torrentium* and these were all recorded as *Cx. pipiens s.l.* (Rees & Snow, 1992). The tribe Aedini is one of the largest groups of British mosquitoes comprising 15 species in three subgenera, *Aedes, Dahliana* and *Ochlerotatus* (Reinert *et al.,* 2004; Reinert *et al.*, 2006). They range from the south coast of England to as far north as Edinburgh and into the Highlands of Scotland (Rees & Snow, 1992; Rees & Snow, 1994; Rees & Snow, 1995; Rees & Snow, 1996; Snow *et al.,* 1998) (Figure 1.4 a, b, c).

1.2.2.2 Distribution of the genus *Anopheles* in the UK

Six species of *Anopheles* (subgenus *Anopheles*) species have been recorded in Britain: *An. (An.) algeriensis*, *An. (An.) claviger and An. (An.) plumbeus* and three members of the *An. (An.) maculipennis* complex: *An. atroparvus, An. messeae* and *An. daciae* (Rees & Snow, 1990; Snow, 1990; Snow, 1998; Linton *et al.,* 2002a; Linton *et al.,* 2005) (Figure 1.5 a, b). However, the distribution maps tend to be somewhat biased, reflecting the locality of entomologists as much as that of the species studied (Snow, 1998). As current molecular techniques have improved species level identification, more up-to-date maps reflecting species distribution can and should be generated.

1.2.3 British Mosquito Species Complexes

1.2.3.1 The *Anopheles maculipennis* Species Complex

Anopheles maculipennis, the historical vector of human malaria in Europe was the first *Anopheles* species to be exposed as comprising cryptic sibling species (Falleroni, 1926; van Thiel, 1927). Studies to elucidate component members of the group included egg morphology (Falleroni, 1926; Falleroni, 1932; Corradetti, 1934; de Buck & Swellengrebel, 1934a; Hackett & Lewis, 1935; Korvenkontio *et al*., 1979), hybridisation experiments (de Buck & Swellengrebel, 1934b; Kitzmiller *et al*., 1967), ecology studies (van Thiel, 1927; de Buck & Swellengrebel, 1934b; Hackett & Missiroli, 1935), larval chaetotaxy (La Face, 1931; Diemer, 1935; Bates, 1939; Buonomini, 1940; Işfan, 1952; Suzzoni-Blatger & Sevin, 1981; Boccolini *et al*., 1986; Suzzoni-Blatger *et al*., 1990;

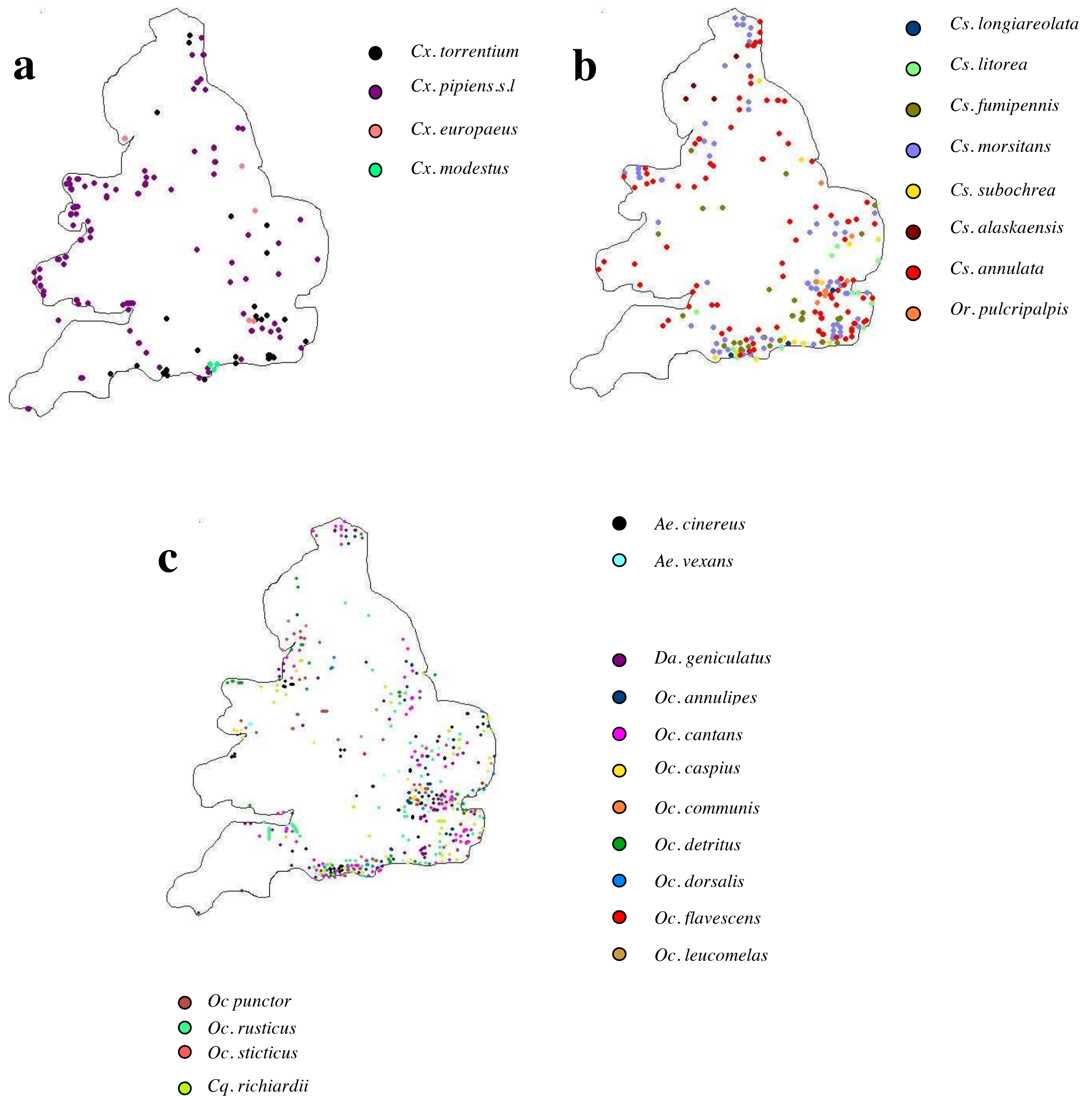

Figure 1.4 Distribution of Culicine mosquitoes in the UK. **(a)** Distribution of genus *Culex* (modified from Rees & Snow *et al*., 1992; Snow *et al.*, 1998); **(b)** Distribution of genera *Culiseta* and *Orthopodomyia* (modified from Rees & Snow, 1994; Snow *et al.,* 1998) and **(c)** Distribution of the *Aedes*, *Coquillettidia, Dahliana* and *Ochlerotatus* genera (modified from Rees & Snow, 1994; Rees & Snow, 1995; Rees & Snow, 1996; Snow *et al.,* 1998). Distribution data for *Ae. geminus* was not previously published as detection of this species was done using museum specimens (Medlock & Vaux, 2009).

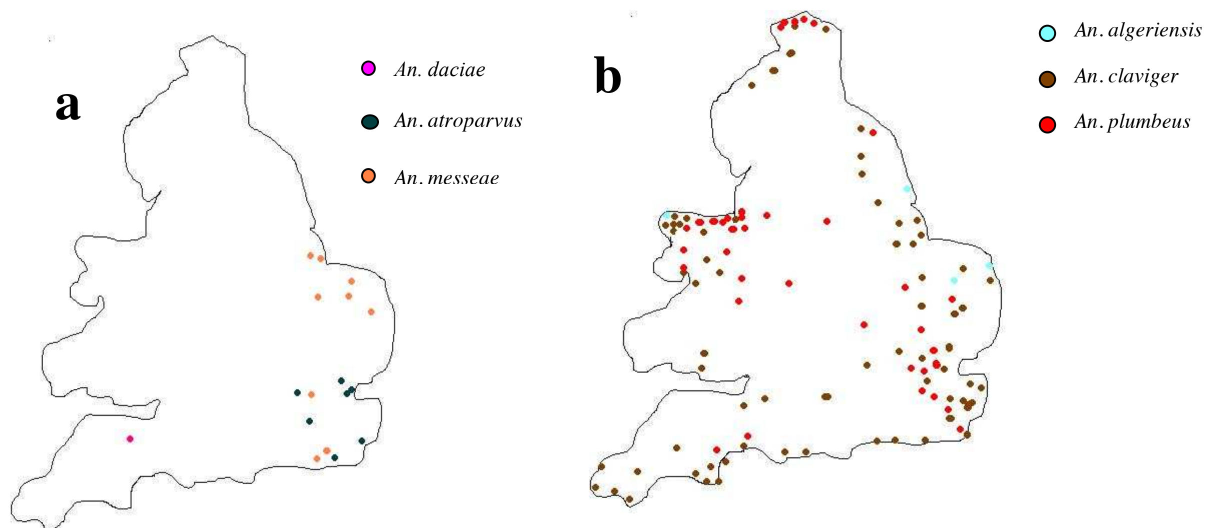

Figure 1.5 Distribution of *Anopheles* mosquitoes in the UK. **(a)** Distribution of *An. atroparvus*, *An. daciae* and *An. messeae* (modified from Rees & Snow, 1990; Snow, 1998; Linton *et al*., 2002a; Linton *et al*., 2005) and **(b)** Distribution of *An. algeriensis*, *An. claviger* and *An. plumbeus* (modified from Snow, 1990; Snow, 1998).

Deruaz *et al.*, 1991), pupal chaetotaxy (Diemer, 1935; Işfan, 1952), adult morphology (Diemer, 1935; Ungureanu & Shute, 1947; Linton *et al.*, 2003; Sedaghat *et al.*, 2003a; Nicolescu *et al.*, 2004), chromosomes (Frizzi, 1952; Frizzi, 1953; Kitzmiller *et al.*, 1967; Stegnii, 1976; Stegnii & Kabanova, 1976; White, 1978), zymotaxonomy (Korvenkontio *et al.*, 1979; Bullini & Coluzzi, 1982; Cianchi *et al.*, 1987; Jaenson *et al.*, 1986a; Suzzoni-Blatger *et al.*, 1990), cuticular hydrocarbons (Phillips *et al.*, 1990) and, most recently, DNA sequences (Marinucci *et al.*, 1999; Proft *et al.*, 1999; Linton *et al.*, 2001a; Linton *et al.*, 2002a,b,c; Sedaghat *et al.*, 2003a, b; Gordeev *et al.*, 2004; Linton, 2004; Nicolescu *et al.*, 2004; Linton *et al.*, 2005; Gordeev *et al.*, 2005; Linton *et al.*, 2007). These works contributed to the current recognition of eleven Palaearctic members within the Maculipennis Complex: *An. artemievi* Gordeev *et al.*, *An. atroparvus* van Thiel, *An. beklemishevi* Stegnii & Kabanova, *An. daciae* Nicolescu *et al.*, *An. labranchiae* Falleroni, *An. maculipennis s.s.* Meigen, *An. martinius* Shingarev, *An. melanoon* Hackett, *An. messeae* Falleroni, *An. persiensis* Linton *et al*. and *An. sacharovi* Favre (Guy *et al.*, 1976a (review through 1975); White, 1978; de Zulueta *et al.*, 1983; Cianchi *et al.*, 1987; Ribiero *et al.*, 1988; Linton *et al.*, 2002a; Sedaghat *et al.*, 2003b; Nicolescu *et al.*, 2004; Gordeev *et al.*, 2005).

In the UK and Ireland, the Maculipennis Group was thought to consist of two species – *An. atroparvus* van Thiel and *An. messeae* Falleroni (Ashe *et al.*, 1991; Ramsdale & Snow, 2000; Linton *et al.*, 2002a). Ecological and biological differences can be used to differentiate the two species found in England. *Anopheles messeae* can be found breeding preferentially in inland fresh waters that are either stagnant or slow moving, whereas *An. atroparvus* is more commonly found in brackish-water pools and ditches in coastal regions. Differences in hibernation conditions have also been used to discriminate between the two species. *Anopheles atroparvus* is normally found hibernating in warm animal shelters where it will periodically feed on the inhabitants, whereas *An. messeae* is usually found in cold shelters where it undergoes complete hibernation, surviving the winter on its food reserves (Rees & Snow, 1990).

Linton *et al.* (2002a) showed they were able to discriminate between *An. atroparvus* and *An. messeae* in the UK using the second nuclear Internal Transcribed Spacer (ITS2) gene and the mitochondrial Cytochrome c Oxidase I (COI) gene. Based on the sequence variation in the ITS2 and COI gene, Linton *et al*. (2005) also positively identified *An. daciae* (a new

member of the Maculipennis Group described from Romania), in the Somerset Levels (southwest England) for the first time.

Anopheles daciae is most closely related to and often sympatric with *An. messeae* (Nicolescu *et al.,* 2004; Linton *et al*., 2005). *Anopheles messeae* has a wide distribution being reported from Ireland and Portugal through to China. Although originally described from Romania, *An. daciae* has since been detected, by correlation of COI data with type specimens of *An. daciae* (Linton *et al*., 2005), in England (Linton *et al*., 2005), Italy, The Netherlands, Former Yugoslavia and Kazakhstan (as *An. messeae,* Di Luca *et al.,* 2004); suggesting that its distribution is also extensive. There are also conflicting reports of the vector status and biting preferences in *An. messeae* populations across Europe that may be attributed to *An. daciae* (Lee *et al*., 2002; Nicolescu *et al.,* 2004; Linton *et al.,* 2005). Since *An. daciae* has only so far been found in the Somerset Levels in the UK (Linton *et al*., 2005), its presence may also be masked by its sibling species, *An. messeae,* in this country. Currently, little is known about its distribution, ecology and malaria vectorial status in the UK or Europe. The known distribution of the members of the Maculipennis Complex prior to this study is shown in Figure 1.5a.

1.2.3.2 The *Culex pipiens* species complex

Despite its global distribution and proven roles in disease transmission, the component members of the *Cx. pipiens* species complex cannot be reliably identified (Smith & Fonseca, 2004). *Culex pipiens s.l.* can be divided into two main groups: *Cx. pipiens*, which occurs in temperate regions with a Holarctic distribution and *Cx. quinquefasciatus* which occurs in subtropical and tropical areas as well as temperate regions (Smith & Fonseca, 2004). In the United States, *Cx. quinquefasciatus* is a vector of major diseases such as Saint Louis Encephalitis, Japanese Encephalitis and West Nile virus (WNv) Fever.

Culex pipiens has been widely reported in the UK (Figure 1.4a). To date, there are two known forms of this species: *Cx. pipiens* f. *pipiens*, which is believed to be ornithophilic and predominantly rural in localised distributions and *Cx. pipiens* f. *molestus,* which feeds on humans and is found mainly in semi-urban environments (Smith & Fonseca, 2004). *Culex pipiens* f. *molestus* was traditionally differentiated from *Cx. pipiens* f. *pipiens* by its autogenous behaviour (ability to lay at least one batch of eggs without taking a blood meal),

but recent studies in Portugal showed some populations of genetically confirmed *Cx. pipiens* f. *pipiens* also display this character trait (Diaz *et al.,* 2006). A species-diagnostic molecular assay based on microsatellites recently developed to differentiate these forms (Bahnck & Fonseca, 2006) has positively identified the presence of both forms of *Cx. pipiens* here in the British Isles (A. Curtotti, pers. comm.). *Culex pipiens* f. *pipiens* has been incriminated as a vector of WNv in the US and in a recent outbreak of the disease in Romania (as *Cx. pipiens s.l.*) (Savage *et al*., 1999). The role of members of the *Cx. pipiens* complex in disease transmission in Britain remains unknown.

1.3 Mosquitoes as vectors of disease

Although there are 3,508 recognised species of mosquitoes in the world (Mosquito Taxonomic Inventory, 2008), fewer than 100 are vectors of diseases (Tyagi, 2003). Mosquitoes belonging to genera *Anopheles*, *Culex* and *Stegomyia* are of biomedical importance worldwide, transmitting diseases such as Malaria, Dengue Fever, WNv Fever, Yellow Fever and Japanese Encephalitis (Tyagi, 2003).

Transmission of an arthropod-borne (arbo) pathogen or viruses can occur vertically or horizontally. Vertical transmission is the passage of pathogens either directly to offspring within vector populations or between males and females (Mullen & Durden, 2002). However, horizontal transmission is essential for the maintenance of pathogens in the environment and can occur either mechanically or biologically (Mullen & Durden*,* 2002). Mechanical transmission does not require the pathogen to amplify or undergo any development in the vector. In this case, the role of the arthropod is an extension on contact transmission between vertebrate hosts (Mullen & Durden, 2002), whereas it is essential for the pathogen to undergo development or reproduction in the insect vector (Carn, 1996), when biological transmission occurs (Figure 1.6).

Transmission of mosquito-borne diseases is often horizontal. Mosquitoes are efficient biological vectors of both avian and human malaria, dengue fever and WNv enabling the respective parasites and viruses to reproduce and amplify to high levels prior to transmission (Mullen & Durden*,* 2002). However, they have also been implicated in the mechanical transmission of the avian poxvirus. By feeding on the lesions and papules of infected birds, the mouthparts of the mosquitoes become infected. As the virus is able to persist for long

periods of time on the mosquito mouthparts, they are able to effectively transmit the poxvirus to uninfected birds during feeding (Carn, 1996). Mosquito-borne diseases can be debilitating and lethal diseases to both humans and animals, thus the management of these arbo-diseases lies not only in the eradication of the pathogen, but also with the control of the vector (Wikelski *et al.,* 2004).

1.4 Mosquito-borne Diseases in the UK

1.4.1 Avian Malaria

Avian malaria, caused by intracellular blood parasites belonging to the family *Plasmodiidae*, comprises of three genera: *Plasmodium, Haemoproteus* and *Leucocytozoon* (Atkinson, 1991; Remple, 2004). There are 25 species of Avian *Plasmodium*. Of these, *P. durae*, *P. elongatum*, *P. gallinaceum*, *P. juxtanucleare* and *P. relictum* are most common infections in birds (Atkinson, 1991). Infection by known strains of *P. elongatum* and *P. relictum* often are fatal (Remple, 2004). The extent of infection, however, varies depending on host and parasite species (Schrenzel *et al.,* 2003; Remple, 2004; Wood & Cosgrove, 2006). Parasites have been isolated from the following bird groups: Passerines (which includes warblers and sparrows), domestic fowl and raptors (including owls, falcons, hawks & kestrels) (Atkinson, 1991; Schrenzel *et al.,* 2003; Remple, 2004; Tavernier *et al.,* 2005).

Avian malaria is transmitted mainly through the bite of haematophagus arthropods such as *Culicoides* biting midges, ticks, blackflies and mosquitoes. Mosquito species belonging to the genus *Culex* have been incriminated as the main vectors of avian *Plasmodium* parasites (van Riper *et al.,* 1993). Despite their role in human malaria, no natural infections of avian malaria in *Anopheles* have been reported, although some species have been shown to be susceptible to avian *Plasmodium* infection (Huff, 1965) and to be good laboratory vectors (Atkinson, 1991).

In the exceptionally hot summer of 1998, avian malaria was reported at zoos across England. All 27 penguins at Marwell Zoo succumbed to the infection and cases were also noted in Bristol Zoo, with 2 recorded mortalities (BBC news online, 1999). Indeed, lack of innate immunity to alien avian malaria in exotic penguin species in British Zoos has resulted

in a controlled programme of chemical prophylaxis against malarial parasites (A. Hartley, pers comm).

1.4.2 West Nile virus (WNv)

West Nile virus is a RNA virus within the Japanese Encephalitis serological group of the family *Flaviviridae* (Buckley *et al.,* 2003; Petersen *et al.,* 2003). It is maintained in the environment through a sylvatic arthropod-bird cycle (Figure 1.7). The virus has been isolated from more than 150 species of wild and domestic birds globally (Van der Meulen *et al.,* 2005). Two lineages of the WNv are known to occur: WNv lineage 1, which circulates amongst the human population and has been isolated from the north eastern United States, Israel, Africa, India and Russia; and WNv lineage 2 from African and Madagascan isolates which is predominantly maintained in the zoonotic cycle (Petersen *et al.,* 2003; Van der Meulen *et al.,* 2005). Genetic analysis of the virus causing an outbreak in North America in 1999, which resulted in deaths of many native and exotic birds, showed it to be similar to the strain found in Israel though it differed from any strains that were isolated prior to the outbreak (Lanicotti *et al.,* 1999).

Bird species of the order Passeriformes (which includes warblers and sparrows) are the most susceptible to the WNv and act as highly competent reservoir hosts, as they are able to develop the highest amount of virus in their blood (viraemia) (Peterson *et al.,* 2004; Van der Meulen *et al.,* 2005). Bird species from the order Chadriiformes as well domestic geese are highly susceptible to infection and disease (Van der Meulen *et al.,* 2005). The extent of infection however, can range from benign, in many European birds, to universally fatal (Peterson *et al.,* 2004). This could be attributed to the age of the birds and genetic variability of the birds and virulence of the viral strains (Van der Meulen *et al.,* 2005).

Mosquitoes are the primary arthropod vectors incriminated in the transmission of the WNv. The virus has been isolated from at least 43 mosquito species; most belonging to the genus *Culex* (Hubalek & Halouzka*,* 1999). The WNv has also been isolated from *An. maculipennis s.l.* in Portugal in 1971 (Filipe, 1972 in Esteves *et al*., 2005), *Culex modestus* in southern France (Hannoun *et al*., 1964) and *Culex pipiens s.l.* in Romania (Tsai *et al*., 1998). Currently in Europe, the main vectors of WNv are *Cx. modestus, Cx. pipiens* and *Cq. richardii* (Higgs *et al*., 2004). *Aedes cinereus, Ae. vexans, An. maculipennis, Oc. cantans* and

Oc. caspius have been linked to WNV transmission in other parts of Europe and USA (Tsai *et al.*, 1998; Higgs *et al.*, 2004; Fyodorova *et al.*, 2006). It has long been suspected that migratory birds play a significant role in the transmission of WNv to new regions (Rappole *et al.*, 2000). According to Medlock *et al.* (2005), outbreaks of WNv in southern Europe have been attributed to the introduction of the virus from infected African migratory birds to the local mosquito population. Higgs *et al.* (2004) suggested that based on the predicted changes in climate and increased movement of livestock and man that more virulent strains of the WNv could establish in Europe in the future. They also suggested that the range of the virus could extend northwards, with a possibility of introduction into the UK.

1.4.3 Tahyna and Inkoo Viruses

Both the Tahyna and Inkoo viruses belong to the family Bunyaviridae and are known to occur in Western Europe (Hubalek & Halouzka, 1996). Of the two, Tahyna is the more widespread (Gould *et al.,* 2006); reported in France, Russia (L'vov *et al.,* 1972), Italy, Spain, Germany, Austria (Pilaski & Mackenstein, 1985), Sweden (Lundström, 1999), Finland and Norway. It was first isolated in *Ae. vexans* and *Oc. caspius* (Bardos & Danielova, 1959); both of which occur in the UK. Although found mainly in non-human hosts such as horses, reindeer and rabbits, human cases of the Tahyna virus have also been reported (Pilaski, 1987; Lundström, 1999). While no reports of human incidence of the virus have been made in the UK, the presence of Tahyna virus was detected (Chastel *et al.* 1985 cited in Ramsdale and Gunn, 2005) in Devon in two species of rodents. Although this was a single observation in 1985, it suggests the virus was being transmitted among the local mosquitoes; however, virus isolation from British mosquitoes has not been documented.

The Inkoo virus, though less prevalent in Europe, is primarily transmitted by *Oc. communis* (Hubalek & Halouzka, 1996). It was reported in Finland and subsequently in Sweden (Francy *et al.,* 1989), Norway (Traavik, 1979 in Hubalek & Halouzka, 1996) and Russia; occurring in cows, reindeer as well as humans (Hubalek & Halouzka, 1996; Ramsdale & Gunn, 2005). While circulating primarily in non-human mammals, Ramsdale & Gunn (2005) have suggested the possibility of transmission of both Inkoo and Tahyna to ground-nesting birds in the UK. The viruses could then be maintained in the environment through the local bird populations. Given the presence of all three vector species and the detection of

Tahyna in UK, low-level transmission as well as enzoonotic transmission of both viruses is a possibility.

1.4.4 Sindbis Virus

Originally isolated from a *Culex* mosquito in Cairo, Egypt in 1952 (Nikolassen, 1989), the Sindbis virus (Alphavirus) is now understood to be part of a complex of 5 viruses (Sindbis complex) of which infection with Sindbis and Sindbis-like viruses have been reported in Russia, Sweden, Finland as well as eastern Europe (Nikolassen *et al.,* 1984; Hubalek & Halouzka, 1996; Lundström *et al.,* 2001). The virus has been isolated primarily from *Cx. torrentium* (serving as a principal vector in Sweden, Lundström *et al.,* 2001), although *Cx. pipiens*, *Cs. morsitans* and *Ae. cinereus* are also thought to be secondary vectors of Sindbis (Nikolassen, 1989; Lundström *et al.,* 1990a, b; Lundström *et al.,* 2001), with birds (mainly Passerines) acting as the main reservoir host for this virus (Lundström, 1999; Lundström *et al.,* 2001).

1.4.5 Arbo-disease transmission in the UK

While these arboviruses are prevalent in Europe, only a suspected low-level transmission of WNv, Sindbis and Tahyna have been suggested in the UK. This could be due to the presence of the European vectors such as *Culex pipiens*, *Oc. communis*, *Oc. caspius*, and *Ae. vexans* in Britain. As the British Isles form part of the migratory route of many species of birds between Africa and North America, there is a real chance of any of the above-mentioned pathogens being introduced into the UK (Higgs *et al*., 2004). Buckley *et al*. (2003) showed seroconversion to the WNv and Sindbis in native British birds and have detected antibodies to the virus in both migratory and native birds; suggesting that at least some species of native British birds have already been exposed to the virus. No local human or animal cases have been detected thus far (DEFRA, 2008), so if the virus is indeed currently present in the UK, it is only cycling within birds. Based on both vector status in the US and Europe and known feeding behaviour, a total of 11 species and 2 species complexes have been identified as potential vectors of the WNv in the UK (Higgs *et al.,* 2004; Medlock *et al.,* 2005): *Aedes cinereus*, *Ae. vexans, An. plumbeus, Cs. annulata, Cs. morsitans, Cs. litorea, Cx. modestus, Cq. richardii, Oc. cantans, Oc. caspius, Oc. detritus, Oc. punctor, An. maculipennis s.l.* and *Cx. pipiens s.l.* Of these, *Ae. cinereus* and *Cx. pipiens* can also transmit

both Sindbis and Tahyna. Susceptibility of British birds to infection by other arboviruses and their ability to maintain and replicate them and the competency of British mosquito vectors to transmit them to other vertebrate hosts is still uncertain (Gould *et al.,* 2006).

Aside from the presence of vectors and reservoir hosts, the transmission of disease is also dependent on changes in climatic conditions and the herd immunity of a population. Global temperatures are expected to increase by at least 6°C by the end of the 21st century (Meteorological Office UK, 2008). This concomitant increase in humidity and alteration of rainfall patterns are predicted to be conducive to the spread of both vectors and pathogens outside their natural ranges (Khasnis *et al.*, 2005; Haines *et al.,* 2006). For example, WNv or Sindbis virus could become fully established in the UK, given the suspected low-level transmission of these viruses within the bird population of the UK (Buckley *et al.,* 2003). Potential bridge vectors present in the area could then spread the viruses into the local human population where immunity to these viruses is low. To circumvent this transmission cycle, a greater knowledge of mosquito species present as well as their selection on hosts is essential.

In 2004, England's Chief Medical Officer (CMO) outlined a prevention and control plan for the possible introduction of West Nile virus into the UK (Department of Health, 2004). Although the threat of arthropod-borne diseases, such as WNv and Chikungunya, entering the UK is low, the CMO reiterated the need for constant vigilance and a strong surveillance system for patients, birds and vectors. Unfortunately, the current knowledge on the ecology and distribution of British mosquitoes does not allow for the establishment of proper surveillance systems. It is hoped that data generated in this study could help to fill in some knowledge gaps, providing comprehensive baseline information on the current distribution, ecology and host selection of mosquitoes in southern England and Wales.

1.5 Overall project aims

This study addresses the habitat preference of British mosquitoes sampled as well as determining the presence of members of species complexes and elucidating their role in disease transmission by:

a) Ascertaining species of mosquitoes present and their habitat preferences (larval habitats, adult resting) in southern England and Wales,

b) Ascertaining the presence of members of the *Anopheles maculipennis* and *Culex pipiens* species complexes in southern England and developing species-specific molecular diagnostic tools to facilitate accurate identification, and

c) Identifying host selection of British mosquitoes by developing a CytB PCR assay and thereby identifying potential vectors and bridge vectors of arbo-diseases

Chapter 2

Field collection of British Mosquitoes

2 Field Collection of British Mosquitoes

2.1 Introduction

2.1.1 British mosquitoes

In 1918 and 1920, Lang published articles detailing aspects of both the morphology and distribution of the 20 known mosquito species recorded in England at that time. These included: *Anopheles maculipennis* Meigen, *An. claviger* (Meigen) (as *An. bifurcatus*), *An. plumbeus* Stephens, *Aedes cinereus* (Meigen), *Ae. vexans* (Meigen) (as *Ochlerotatus vexans)*, *Oc. caspius* (Pallas), *Oc. dorsalis* (Meigen) (*as Oc. curriei*), *Oc. cantans* (Meigen) (as *Oc. waterhousei*), *Oc. annulipes* (Meigen), *Oc. detritus* (Haliday), *Oc. punctor* (Kirby) (as *Oc. nemorosus*), *Oc. rusticus* (Rossi), *Dahliana geniculata* (Olivier) (as *Finlaya geniculata*), *Coquillettidia richiardii* (Ficalbi) (as *Taeniorhynchus richiardii)*, *Culiseta annulata* (Shrank) (as *Theobaldia annulata*), *Cs. morsitans* (Theobald) (*as Culicella morsitans*), *Cs. fumipennis* (Stephens) (as *Culicella fumipennis*), *Culex pipiens* Linneaus, *Cx. territans* Walker (as *Cx. apicalis*) and *Orthopodomyia pulcripalpis* (Rondani) (as *Orthopodomyia albionensis*). Close scrutiny of *Anopheles maculipennis* across Europe at the time, revealed differences in egg morphology (Falleroni, 1926; van Thiel, 1927) and two species, *An. atroparvus* and *An. messeae* were first reported in the UK in 1934 (in Marshall, 1938). Marshall (1938) also updated these earlier works and produced comprehensive descriptions of morphology, ecology and distributions of British mosquitoes, including data on larval habitats and methods of overwintering (diapause) (Table 5.1). This publication added a further nine new species records for UK as follows: *An. algeriensis* Theobald, *Oc. sticticus* (Meigen) (as *Aedes sticticus*), *Oc. communis* (De Geer) (as *Aedes communis*), *Oc. leucomelas* (Meigen) (as *Aedes leucomelas*), *Oc. flavescens* (Müller) (as *Aedes flavescens*), *Cs. subochrea* (Edwards) (as *Theobaldia subochrea*), *Cs. alaskaensis* (Ludlow) (as *Theobaldia alaskaensis*), *Cs. litorea* (Shute) (as *Theobaldia litorea*) and *Cx. molestus* Forskål, increasing the British mosquitoes to 30.

Species	Larval Habitat	Adult behaviour	Environment
Aedes cinereus	Flooded margins of permanent ponds	Outdoor resting	Rural
Aedes geminus	Freshwater	?	?
Aedes vexans	Open, unshaded pools of temporary water, flooded grasslands (fresh/brackish)		Rural
Anopheles algeriensis	Shallow freshwater pools and swamps in calcarious fenland	Outdoor resting	Rural
Anopheles atroparvus	Brackish water pools and ditches in salt marshes	Indoor resting	Rural/ Domestic
Anopheles claviger	Along shady margins of ponds & lakes; fresh/brackish water	Outdoor/animal shelters	Rural
Anopheles daciae	?	Animal shelters	Rural
Anopheles messeae	Fresh water stream margins and ditches	Animal shelters/ Indoor resting	Rural/ Domestic
Anopheles plumbeus	Treeholes (rots and pans); fresh water	?	Arboreal
Coquillettidia richiardii	Permanent ponds, larvae attached to roots and stems of aquatic plants, including *Typha*, *Acorus*, *Glyceria* & *Ranunculus* spp.	Indoors & outdoors; adults fly late in the evening	Rural/Domestic
Culex europaeus	Cool freshwater, also brackish water	?	?
Culex modestus	Fresh / brackish water	?	Rural
Culex pipiens (f. *molestus*)	Subterranean freshwater	Indoor/ cellars	Rural/Domestic
Culex pipiens (f. *pipiens)*	Shallow ground pools, artificial containers with rainwater	Farm shelters/Indoors	Rural/domestic
Culex torrentium	Predominantly artificial containers, occasionally tree holes; fresh water	Farmland	Rural/Arboreal
Culiseta alaskaensis	Freshwater	?	?
Culiseta annulata	Shallow flooded grassland & woodland localities, ditches, sometimes subterranean; artificial containers; fresh/brackish water	Rests indoors (cellars); continues to feed all through the year	Rural/Domestic
Culiseta fumipennis	Temporary woodland pools, or edges of weedy permanent open pools	?	Rural
Culiseta litorea	Coastal species, open sunlit slightly brackish pools	?	Rural

Species	Larval Habitat	Adult behaviour	Environment
Culiseta longiareolata	Foul brackish water pools, fresh water butts	?	?
Culiseta morsitans	Small permanent woodland pools; one record in treeholes	Outdoor resting on vegetation; overwinter as larvae	Rural
Culiseta subochrea	Shallow flooded grassland & woodland localities & ditches; artificial containers (fresh/brackish)	Indoor resting; autogenous	Rural/Domestic
Dahliana geniculata	Treeholes (rots and pans)	Outdoor resting	Arboreal
Ochlerotatus annulipes	Open / partially shaded ditches and pools and depressions in marshy land	?	Rural
Ochlerotatus cantans	Densely shaded temporary woodland pools, roadside ditches	Outdoor resting in low vegetation	Rural
Ochlerotatus caspius	Brackish water pools, salt marshes	Outdoor resting	Rural
Ochlerotatus communis	Temporary woodland pools	Indoor resting	Domestic
Ochlerotatus detritus	Brackish water pools, salt marshes	Indoor resting	Rural
Ochlerotatus dorsalis	Temporary pools of fresh/Brackish water pools	?	Rural
Ochlerotatus flavescens	Temporary pools of fresh/Brackish water	?	Rural
Ochlerotatus leucomelas	Temporary and permanent pools of fresh/brackish water	?	Rural
Ochlerotatus punctor	Ditches and temporary woodland depressions, mainly in areas with acidic, sandy/gravely soils	Indoor resting	Rural/Domestic
Ochlerotatus rusticus	Ditches and shaded temporary pools in deciduous woodlands with plenty of leaf litter	Outdoor resting	Rural
Ochlerotatus sticticus	Temporary woodland pools of open water	?	Rural
Orthopodomyia pulcripalpis	Tree holes (rots and pans)	Resting in rot holes or pans of old beech trees	Arboreal

Table 2.1 Preferred larval habitats and adult resting sites for currently recognised British Culicidae (Shute, 1930; Marshall, 1938; Staley, 1940; Nye, 1954; Wallace, 1958; Service, 1968; Rees & Rees, 1989; Snow, 1990; Linton *et al.*, 200a2; Linton *et al.*, 2005; Snow & Medlock, 2008). ? Indicates no data are available in the literature.

The first record of *Cs. longiareolata* in southern England (Staley, 1940), brought the total number of UK mosquito species to 31. In summer 1945, *Culex modestus* Ficalbi was reported from three separate larval collections in Portsmouth, Gosport and on Hayling Island (all within a 5km radius) (Marshall, 1945). Field specimens of *Culex torrentium* Martini was first reported in the UK by Mattingly (1951), although by revisiting museum specimens held in the Natural History Museum, London (BMNH), Service confirmed the presence of the species some 50 years prior to this record (in Gillies & Gubbins, 1982). Thus, by 1951, 33 species were recorded in the British Isles.

The proposal of *Cx. molestus* as a separate species from *Cx. pipiens* sparked much debate amongst UK entomologists (Marshall & Staley, 1937; Barr, 1957; Stone *et al.*, 1959). It was initially differentiated from the closely related *Cx. pipiens*, based on its adult morphology and biting behaviour (Forskål, 1775, in Harbach *et al.,* 1984) and subsequently differences in larval siphonal index and in aspects of both male and female morphology were detailed by Marshall & Staley (1937) (see Chapter 4, Table 4.1). Following the neotype designations of *Cx. molestus* (Harbach *et al.*, 1984) and *Cx. pipiens* (Harbach *et al.,* 1985), Harbach *et al.* (1984) proposed that due to the lack of distinctive morphological characters between the two species, *Cx. molestus* was to be considered as a physiological variant of *Cx. pipiens*. Thus together with the two forms of *Cx. pipiens* (*Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*) 32 species were included in the morphological identification keys of British mosquitoes by Cranston *et al*. (1987) and Snow (1990).

No additional taxa or taxonomic changes were noted in the review paper of Snow *et al.* (1998) and the British species list appeared to be stabilising. However, the re-elevation of the subgenus *Ochlerotatus* to generic status (Reinert *et al*., 2000), resulted in generic nomenclatural changes to all former British *Aedes*, except *Ae. cinereus* and *Ae. vexans*. By careful examination of specimens of *Cx. territans* Walker from Portugal against those from the type locality in USA, Da Cunha Ramos *et al.* (2003) determined that the European taxa were in fact a distinct species: *Culex (Neoculex) europaeus* Da Cunha Ramos, Ribiero & Harrison, thus it is now accepted that all previous records of *Cx*. *territans* in Europe actually refer to *Cx. europaeus.* Following further revisions on the Tribe Aedini, *Ae. geniculatus* (changed to *Oc. geniculatus*, after Reinert *et al*., 2004), was reclassified as *Dahliana geniculata* (Reinert *et al*., 2006).

With the recent description of the newest member to the Maculipennis Group, *An. daciae* Linton, Nicolescu & Harbach from Romania (Nicolescu *et al*., 2004), Linton *et al*. (2005) retrospectively detected this particular species in collections made in the Somerset Levels in 2001. Similarly the reappraisal of male genitalia of *Ae. cinereus* museum specimens by Medlock & Vaux (2009) revealed the presence of morpholigcal twin species *Ae. geminus* in the UK. It is suspected that the fairly recent description of *Ae. geminus* (Peus, 1970 cited in Medlock & Vaux, 2009) coupled with its morphological similarity to *Ae.cinereus* could have masked the presence *Ae. geminus* in the UK (Medlock & Vaux, 2009) which is now thought to have been in Britain as early as the 1920s (Medlock & Vaux, 2009). Recently identified specimens from Devon have confirmed its continued presence in Britain (Medlock, J. pers comm.). Thus with the addition of these two species, the British mosquito taxa currently stands at 34 species (Table 2.1).

2.1.2 Ecology of British mosquitoes

The ecological niches of the 34 British mosquitoes exhibit marked differences in both immature and adult ecology. Aquatic niches are particularly diverse. For example, three species of endemic mosquitoes, *Anopheles plumbeus*, *Dahliana geniculata* and *Orthopodomyia pulcripalpis,* rear out in tree holes (Snow, 1990; Snow & Medlock, 2006), while aquatic stages of *An. atroparvus* and *Ochlerotatus* species such as *Oc. caspius* and *Oc. detritus*, thrive in brackish waters (Cranston *et al*., 1987; Medlock & Snow, 2006). Immature stages of *Cx. pipiens* and *Cx. torrentium* are often found in artificial containers, while those of *An. claviger*, *Cq. richiardii*, *Cs. fumipennis*, *Cs. litorea*, *Cs. morsitans* and *Cx. europaeus* can be found in groundpools, ponds and streams often amongst dense vegetation (Medlock & Snow, 2006; Snow & Medlock, 2008). Larvae of *Cq. richiardii* have been known survive for long periods of time below the surface of the water (Cranston *et al.,* 1987), obtaining oxygen by piercing the roots of submerged plants such as reed mace (*Typhya* sp.) and sweet mace (*Acora* sp.) (Snow & Medlock, 2008). Adult mosquitoes can either be found resting indoors in houses or in animal shelters as observed for *An. atroparvus*, *An. messeae*; *Cs. annulata*, *Cx. pipiens* and *Cx. torrentium* (Cranston *et al.,* 1987; Snow, 1990), while adults of *Cs. annulata*, *Cx. pipiens, Da. geniculata, Oc*. *cantans*, *Oc. caspius* and *Oc. detritus* are usually found resting outdoors amongst vegetation (Snow, 1990).

Given the temperate climate of the British Isles, all local species of mosquitoes overwinter. Eggs of *Ochlerotatus* are generally resistant to desiccation (Marshall, 1938), therefore species from this genus normally overwinter as eggs (Snow, 1990). This holds true for species such as *Oc. annulipes*, *Oc. cantans*, *Oc. caspius*, *Oc. detritus*, *Oc. punctor*, *Oc. rusticus*, Oc. *sticticus*, including *Cs. morsitans* (Cranston *et al.,* 1987). Other British taxa, including *An. algeriensis*, *An. claviger*, *An. plumbeus*, *Cq. richiardii*, *Da. geniculata* and *Or. pulcripalpis* overwinter as larvae (Cranston *et al.,* 1987; Snow, 1990; Snow & Medlock, 2008). *Anopheles atroparvus* and *An. messeae* overwinter as fertilised females (Snow, 1990), although *An. atroparvus* is known to periodically break out of hibernation to feed on the occupants of the shelter. Reports have shown the susceptibility of *An. atroparvus* and *An. messeae* to a short day photoperiod which induces ovarian diapause (Cranston *et al.,* 1987), similar to that observed in females of *Cx. pipiens* (Clements, 1963 in Cranston *et al.,* 1987). Hibernation is not noted in *Cs. annulata*, where no effect of photoperiod on diapause was also observed (Service, 1968 in Cranston *et al.,* 1987).

2.2 Materials and Methods

This chapter describes in detail materials and methods used in the collection of larval and pupal stages as well as resting and host seeking adult mosquitoes.

The field collections were carried out according to the standardised Natural History Museum procedure, following the recommendations of Belkin *et al*. (1962). Field data recorded for each capture included: collection type (e.g. immature, resting, human landing etc), assessment of vegetation present in larval habitat (floating/emergent and abundance of algae) and the potential hosts present (Figure 2.1). Data from each collection site were later correlated with the mosquito identifications (Figure 2.2) in order to more fully characterize the ecological parameters of mosquito species collected.

Collection No.	Nearest Town	Date
Country	Specific Locality	Time
Province	Latitude/Longitude	Collector(s)
Second Administrative Division	Elevation	Organisation

COLLECTION TYPE
- Immature
- Resting -
  - House
  - Animal Shelter
  - Cave
  - Tree Hole
  - Vegetation
  - Other: ____________
- Biting/ Landing -
  - Human
  - Animal: ___________
- Net
- Light Trap: ____________
- Bait Trap
- Swarming
- At Light
- Other: ____________

TERRAIN
- Mountain
- Hill
- Valley
- Plateau
- Plain

DISTANCE FROM HOMES

m

SKY
- Clear
- Partly Cloudy
- Overcast
- Fog
- Mist
- Light Rain
- Heavy Rain

HOST
- Human
- Horse
- Cow

ENVIRONMENT
- Woodland
- Evergreen Forest
- Deciduous Forest
- Grassland
- Island
- Swamp
- Salt Marsh
- Beach
- Orchard - Plantation
- Cultivated Field: ____________
- Urban
- Village
- Other: ____________

ENVIRONMENTAL MODIFIERS
- Primary
- Secondary
- Agriculture
- Pasture
- Grove/Plantation: ____________
- Other: ____________

WIND
- None
- Light
- Gusts
- Strong

HEIGHT ABOVE GROUND

m

SHADE
- None
- Partial
- Heavy

LARVAL HABITAT
- Pond – Lake
- Ground Pool
- Swamp
- Marshy Depression
- Stream Margin
- Stream Pool
- Rock Pool
- Seepage - Spring
- Ditch
- Well
- Artificial Container
- Hoof Print
- Rut
- Other: ____________

ALGAE
- Filamentous
- Green
- Blue-Green
- Brown
- Other: ____________

ALGAL DENSITY
- None
- Scarce
- Moderate
- Abundant

DIMENSIONS OF SITE

m X m

Depth m

WATER:
- Permanent
- Temporary

WATER MOVEMENT
- Stagnant
- Slow
- Moderate
- Fast

SALINITY
- Fresh
- Brackish

TURBIDITY
- Clear
- Coloured
- Turbid
- Polluted

PHYSICAL FACTORS
- pH
- Conductivity
- Temperature (ºC)
- TDS

AQUATIC VEGETATION
- Submerged
- Floating
- Emergent
- Submerged and Floating
- Submerged and Emergent
- Floating and Emergent
- All Types

QUANTITY OF AQUATIC VEGETATION
- None
- Scarce
- Moderate
- Abundant

REMARKS

Figure 2.1: Collection form.

Collection Number **EN12**				Country **England**
Number	Le	Pe	Sex	Identification / Notes
-1		√	♂	*Anopheles (An.) claviger* (Pinned)
-2	√	√	♀	*Culex* spp. (in EtOH)

Figure 2.2: The reverse side of the field collection forms (see Figure 5.1) showing an example of the detailed notes kept of each individual larval rearing.

County	Nearest Town	Co-ordinates (Degree decimal)	Habitat
Anglesey	Benllech	53.2011838'N, 04.3714482'W	Ditch
		53.3153544'N, 04.2379822'W	Stream pool
		53.3173278'N, 04.2382357'W	Nr horse stables[MT]
			Bath Tub
			Bucket
			Water trough
		53.3178668'N, 04.2382639'W	Bath Tub
			Water trough
	Brynteg	53.3065719'N, 04.2649949'W	Bath Tub
	Llangferfechan	53.2489805'N, 03.9958504'W	Bucket
			Water trough
	Newborough	53.1635733'N, 04.3316682'W	Bucket Rim
		53.1638428'N, 04.3316828'W	Shed[R, MT]
			Pond, Bird World[R, MT]
	Penraeth	53.2942853'N, 04.1911069'W	Bucket
			Plastic Barrel
	Mallraeth	53.2969830'N, 04.1910949'W	Tarpaulin Sheet
		53.2000146'N, 04.3396364'W	Tyre
		53.2009233'N, 04.3709845'W	Ditch
		53.2017891'N, 04.3454238'W	Stream pool
		53.2485910'N, 03.9976312'W	Ditch
		53.2728030'N, 04.2438583'W	Ditch
		53.2736646'N, 04.2458535'W	Ground Pool
		53.2738329'N, 04.2464624'W	Ditch
Caernarfonshire	Betws-y-Coed	53.0540031'N, 03.8307297'W	Water trough
		53.0552657'N, 03.8304847'W	BBQ Bowl
		53.0555421'N, 03.8300487'W	Bucket
		53.0555731'N, 03.8294598'W	Bucket
Devon	Belstone	50.7274548'N, 03.9508172'W	Ditch
		50.7358999'N, 03.9674681'W	Horse Stables[R]
			Bucket
			Plastic Barrel

County	Nearest Town	Co-ordinates (Degree decimal)	Habitat
Devon	Bridestowe	50.6830244'N, 04.0956511'W	Bucket
		50.6837282'N, 04.0965319'W	Human
		50.6839079'N, 04.0965399'W	Bucket
		50.6839978'N, 04.0965439'W	Black Bins
		50.6840673'N, 04.0976797'W	Bucket
			Forklift bucket
		50.6842216'N, 04.0991025'W	Bucket
	Cheriton Bishop CP	50.7114499'N, 03.7098920'W	Boat
		50.7130395'N, 03.7422498'W	Ground Pool
	Exminster Marshes	50.6742739'N, 03.4679099'W	Stream Margin
		50.6752617'N, 03.4751598'W	Brick Shelter[R]
		50.6756321'N, 03.4743221'W	Brick Shelter[R]
		50.6765270'N, 03.4675556'W	Stream Margin
		50.6773582'N, 03.4729606'W	Stream Margin
		50.6784496'N, 03.4720039'W	Ditch
		50.6786812'N, 03.4679059'W	Ditch
		50.6789962'N, 03.4714547'W	Ditch
	Launceston	50.5812099'N, 04.3317238'W	Pond
	Moretonhampstead	50.6463599'N, 03.8149006'W	Boat
		50.6466229'N, 03.8153351'W	Water trough
			Near Chicken coop[MT]
		50.6488266'N, 03.8124491'W	Pond
		50.6469605'N, 03.8167628'W	Horse Stables[MT]
	Okehampton	50.6831143'N, 04.0956551'W	Stream pool
		50.7291615'N, 04.0607206'W	Lake
		50.7335964'N, 03.9971353'W	Stream pool
	St Erney	50.4011439'N, 04.2806501'W	Tyre
		50.4026551'N, 04.2815672'W	Pond
	Trevollard	50.3859807'N, 04.2555818'W	Ground Pool
	Two Bridges (Dartmoor Forest)	50.5588627'N, 03.9685579'W	Bath Tub
Kent	Cliffe Marshes	51.4537556'N, 00.4782834’E	Brick shelter 1[R]
		51.4671890'N, 00.4895240’E	Salt Lane Bunker[R]

County	Nearest Town	Co-ordinates (Degree decimal)	Habitat
Kent		51.4783052'N, 00.4827834'E	Sheep corral[R]
Norfolk	Ranworth	52.6814994'N, 01.4881677'E	Boat
	Reedham	52.4656741'N, 01.5704780'E	Petting Area 1[MT]
		52.5659393'N, 01.5805442'E	Pond
		52.5659609'N, 01.5768554'E	Goat Stables[R]
			Petting area[R, MT]
			Reindeer Stables[R]
			Rhea Stables[R]
		52.5661226'N, 01.5774591'E	Petting area 2[MT]
			Stream pool
		52.5661404'N, 01.5768701'E	Horse/donkey stable[R]
			Petting area[R]
	West Somerton	52.7214148'N, 01.6559478'E	Stream Margin
		52.7215319'N, 01.6550687'E	Boat
			Stream Margin
Somerset	Godney	51.1816487'N, 02.7273050'W	Pillbox 1[R]
			Ground Pool
		51.1817652'N, 02.7230142'W	Horse Stables[R]
		51.1822719'N, 02.7283164'W	Pillbox 2[R]
	Highbridge	51.1873104'N, 03.0173270'W	Ditch
	Otterhampton	51.2106327'N, 03.0344497'W	Lake
	Stockland Bristol	51.1879387'N, 03.0777330'W	Ditch
Suffolk	Beccles	52.4658536'N, 01.5704926'E	Stream pool
		52.4677069'N, 01.5716738'E	Ditch
		52.4693843'N, 01.5697476'E	Ditch
	Oulton	52.4682112'N, 01.6927924'E	Ditch
		52.4683907'N, 01.6928074'E	Ditch

Table 2.2 The localities, co-ordinates and habitats of mosquitoes collected in this study. [R] and [MT] Indicate Resting and Mosquito Magnet Trap ® collections and the rest are larval collections.

a

b

c

d

e

f

g

Figure 2.3: Collection of aquatic stages in natural habitats (a, b & d) streams and (e) a pond and artificial habitats including (c) disused bathtub, (f) moored boat and (g) fire buckets. Methods of collection for aquatic stages are shown in 2.3d.

2.2.1 Collection of immature stages

Larvae and pupae were collected from breeding sites - both natural and artificial (Figure 2.3) - (Table 2.2) using standardised mosquito dippers (Figure 2.3d, BioQuip, LA, USA). Larvae and pupae were carefully isolated using wide-mouthed plastic pipettes and transferred to labelled plastic Whirl-Pak® (Nasco) bags, containing ample water and fine debris from the original breeding site. Bags were sealed for transportation. Once in the laboratory, the contents of each Whirl-pak® were individually transferred into bowls labelled with the corresponding collection number (e.g. EN101) and the immatures were fed Tetramin® powdered baby fish food once daily. Immatures were reared collectively until reaching the fourth instar, whereby each specimen was individually reared.

2.2.1.1 Individual rearing

Individual rearing were carried out from the fourth larval instar and on those collected as pupae in the field. All fourth instar larvae / pupae were transferred individually into plastic vials containing 2-3 cm of original habitat water. Plastic vials were marked with the collection number using a wax pencil. Vials containing isolated larvae, marked with collection numbers, were examined twice a day - early morning and late afternoon. Larval skins were removed with an applicator stick and transferred to a small glass storage vial with 80% alcohol and attached to the rearing vial containing the pupa using an elastic band. A lid was placed on the rearing vial to prevent escape of the adult mosquito after emergence.

The pupal exuviae was then placed into the same vial as the associated larval skin and both the adult and linked exuviae were labelled with the same unique number (e.g. EN101-07, where EN101 indicates English collection 101 and -07 the individual in that collection). Viable adults were pinned for taxonomic studies as described below and associated numbers added to the pinned specimen so both exuviae and specimen were linked. Pupae, larvae and partially emerged adults that died during rearing were preserved in 80% ethanol for DNA studies.

2.2.1.2 Preparation of link-reared adults

Adults were transferred from the rearing vials to killing tubes containing plaster infused with ethyl acetate. The adult mosquito and its corresponding identification label were transferred from the killing tube onto clean white card. Using fine forceps, the adult was picked up by one leg and placed on an elevated surface (e.g. postal box) with a white background. The specimen was oriented with proboscis facing right and moved to the edge of the elevated surface with its legs projecting beyond the edge. A card point was fixed an appropriate distance from the head of an insect pin and a tiny droplet of Ambroid® cement put on the upper apical angle of the point. Holding the pin so that the point is upside down, the droplet of glue was gently touched to the thorax of the mosquito. Final orientation of the mosquito on the upper surface of the point was with the left side up, head facing left and the legs extended toward the pin. This orientation protects the specimen from damage and corresponds to the preferred orientation of illustrations in key taxonomic publications (e.g. Harbach & Knight, 1980). The label with the collection and rearing number was attached to the pin. Once pinned, specimens were kept in postal boxes in zip-sealed large plastic bags, to avoid destruction by insectivorous pests. Pinned insect also underwent freezing at -80°C for 5 days after processing to kill fungal spores and bacteria, which can also damage the specimens. Rearing records (Figure 2.2) are maintained on the back of the original field collection records.

2.2.2 Field collections of adult mosquitoes

Collections of resting mosquitoes were carried out manually (Table 2.2) inside animal stables, in abandoned war bunkers and from the walls and ceilings of disused outhouses (Figures 2.4d, e) with an aspirator (Figure 2.4a). Pootered mosquitoes were transferred to collection cups (Figure 2.4b), labelled with a unique collection number. In addition, the propane-powered Mosquito Magnet® Liberty Pro (Pennsylvania, USA; referred to as Mosquito Magnet Trap ®) trap was used to collect host seeking adults (Figure 2.4c).

Collections were killed either by placing the cup containing the mosquitoes directly into the freezer for 40 minutes or by placing the cup or Magnet net into a sealed plastic bag, containing tissue paper saturated with ethyl acetate. Once the mosquitoes were killed, they were visually sorted to genera and minimums of 3-5 representatives, per species per

collection, were pinned for morphology (see section 2.2.1.2). All other mosquitoes were preserved for DNA studies by individually placing in Beem® capsules with their unique labels, before closing. A mounted needle was used to pierce a hole in the capsule to allow escape of moisture. Beem® capsules were stored sealed in plastic bags containing sachets of silica gel at room temperature prior to DNA extraction.

a

b

c

d

e

Figure 2.4: Adult collection methods and resting habitats: (a) aspiration of adults resting on walls of a Brick Shelter in Kent, (b) collection cups used to transport collected resting adults, (c) Mosquito Magnet trap ® uses carbon dioxide, moisture and heat (bi-products of propane) and a chemical lure (1-Octen-3-ol) to attract host-seeking females (picture from http://amazon.com) (d) a brick shelter in Exminster Nature Reserve, Devon and (e) goat stable in Pettits Animal Farm, Norfolk.

2.2.3 Mosquito identification

Using a stereoscope and the morphological identification keys of Cranston *et al.* (1987) and Snow (1990), both link-reared and resting adult mosquitoes were identified to species in the adult stage, where possible. During the process of collection and transportation, some adult specimens were so badly damaged that species identification was impossible. In addition, not all larvae/pupae collected were successfully reared to adults, these immatures were not identified to species in this study. In such cases, specimens were identified to the genus level only.

A total of 1,463 adult mosquitoes and 1,601 immature stages were collected from over 73 unique sites (Table 2.2) in this study. Species of the Maculipennis Complex were used in the development of an ITS2 PCR-RFLP assay described in Chapter 3 and specimens were then identified as *An. atroparvus*, *An. daciae* and *An. messeae*. *Culex pipiens s.l.* specimens were used to determine the accuracy of two published assays developed to differentiate between the two forms (Chapter 4).

Chapter 3

Molecular differentiation of the Maculipennis Group in the UK

3. Molecular differentiation of the Maculipennis Group in the UK

3.1 Introduction

3.1.1 The taxonomic status of the Palaearctic Maculipennis Group

Anopheles maculipennis was exposed as the first mosquito sibling species complex, comprising several species on the basis of egg morphology (Falleroni, 1926; van Thiel, 1927; Falleroni, 1932; Corradetti, 1934; de Buck & Swellengrebel, 1934a; Hackett & Lewis, 1935; Weyer, 1942; Angelucci, 1955; Gutsevich *et al*., 1974; White, 1978; Korvenkontio *et al*., 1979; Pichot & Deruaz, 1981; Jaenson *et al*., 1986a; Jetten & Takken, 1994; Sedaghat *et al*., 2003a; Nicolescu *et al*., 2004). Despite several other techniques that were later employed to differentiate the component species, including hybridisation experiments (de Buck & Swellengrebel, 1934b; Kitzmiller *et al*., 1967), detailed morphology (La Face, 1931; de Buck *et al*., 1933; Diemer, 1935; Bates, 1939; Buonomini, 1940; Ungureanu & Shute, 1947; Işfan, 1952; Suzzoni-Blatger & Sevin, 1981; Boccolini *et al*., 1986; Suzzoni-Blatger *et al*., 1990; Deruaz *et al.*, 1991), ecology (van Thiel, 1927; de Buck & Swellengrebel, 1934b; Hackett & Missiroli, 1935), cytotaxonomy (Frizzi, 1952; Frizzi, 1953; Kitzmiller *et al*., 1967; Stegnii, 1976; Stegnii & Kabanova, 1976; White, 1978), zymotaxonomy (Korvenkontio *et al*., 1979; Bullini *et al*., 1980; Bullini & Coluzzi, 1982; Jaenson *et al*., 1986a; Cianchi *et al*., 1987; Suzzoni-Blatger *et al*., 1990) and cuticular hydrocarbons (Phillips *et al*., 1990), egg morphology remained the golden standard for differentiating species within the complex. However several authors have pointed out that intraspecific variation in egg morphology can result in incorrect identifications (Guy *et al*., 1976b; Jaenson *et al*., 1986a; Alten *et al*., 2000; Linton *et al*., 2002b).

The relatively recent application of DNA sequence analysis to the Maculipennis Group has proven to be the most reliable method of differentiating the component taxa (Marinucci *et al*., 1999; Proft *et al*., 1999; Romi *et al*., 2000; Linton *et al*., 2001a; Linton *et al*., 2002a,b, c; Linton, 2004; Sedaghat *et al*., 2003a,b; Nicolescu *et al*., 2004; Gordeev *et al*., 2004, Gordeev *et al*., 2005; Linton *et al*., 2007; Djadid *et al*., 2007). Indeed DNA sequences were used to prove the synonymy of *An. subalpinus* with *An. melanoon* (Linton *et al*., 2002b) and have revealed three new taxa in the Maculipennis Complex: *An. persiensis* Linton, Sedaghat &

Harbach (Sedaghat *et al*., 2003b), *An. daciae* Linton, Nicolescu & Harbach (Nicolescu *et al*., 2004) and *An. artemievi* Gordeev, Zvantsov, Goriacheva, Shaikevich & Ezhov (Gordeev *et al*., 2005).

The Palaearctic Maculipennis Group currently comprises eleven formally recognised species: *Anopheles artemievi*, *An. atroparvus* van Thiel, *An. beklemishevi* Stegnii & Kabanova, *An. daciae*, *An. labranchiae* Falleroni, *An. maculipennis s.s*, Meigen, *An. martinius* Shingarev, *An. melanoon* Hackett, *An. messeae* Falleroni, *An. persiensis* and *An. sacharovi* Favre (White, 1978; Linton *et al*., 2002b; Sedaghat *et al*., 2003b; Nicolescu *et al*., 2004; Gordeev *et al*., 2005).

3.1.2 Molecular differentiation of species in the Maculipennis Group

Molecular methods using the sequence of the second nuclear internal transcribed spacer (ITS2) region of the ribosomal DNA (rDNA) have been widely employed to identify the isomorphic Palaearctic members (Marinucci *et al*., 1999; Proft *et al*., 1999; Linton *et al.,* 2001a; 2002a,b,c; Sedaghat *et al.,* 2003a,b; Nicolescu *et al*., 2004; Gordeev *et al*., 2004; Gordeev *et al*., 2005; Kampen, 2005a,b; Linton, 2004; Linton *et al*., 2005; Linton *et al*., 2007; Djadid *et al*., 2007) and to investigate the internal phylogenetic relationships within the Maculipennis Group (Marinucci *et al*., 1999; Linton, 2004; Kampen, 2005b; Djadid *et al*., 2007). The high inter-specific divergence of the ITS2 region makes it a useful marker in accurately identifying the members of species complexes (Section 2.1.1).

Proft *et al*. (1999) developed an ITS2-PCR assay that could differentiate six members of the Maculipennis Group (*An. atroparvus*, *An. labranchiae*, *An. maculipennis*, *An. melanoon*, *An. messeae* and *An. sacharovi*) using species-specific primers. Recently Kampen (2005a) incorporated another species-specific primer for *An. beklemishevi* into the original assay. Linton *et al*. (2005) reported that the purported *messeae*-specific primer could also amplify *An. daciae*, a recently described member in the Maculipennis Group (Nicolescu *et al.,* 2004). *Anopheles daciae* and *An. messeae* are the two most closely related sister taxa within the Maculipennis Group, sharing 99.0% sequence homology in the 485bp ITS2 amplicon, with five fixed variable sites (Linton, 2004; Nicolescu *et al.,* 2004; Linton *et al.,* 2005). In Romania, where *An. daciae* was originally described, the two were often found in sympatry

(Nicolescu *et al.,* 2004). Correlation of mitochondrial sequence data from the cytochrome oxidase I (COI) gene shows the purported presence of *An. daciae* in Italy, The Netherlands, Former Yugoslavia and Kazakhstan (as *An. messeae,* Di Luca *et al*., 2004), suggesting that its Eurasian distribution is extensive (Linton *et al*., 2005). With the exception of DNA assays, there are currently no other reliable means of differentiating these two species.

3.1.3 Maculipennis Group and malaria transmission

Interest in the Maculipennis Group has been sustained due to their role in malaria transmission in Europe and the Middle East. Three species of the Maculipennis Group, *An. atroparvus*, *An. sacharovi* and *An. labranchiae*, are known to be efficient current or historical malaria vectors in Europe (Hackett & Missiroli, 1935; Bruce-Chwatt & de Zulueta, 1980; Jaenson *et al*., 1986a; Ribeiro *et al*., 1988; Kasap, 1990; Jetten & Takken, 1994; Fantini, 1994; Romi *et al*., 1997; Romi, 1999; Alten *et al*., 2000; Romi *et al*., 2001; Romi *et al*., 2002; Sedaghat *et al*., 2003a). *Anopheles maculipennis s.s.* and *An. melanoon* (as *An. subalpinus*) were recently incriminated as secondary vectors in the Biga Plains of Turkey (Alten *et al.*, 2000), perhaps indicating an increased role in malaria transmission. There is still debate over the role of *Anopheles messeae* as a vector. It is reported to be an efficient vector in western Asia (Bruce-Chwatt & de Zulueta, 1980), Ukraine and Russia (Nikolaeva, 1996) but in Europe.

Despite the eradication of malaria from Europe following WWII (Ramsdale & Gunn, 2005), increasing numbers of malaria cases are now being reported (Sartori *et al*., 1989; Nikolaeva, 1996; Baldari *et al*., 1998; Romi *et al*., 2001) heightening concern for the reintroduction of malaria in regions, such as the UK where competent mosquito vectors still exist (Jetten *et al*., 1996; Romi *et al*., 1997; Lindsay & Birley, 1996; Romi, 1999; Snow, 1999; Romi *et al*., 2001; Linton *et al*., 2001b).

3.1.4 The Maculipennis Group in the UK

Based on egg morphology, Edwards (1936) suggested the presence of three species of the Maculipennis Group in the UK: *An. atroparvus*, *An. messeae* and *An. maculipennis s.s.* However, subsequent morphological studies confirmed the presence of only two species,

namely *An. atroparvus* and *An. messeae* (Marshall, 1938; Mattingly, 1950; Wallace, 1958; Cranston *et al*., 1987; Snow, 1990; Snow *et al*., 1998). Despite the presence of *An. maculipennis s.s.* in The Netherlands, France, Germany and Belgium, it has not been confirmed in the UK (Cranston *et al*., 1987; Snow & Ramsdale, 1999; Ramsdale & Snow, 2000), despite a tentative report from the Channel Islands (Ramsdale & Wilkes, 1985 , cited in Cranston *et al.,* 1987). Although *An. maculipennis s.l.* has been reported from as far north as the Grampians in Scotland (Ashworth, 1927) and in Ireland (Ashe *et al*., 1991), no formal studies have been carried out to determine which species are actually present (Rees & Snow, 1990). The presence of *An. messeae*, however, was confirmed in County Galway in Ireland (F. Geraghty & Y.-M. Linton, pers comm), using ITS2 DNA sequence data. Employing the same nuclear gene region, Linton *et al*. (2002a) verified the presence of both *An. atroparvus* and *An. messeae* in Kent and *An. messeae* in Yorkshire.

Linton *et al.* (2005) also confirmed the presence of a third member of the Maculipennis Group in the Somerset Levels, UK, *Anopheles daciae*, through the comparison of ITS2 sequence data from five adults (collected resting in a horse stable in Godney Farm, Godney), with those from the type locality of Budeni, Romania (Nicolescu *et al*., 2004).

3.1.4.1 Distribution and ecology of the Maculipennis Group in the UK

Anopheles messeae is widely reported in Europe, occurring also in northern China, Mongolia and former USSR states (WRBU, 2008). In the UK it is reported from Wales, England and as far north as central Scotland (Walter, 1927 in Morgan, 1978; Wallace, 1958; Cranston *et al*., 1987; Rees & Snow, 1990; Snow *et al*., 1998; Ramsdale & Snow, 2000). The species has been reported in the London area (Epsom, Esher, Dartford, Bexley, Romford, Richmond, Wimbledon Common, Putney and Barking (Nye, 1955) and from the English counties of Berkshire, Cambridgeshire, Cheshire, Devon, Kent, Norfolk, Northumberland, Suffolk, Surrey, Sussex and Yorkshire (Evans, 1934; Carter, 1978; Cranston *et al*., 1987; Ramsdale & Snow, 2000; Linton *et al*., direct submission to GenBank, 2001; Linton *et al*., 2002a).

Anopheles atroparvus has been recorded from Berkshire, Cheshire, Dartford, Devon, Dorset, Essex rivers, Hayling Island, Kent, Pevensey Levels, Romney Marsh, Surrey, Thames

Estuary and in the lower reaches of Sussex rivers in England (James, 1929; Shute, 1933; Marshall & Staley, 1933; Killington, 1946; Ramsdale & Snow, 2000; Linton *et al*., 2002a) and around the London area (Bexley, Dartford, Epsom, Esher, Epping & Romford (Nye, 1955). In northern Wales, *An. atroparvus* has been recorded from Anglesey, Llanfaglan, the southern coast of the Menai Straits and at Gwyrfai on the River Afon estuary (Wright, 1924; Evans, 1934; Ramsdale & Snow, 2000).

Immatures of *An. messeae* can be found in inland fresh water pools, such as ponds and ditches, which are either slow moving or stagnant (Cranston *et al*., 1987; Snow, 1990). In contrast, *An. atroparvus* immatures are tolerant to high levels of salinity (Wallace, 1958), thus it is predominantly found in brackish open water or weedy ditches in coastal or estuarine locations (Marshall, 1938; Wallace, 1958; Rees & Snow, 1990; Snow, 1990). Although *An. atroparvus* has also been reported from fresh water (Rodhain & van Hoof, 1942) and has an extensive inland distribution in the Iberian Peninsula (Romeo Viamonte, 1950).

Both *An. messeae* and *An. atroparvus* overwinter as nulliparous females (Cranston *et al*., 1987). Adults of *An. messeae* are commonly found resting in animal shelters, such as horse stables and abandoned outhouses (Linton *et al*., 2002a). In winter, they enter complete hibernation, seeking refuge in cold, uninhabited shelters (Marshall, 1938; Cranston *et al*., 1987) and surviving off their fat body reserves. Capable of entering full diapause (Mohrig, 1969), *An. atroparvus* tends to hibernate in warmer sheltered spots such as sheds and stables and will periodically break out of hibernation to feed (Cranston *et al*., 1987; Rees & Snow, 1990).

Anopheles daciae has only been reported from Godney, in the Somerset Levels, to date (Linton *et al*., 2005). Five females were collected as resting adults within a horse stable, thus apart from adult resting habitat, little is known of the specific ecology of *An. daciae* in the UK (Linton *et al*., 2005). However, in the type description, Nicolescu *et al.* (2004) did report sympatric larval collections of *An. messeae* and *An. daciae* in several localities in Romania, suggesting that the habitat requirements for *An. daciae* and *An. messeae* immatures could be similar.

3.1.4.2 Role of Maculipennis Group in malaria transmission in the UK

Early surveys of mosquitoes in the UK (Nutall *et al*., 1901; Lang, 1918) showed that the distribution of *Anopheles* mosquitoes was much more extensive than the regions affected by malaria (Cranston *et al*., 1987). However, a review of historical documents detailing malaria cases in England and Wales from 1840 to 1910 by Kuhn *et al.* (2003) showed that although malaria was documented across southern Britain, the highest incidence of malaria was documented in the inland county of Cambridgeshire and the coastal county of Kent where up to 96-114 cases per 100,000 inhabitants were reported annually (Figure 3.1).

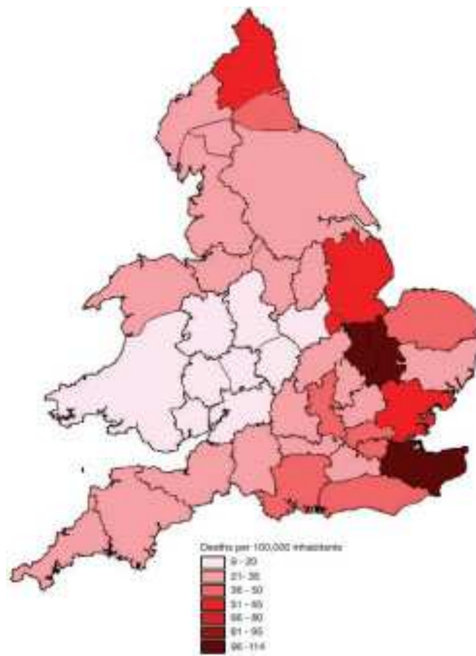

Figure 3.1 Distribution of benign tertiary malaria in England from 1840 to 1910 (Kuhn *et al*., 2003). Intensity of colour signifies the annual number of cases per 100,000 inhabitants by county: Maroon =96-114, bright red =51-65, salmon pink =36-50 and light pink =9-20.

Post World War I, the transmission of *Plasmodium vivax* malaria from soldiers quarantined on the Isle of Grain and Sheppey to the locals in Kent inextricably linked *An. atroparvus* to the transmission of malaria (Shute, 1963). Although *An. atroparvus* was regarded as the major vector of *P. vivax* in the UK (Shute & Maryon, 1974; reviewed in Cranston *et al*., 1987 & Rees & Snow, 1990), its propensity for coastal sites and the intensity of malaria in other more inland localities (Figure 3.1), suggests that other native species must also have played a role.

Anopheles plumbeus, a tree-hole breeder, was implicated in both an outbreak of *P. falciparum* in a "northern health resort" (Blacklock & Carter, 1920) and in a *P. vivax* malaria outbreak in Lambeth, London (Shute, 1954). The efficacy of this species as a vector of *Plasmodium* was later verified in the laboratory (Marchant *et al.*, 1998; Eling *et al*., 2003). Although *An. plumbeus* is highly anthropophilic (Cranston *et al*., 1987), it seems unlikely that it played a major role in the UK malaria transmission, except in localized areas.

To date, *Anopheles messeae* is not considered a vector of malaria transmission in the UK (Snow, 1998; Marchant *et al*., 1998). Its occurrence in Kent (Linton *et al.,* 2005), where the burden of malaria was high and its susceptibility to *P. vivax* infection (Curtis & White, 1984) was evident, presented itself as a potential vector in the UK. It is thought to be a highly competent vector in Russia and the Ukraine, Eastern Europe and western Asia (Detinova, 1953; Bruce-Chwatt & de Zulueta, 1980; Nikolaeva, 1996), yet it is not considered an efficient vector in northwestern Europe (Jetten & Takken, 1994). Linton *et al.* (2005) suggested that this conflict may in part be due to the discovery of the sympatric and closely related species, *An. daciae*, whose true distribution could be masked by *An. messeae*. They proposed that if only one of these isomorphic taxa were to be a good vector of *P. vivax*, then this could account for the patchy endemic malaria in regions where only *An. messeae* has been reported and indirectly incriminated (Linton *et al*., 2005). *Anopheles daciae* was recently circumstantially incriminated, as it was the only species collected in close proximity to an indigenous malaria case in southern Romania (Vladimirescu *et al*., 2006). Given the genetic similarity and reported geographical sympatry of *An. messeae* and *An. daciae* and the closely related *An. atroparvus*, it is important to obtain a robust identification method that will allow for future study of these species to determine vector capacity and define effective control programs both in the UK and across Europe.

3.2 Aims

The aims of this study were:

[1] To carry out field collections of the Maculipennis Group in five counties in southern England (Devon, Kent, Norfolk, Somerset and Suffolk) and in Anglesey, North Wales,

[2] To design a molecular assay to reliably differentiate between the three British members of the Maculipennis Group and

[3] To use molecularly identified specimens to determine the current presence of *An. atroparvus*, *An. daciae* and *An. messeae* in six regions of southern England and Wales.

3.3 Materials and Methods

3.3.1 Collection and identification of field-caught specimens

Adults and immatures of *An. maculipennis s.l.* were collected in various sites across southern England and Wales in July 2006: Devon, Somerset, Norfolk, Suffolk and on the Welsh island of Anglesey and in August 2006, in Kent (Table 3.1). Specimens of the Maculipennis Group were collected as immatures in four sites - in the Exminster Marshes (Devon), in a fenland near Reedham (Norfolk), Carlton Marshes in Oulton (Suffolk) and in the Mallraeth Marshes near Llangristriolus (Anglesey). Resting adults of *An. maculipennis s.l.* were manually collected from the walls and ceilings of animal stables in Norfolk and Somerset and abandoned pillboxes, brick shelters and war bunkers in Devon, Kent and Somerset (Table 3.1) and processed according to methods outlined in Chapter 2 Section 2.2.

County	Exact Locality	Co-ordinates	Date
Devon	*Stream margin, Exminster Marshes	50.6773582'N, 03.4729606'W	06.07.06
	Brick Shelter 1, Exminster Marshes	50.6756321'N, 03.4743221'W	06.07.06
	Brick Shelter 2, Exminster Marshes	50.6752617'N, 03.4751598'W	06.07.06
Kent	Brick shelter, Cliffe Marshes	51.4537556'N, 00.4782834'E	11.08.06
	Bunker, Cliffe Marshes	51.4783052'N, 00.4827834'E	11.08.06
	Sheep Corral, Cliffe Marshes	51.4671890'N, 00.4895240'E	11.08.06
Norfolk	Pettits Animal Farm, Reindeer and Miniature Horse stables	52.5659609'N, 01.5768554'E	20.07.06
	Pettits Animal Farm, Goat and Rhea Stables	52.6614040'N, 01.5768701'E	16.07.06
	*Stream margin Horsey Road, Reedham	52.7215319'N, 01.6550687'E	16.07.06
Somerset	Pillbox 1, Godney	51.1822719'N, 02.7283164'W	07.07.06
	Pillbox 2, Godney	51.1816487'N, 02.7273050'W	07.07.06
	Horse stable, Godney	51.1817652'N, 02.7230142'W	07.07.06
Suffolk	*Carlton Marshes, Oulton	52.4683907'N, 01.6928074'E	19.07.06
Anglesey	*Mallraeth Marsh, Llangristriolus C	53.2017891'N, 04.3454238'W	14.07.06

Table 3.1 Dates and exact collection localities of *An. maculipennis s.l.* collected across southern England and Wales in June and August 2006. *Indicates immature collections, link-reared to adults; all others were collected as resting adults.

Specimens were morphologically identified as belonging to *An. maculipennis s.l.*, primarily by their distinctive spotted wings, using the morphological key of Snow (1990), then identified to species either by direct sequencing of the nuclear ribosomal ITS2 (second internal transcribed spacer) region (see section 3.3.3), or using the ITS2 PCR-RFLP assay designed herein (see section 3.3.4). Voucher specimens (morphological specimens and/or DNA extractions) are available in the Natural History Museum, London, for future reference.

3.3.2 DNA extraction and ITS2 PCR amplification

DNA was extracted from individual specimens following the phenol-chloroform extraction protocol of Linton *et al*. (2001b). PCR amplification of the ITS2 region was achieved using the 5.8SF and 28SR primers of Collins & Paskewitz (1996). PCR reaction mixes and thermocycler parameters used in this study were those previously developed and detailed in Linton *et al.* (2001b). PCR products were amplified using either 2μl of template DNA or using a single mosquito leg placed directly in the PCR mix (Scott *et al*., 1993). PCR products destined for sequencing were first cleaned using the commercially available QIAgen PCR Purification Kit (QIAgen Ltd, Sussex, England), following the manufacturers instructions.

3.3.3 Direct sequencing of the ITS2 amplicon

Amplicons of ITS2 were directly sequenced for specimens of *An. maculipennis s.l.* collected in Norfolk (n=77), Devon (n=21), Kent (n=9) and Somerset (n=10). Purified ITS2 PCR products were sent to the Zoological Sequencing Facility in the Natural History Museum for sequencing. Resultant sequences were edited and aligned using Sequencher^{TM} version 4.6 (Genes Codes Corporation, Ann Arbor, Michigan) and CLUSTAL W (http://align.genome.jp/, Thompson *et al*., 1997). The FASTA search engine (http://www.ebi.ac.uk/fasta33/) was used to assess the similarity of ITS2 sequences generated in this study with those in GenBank. ITS2 sequences generated in this study were identified by comparison with those available in GenBank (*An. atroparvus* from England and Romania (487 bp; Linton *et al*., 2002a; Nicolescu *et al*., 2004), *An. daciae* from England and Romania (485bp; Linton, 2004; Nicolescu *et al*., 2004; Linton *et al*.,

Species	Country	Locality	GenBank accession Numbers	References
***An. atroparvus* (42)**	England (12)	Kent (12)	AF504237-248	Linton *et al*., 2002a
	Romania (30)	Budeni (18)	AY634505-522	Nicolescu *et al*., 2004
		Saftica (12)	AY634523-534	Nicolescu *et al*., 2004
***An. daciae* (103)**	England (5)	Somerset (5)	AY822585-589;	Linton *et al*., 2005
	Romania (98)	Budeni (63)	AY634407-469	Nicolescu *et al*., 2004
		Constanta (13)	AY634470-482	Nicolescu *et al*., 2004
		Giurgiu (1)	AY634406	Nicolescu *et al*., 2004
		Mehedinti (2)	AY634483-484	Nicolescu *et al*., 2004
		Saftica (19)	AY634485-503	Nicolescu *et al*., 2004
***An. messeae* (65)**	England (43)	Kent (40)	AF504197-236	Linton *et al*., 2002a
		London (1)	AF504196	Linton, dir. sub. 2001
		Yorkshire (2)	AF452699-700	Linton, dir. sub. 2001
	Greece (2)	Florina (2)	AF342711-712	Linton *et al*., 2002c
	Romania (17)	Budeni (1)	AY648982	Nicolescu *et al*., 2004
		Mehedinti (16)	AY648984-998	Nicolescu *et al*., 2004
			EF090197	Nicolescu *et al*., 2004
	Sweden (3)	Moja Island (3)	EF090194-196	Linton, dir. sub. 2001

Table 3.2 GenBank accession numbers and locality of 210 ITS2 sequences [*An. atroparvus* from England and Romania (n=42), *An. daciae* from England and Romania (n=103) and *An. messeae* from England, Greece, Romania and Sweden (n=65)] used to design the ITS2 PCR-RFLP assay designed to differentiate the three members of the British Maculipennis Group together with sequences generated in this study.

2005) and *An. messeae* from England, Greece, Romania and Sweden (485 bp; Linton *et al*., direct submission 2001; Linton *et al*., 2002a,c; Nicolescu *et al*., 2004) (Table 3.2).

3.3.4 Development of ITS2 PCR-RFLP assay

The ITS2 sequences (n=117) generated in this study (Table 3.3) were aligned together with the 210 available ITS2 sequences of *An. atroparvus* (487bp), *An. daciae* (485bp) and *An. messeae* (485bp) in GenBank (Table 3.2). Consensus sequences of *An. atroparvus*, *An. daciae* and *An. messeae* were aligned using Clustal W and used to identify appropriate fixed sequence differences within the fragments which could be exploited by restriction enzymes to differentiate between the three members of the British Maculipennis Group. The computer software Mapper, available online at http://arbl.cvmbs.colostate.edu/molkit/mapper/index.html, was used to determine enzyme choice. The optimal enzyme *BstU* I (CG↓CG) was chosen as it resulted in sufficiently different sized fragments that were species-diagnostic for *An. atroparvus* (445 & 42 bp), *An. daciae* (332, 59, 52 & 42bp) and *An. messeae* (332, 109 & 42bp). Restriction sites for each species are indicated in Figure 3.3 and resultant fragment sizes following electrophoresis are shown in Figure 3.2.

RFLP digestions were carried out in 20µl reactions as follows: 4 µl cleaned ITS2 PCR product, 13µl ddH_2O, 2µl Buffer 2* and 1µl *BstU* I enzyme* (*New England BioLabs). The reactions were incubated at the optimal enzyme activity temperature of 60°C in a thermocycler for a minimum of 3 hours to ensure full digestion of the fragments. Restriction fragments were visualised following electrophoresis on a 3% agarose gel containing 1% ethidium bromide for 1 hour at 70V. Fragment sizes were measured using Hyperladder IV (BioLine) (Figure 3.2).

Figure 3.2 Species-diagnostic ITS2 products from the three British Maculipennis Group species following digestion with *BstU* I enzyme (CG↓CG). Lane 1: Hyperladder I 100 bp ladder (BioLine). Lanes 2 & 3: *An. atroparvus* (445 bp, 42 bp). Lanes 4 & 5: *An. messeae* (332bp, 109bp, 42bp). Lanes 6 & 7: *An. daciae* (332bp, 59bp, 52bp, 42bp). Note: fragments under 100bp are generally not visible on this agarose gel.

3.4 Results

A total of 711 specimens of the Maculipennis Complex collected across southern England and northern Wales were molecularly identified to species in this study (Table 3.3). One hundred and seventeen nuclear DNA (nDNA) ITS2 sequences were generated (Table 3.3) and compared with reference sequences from England, Greece, Romania and Sweden available in Genbank (Table 3.2). The remaining 594 samples were identified using the ITS2 PCR-RFLP designed in this study (Table 3.3).

Of the counties sampled all three members were present in two – Kent and Norfolk. In Somerset and Suffolk, *An. messeae* and *An. daciae* were collected in sympatry, as adults and as larvae, respectively. In Somerset and Anglesey, only *An. daciae* was detected, while only *An. messeae* were collected in Devon.

3.4.1 ITS2 sequences

No intra-specific variation was noted within the ITS2 sequences of *An. atroparvus*, *An. daciae* and *An. messeae* generated from UK populations and were identical to those previously sequenced from England, Greece, Romania and Sweden (Table 3.2). *Anopheles daciae* and *An. messeae* share 99% ITS2 sequence identity, with only five fixed species-diagnostic sites at bases 214, 218, 220, 416 and 436 of the alignment with *An. atroparvus* (Figure 3.3). The ITS2 sequence of *An. atroparvus* was more divergent, with 46 divergent bases (90.6% identity) and 47 (90.4% identity) fixed nucleotide differences from *An. daciae* and *An. messeae*, respectively (Figure 3.3).

County	Exact localities	*An. atroparvus* (n=111)	*An. daciae* (n=471)	*An. messeae* (n=129)
Anglesey (n=1)	Mallraeth Marshes, Llangristriolus C.	0	1	0
Devon (n=99)	*Exminster Marshes (Stream margin)	0	0	2
	Exminster Marshes (Brick shelter 1)	0	0	71(19)
	Exminster Marshes (Brick shelter 2)	0	0	26(2)
Kent (n=110)	Cliffe marshes, Cliffe (Brick shelter 1)	5	0	2
	Cliff marshes, Cliffe (Salt Lane Bunker)	4	0	2
	Cliffe marshes (Sheep corral)	88(3)	6(6)	3
Norfolk (n=402)	Goat Stables, Pettits Animal Farm	8(5)	198(43)	2(1)
	Reindeer and miniature Horse stables, Pettits Animal Farm	6(1)	175(27)	11
	Stream on Horsey Road, Reedham	0	2	0
Somerset (n=93)	Godney Village (Pillbox 1)	0	63(1)	5
	Godney Village (Pillbox 2)	0	15	1
	Godney Farm, Godney (Horse stable)	0	8(8)	1(1)
Suffolk (n=6)	*Carlton Marshes, Oulton	0	3	3

Table 3.3 Relative proportions of species of the Maculipennis Group (n=711) collected and molecularly identified in five English counties (Devon (n=99), Norfolk (n=402), Somerset (n=93), Suffolk (n=6) and Kent (n=110) and on the Welsh island of Anglesey (n=1) in this study. *Anopheles atroparvus* (n=111), *An. daciae* (n=471) and *An. messeae* (n=129) Total numbers identified by the ITS2 PCR-RFLP assay and by direct sequencing of the ITS2 fragment (numbers sequenced are shown in parenthesis) are shown. *indicates larval collections.

3.4.2 PCR-RFLP Assay

All previously sequenced (n=210) and newly generated ITS2 sequences (n=117) from a wide range of geographical locations (Table 3.3) were aligned using Clustal W (http://align.genome.jp/). As no intraspecific variation was noted, irrespective of geographical origin of the samples, a representative ITS2 sequence for each of *An. atroparvus*, *An. daciae* and *An. messeae* were aligned. Polymorphic portions of the alignment were identified and screened for potential cutting sites using restriction enzymes (Figure 3.3).

In addition to the 117 specimens of the Maculipennis Group identified by ITS2 sequencing, a further 594 were identified following screening with the ITS2 PCR-RFLP assay designed herein (Table 3.3). Of the 711 specimens identified in this study, 11 individuals identified were collected as immatures, with the remaining 700 individuals were collected as resting adults. *Anopheles daciae* comprised 65.6% of the total number sampled, 17.9% were identified as *An. messeae* and 15.5% were *An. atroparvus* (Table 3.3).

```
                 1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
messeae   atcactcggc tcgtggatcg atgaagaccg cagctaaatg cgcgtcacaa tgtgaactgc aggacacatg aacaccgata
daciae    .......... .......... .......... .......... .......... .......... .......... ..........
atroparvus .......... .......... .......... .......... .......... .......... .......... ..........

                            1 1111111111 1111111111 1111111111 1111111111 1111111111 1111111111
          8888888889 9999999990 0000000001 1111111112 2222222223 3333333334 4444444445 5555555556
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
messeae   agttgaacgc atattgcgca tcgtgcgaca cagctcgatg tacacatttt tgagtgccca tatttgaccc --attcaagt
daciae    .......... .......... .......... .......... .......... .......... .......... --........
atroparvus .......... .......... .......... .......... .......... .......... .......t.a ta.cc....c

          1111111111 1111111111 1111111111 1111111112 2222222222 2222222222 2222222222 2222222222
          6666666667 7777777778 8888888889 9999999990 0000000001 1111111112 2222222223 3333333334
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
messeae   caaactacgt acctccgtgt acgtgcatga tgatgaaaga gtttgga-ac accttccttc -tcttgcatt gaaagcgcag
#daciae   .......... .......... .......... .......... .......-.. ...a...a.t -......... ..........
#atroparvus.....gg... .....acc.. .....g.-.t .........g ..c....t.. g..a.....t c........c ...gt..t..

           2222222222 2222222222 2222222222 2222222222 2222222222 2222222223 3333333333 3333333333
           4444444445 5555555556 6666666667 7777777778 8888888889 9999999990 0000000001 1111111112
           1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
#messeae   cgtgtagcaa ccccaggttt caacttgcaa agtggccatg gggctgacac ctcaccacca tcagcgtgct gtgtagcgtg
#daciae    .......... .......... .......... .......... .......... .......... .......... ..........
#atroparvus .......... .......... .......... .......... .......... .......... .......... ..........

          3333333333 3333333333 3333333333 3333333333 3333333333 3333333333 3333333333 3333333334
          2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
messeae   ttcggcccag taaggtcatc gtgaggcgtc acctaacggg gaagcacaca ctgttgcgcg tatctcgtgg ttctaaccca
daciae    .......... .......... .......... .......... .......... .......... .......... ..........
atroparvus .......... .tc....... .........t .........a .......cag ...c....t. ......a... ..acc-....

          4444444444 4444444444 4444444444 4444444444 4444444444 4444444444 4444444444 4444444444
          0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
messeae   accatagcag cagaggtaca agaccagctc ctagcggcgg gagctcatgg gcctcaaata atgtgtgact accccctaaa
daciae    .......... .....a.... .......... .....c.... .......... .......... .......... ..........
atroparvus .......... .....a.... .......... .....a.... .......... .t........ .....a.... ..........

          444444444
          888888888
          123456789
messeae   tttaagcat
daciae    .........
atroparvus .........
```

Figure 3.3 The 489bp alignment of nuclear ITS2 sequences of *An. messeae* (485bp), *An. daciae* (485bp) and *An. atroparvus* (487bp). Enzyme *BstU* I (CG↓CG) cutting sites are highlighted in yellow. One site is present at 42 bp for all three species, a second site is present for both *An. messeae* and *An. daciae* at 378bp and a third site is present for *An. daciae* only at 437bp**,** thus the following species-diagnostic fragments are generated: *An. atroparvus* (445 & 42 bp), *An. daciae* (332, 59, 52 & 42bp) and *An. messeae* (332, 109 & 42bp).

3.4.3 Ecology

Anopheles daciae was collected as immatures (n=6) in Mallraeth Marshes in Anglesey (n=1, pupae), in Carlton Marsh Reserve, Suffolk (larvae, n = 3) and in a ditch in fenland near Reedham, Norfolk (larvae, n=2) and as resting adults in various animal stables (rhea, goat, reindeer, miniature donkey) in Pettits Animal Farm in Reedham, Norfolk (n=373), in Godney farm and Garslade farm in Somerset (n=86) and in the Cliffe marshes, Kent (n=6).

A single collection consisting of *An. messeae* was collected as immatures (n=5) in a stream margin in Exminster Marshes, Devon (n=2 pupae). *Anopheles messeae* was also collected in the Carlton Marsh Reserve, Suffolk (n=3; 1 larva, 2 pupae) in sympatry with *An. daciae.* Adults of *An. messeae* (n=122) were collected in the Exminster Marshes in Devon (n=97), in adjacent reindeer (n=11) and goat stables (n=2) at Petitts Animal farm in Norfolk, in Godney farm and Garslade farm in Somerset (n=7) and in the Cliffe Marshes in Kent (n=7). The only member of the Maculipennis Group collected as larvae and adults in the Exminster RSPB Nature Reserve, near Exeter, were *An. messeae* (n=99).

Anopheles atroparvus was only collected as adults in two counties in this study - in reindeer stables in Pettits Animal Farm in Norfolk (n=14) and resting in disused bunkers in the Cliffe Marshes in Kent (n=97), being particularly abundant in one bunker currently sused as a sheep corral.

3.5 Discussion

Historically, identification of British members of the Maculipennis Group relied on the indirect method of comparison of egg morphology or ecological nuances, such as incomplete diapause, to differentiate between *An. atroparvus* and *An. messeae*. Linton *et al*. (2002a) were the first to use ITS2 DNA sequencing to identify *An. atroparvus* and *An. messeae* in the Cliffe Marshes in Kent and ITS2 sequencing of specimens of purported "*An. messeae*" in Somerset, revealed the presence of *An. daciae* for the first time (Linton *et al*., 2005). The costs of DNA sequencing is prohibitive when considering a wider study on current distribution of the Maculipennis Group, but herein an accurate, inexpensive method of processing large numbers of specimens is presented. A total of 711 specimens collected in July and August 2006 from

northern Wales (Anglesey) and England (Devon, Norfolk, Somerset, Suffolk and Kent) were identified to species in this study, thus comprising the largest species-level study of the British Maculipennis Group to date. Interestingly this study suggested a widespread occurrence of *An. daciae*, with 65.6% of all specimens collected in southern Britain being of this species.

The most interesting result obtained in this study, aside from the presence of *An. daciae* in Godney, Somerset, is the documented presence of *An. daciae* in Norfolk, Somerset, Suffolk and Kent herein all comprise new distribution records and the collection of a single individual in Anglesey is a new country record for Wales. New distribution records were also established for *An. atroparvus* from Norfolk and *An. messeae* in Somerset. Adults of *An. daciae* were collected resting in sympatry with both *An. messeae* and *An. atroparvus* in animal stables and disused war installations in Kent and in Norfolk and with *An. messeae* only in Somerset. As observed in Romania (Nicolescu *et al*., 2004), adults of *An. daciae* in the current study were collected in sympatry with *An. atroparvus* in Kent and Norfolk and immatures of *An. daciae* were collected in sympatry with *An. messeae* in Suffolk.

The main purpose of this study was to develop a PCR assay that could differentiate the members of the Maculipennis Group. The most important objective was to sample all genetic variation found within each species for the UK in order to ensure that the assay was based on fixed polymorphisms rather than on nucleotides that are polymorphic within UK populations. A broad geographical sampling of the Maculipennis Group was thus undertaken to ensure sufficient material for the development of a robust assay. Specimens of the Maculipennis Group were collected in 14 localities and collections of both resting adults and larvae were aimed at maximum capture rates. The extent of sampling was not designed to establish accurate distribution records, and because collections were not standardised, detailed conclusions on the species abundance, composition and occurrence within and between sites were not appropriate. For example, the presence of a single *An. daciae* individual in Anglesey indicates the presence of the species, but does not exclude the possibility of either *An. messeae* or *An. atroparvus* being present. To measure species abundance and richness in each site and/or to compare the prevalence of species between sites, standardised collection methods such as a predetermined number of dips per larval site, collecting along a transect in an aquatic environment, or to collect resting adults for a predetermined period of time over a day for a set number of days (for example collecting every 15min 3 times a day for one week) (Service,

1998) are required. Data gathered from such collections could then be statistically examined and a quantitative spatial distribution pattern of the Maculipennis Group could be determined.

Prior to this study, ecological studies carried out on the Maculipennis Group did not differentiate between the three members (Snow, 1998; Hutchinson *et al.,* 2007; Snow & Medlock, 2008). As a result, detailed knowledge of the distribution of individual species within the group was undocumented. Results generated from the assay developed here (section 3.3.4) have confirmed the occurrence of these species in southern England, have contributed significantly to the knowledge of resting adult habitats of *An. atroparvus*, *An. daciae* and *An. messeae*, and the assay will enable fast and accurate studies to be carried out in the future. The detection of *An. daciae* in 3 English counties and in Anglesey implies that its presence was masked by that of *An. messeae*, as suggested by Linton *et al*. (2005). It also suggests that *An. daciae* could be widespread, cryptically co-occurring with the currently proposed global distribution of *An. messeae*. Linton *et al* (2005) compared mitochondrial Cytochrome Oxidase I (COI) of *An. daciae* from the type series (Nicolescu *et al*., 2004) with the published study of Di Luca *et al*. (2004) and showed that some purported *An*. *messeae* from The Netherlands, Kazakhstan and Italy were in fact *An. daciae*. This indicates that the species is present in various places across Europe and in the former USSR states; As well as its documented presence in the UK and Romania, *An. daciae* has also recently been detected in Poland and Bulgaria (Y-M. Linton, Pers. Comm.).

A recent study of ITS2 sequences in six purported *An. messeae* populations from Russia (Bezzhonova & Goryacheva, 2008) revealed polymorphism in eight sites along the ITS2 region (including the five *daciae*-specific bases of Nicolescu *et al*. (2004). Also documented was intragenomic variation, whereby one individual which showed both *An. daciae* and *An. messeae* ITS2 haplotypes. Due to this, the authors proposed that ITS2 was not an effective marker to discriminate between *An. daciae* and *An. messeae* and proposed the synonymy of *An. daciae* with *An. messeae*. I disagree with this for several reasons. Firstly, both species are found in sympatry in several areas of their range (Nicolescu *et al*., 2004; Linton *et al*., 2005; herein). Given that *An. daciae* and *An. messeae* are the most closely related species in the Maculipennis Group (Linton *et al.,* 2004; Nicolescu *et al*., 2004; Linton *et al*., 2005) and that they occur in sympatry (Nicolescu *et al*., 2004; herein); it is plausible that these two species have recently undergone ecological speciation. Concerted evolution

within the ITS2 can result in the homogenisation of polymorphic loci within a species in a fairly short period of time (Collins & Paskewitz, 1996) thus allowing for successful differentiation of species over a geographical range (Fritz *et al.,* 1994; Navajas *et al.,* 1998); the presence of intra-individual / intra-population variation in the nuclear ITS2 gene has been attributed to the intermixing of differentiated ITS2 populations (Vogler & DeSalle, 1994). Intragenomic ITS variation, on both the individual and population level, has been reported in other species complexes, e.g. Australian tiger beetles - *Cicindela dorsalis* (Vogler & DeSalle, 1994), in the *Ixodes ricinus complex* (Wesson *et al.,* 1993) and in mosquitoes, *Anopheles albitarsis* complex (Wilkerson *et al*., 2005). Despite this variation, the phylogenetic importance of fixed loci supported the recognition of morphologically distinct subspecies (Vogler & De Salle, 1994), as do the five fixed ITS2 differences of Nicolescu *et al*. (2004). Given the nature of PCR (i.e. amplification of the most prolific haplotype), low level polymorphic intra-individual ITS haplotypes are usually only revealed by cloning, as in the study of Bezzhonova & Goryacheva (2008). However, sequence data of the independent mitochondrial COI gene from individuals identified by their signature ITS2 gene region do corroborate the separate species status of these taxa (Linton, 2004; Nicolescu *et al*., 2004; Di Luca *et al*., 2004). Interestingly, Bezzhonova & Goryacheva (2008) do report the same five fixed base differences between *An. daciae* and *An. messeae* (Nicolescu *et al*., 2004; Linton *et al.,* 2005) and the presence of the diagnostic RFLP restriction site (Section 2.3.4). Thus establishing the validity of *An. daciae* as a species and suggesting its presence in Russia.

The ITS2 PCR-RFLP assay herein was designed specifically for the differentiation of British members of the Maculipennis Group with a particular focus on *An. daciae* and *An. messeae*. With that said, identification of these two species can still be carried out using this RFLP outside the UK. However, due to the short fragment size of the ITS2 (circa 470-490 bp in most members of Palaearctic Maculipennis Group, except *An. beklemishevi* (853bp) (Kampen, 2005b) and the close genetic relationship between the species, other closely related species could produce similarly sized fragments following the restriction with enzyme *BstU* I (CG↓CG). For example, this assay will not work effectively in The Netherlands as *An. maculipennis s.s.* is present in addition to the three British species. Digestion of *An. maculipennis s.s.* ITS2 amplicons (472bp) with this enzyme would yield fragment sizes of 332 & 103 bp which, when visualised on an agarose gel, could be mistaken for *An. messeae*. Thus, to avoid such misidentification, *An. messeae* could first be identified, using the PCR assay of

Proft *et al.* (1999), from the rest of the Palaearctic Maculipennis Group using their purported *messeae*-specific primer. These individuals could then subsequently be differentiated as either *An. messeae* or *An. daciae* (both will be identified as *An. messeae*), using the ITS2 PCR-RFLP described in this chapter.

The spread of emerging and re-emerging diseases such as malaria and WNv into Europe and the impact of global warming have forced scientists to reassess the potential introduction of these diseases into the British Isles (Medlock *et al.,* 2006) and Europe as a whole. Increased global travel results in approximately 2000 cases of malaria imported annually in the UK (HPA, 2008) presenting the possibility of UK mosquitoes becoming infected with imported *Plasmodium* as previously documented in the north Kent marshes (Shute, 1963) and in two cases of airport malaria reported near Gatwick (Whitfield *et al.,* 1984). The widespread presence of *An. daciae* and its sympatry with *An. atroparvus* in Kent and Norfolk detailed in this study presents this species as a potential vector of malaria in the UK.

It is essential that the vector competencies of our endemic species are carefully assessed with wild populations and that the distribution and ecological factors of all British mosquitoes, especially the Maculipennis Group, are documented. This assay allows cheap, accurate identification of members of the Maculipennis Group and provides a solid identification tool for use in such projects in future. Specific host selection of members of the Maculipennis Group is assessed in Chapter 4.

Chapter 4

A critical assessment of molecular identification tools for the Palaearctic members of the Pipiens Group (Diptera: Culicidae)

4. A critical assessment of molecular identification tools for Palaearctic members of the Pipiens Group (Diptera: Culicidae)

4.1 Introduction

4.1.1 Taxonomic status and distribution of the Pipiens Group

The genus *Culex* has a worldwide distribution and comprises more than 762 species in 26 subgenera (Mosquito Taxonomic Inventory, 2008). *Culex* (*Culex*) *pipiens* Linneaus is the nominotypical species of the genus *Culex* and was originally described from Near Lake Krankesjo, Silvakra farm, Veberod, Scania in Sweden by Linnaeus in 1758. The subgenus *Culex* comprises the Pipiens Group (5 species), the Sitiens Group (6 species) (Edwards, 1932; Harbach, 1988) (see Chapter 1, Figure 1.3), the South Pacific Atriceps Group (3 species) (Belkin, 1962) and the Duttoni Group (Harbach, 1988) for the monotypic Afrotropical *Cx. duttoni* Theobald.

The Pipiens Group is further divided into the Pipiens, Trifiliatus, Theileri and Univittatus subgroups (Harbach, 1988). The Pipiens subgroup currently comprises *Cx. pipiens* Linnaeus (with its two forms, *pipiens* and *molestus*), *Cx. quinquefasciatus* Say, *Cx. pallens* Coquillett*, Cx. australicus* Dobrotworsky & Drummon and *Cx. globocoxitus* Dobrotworsky. Of these, three exhibit limited distributions: *Cx. pallens* (Japan, Korea and Mexico) and *Cx. australicus* and *Cx. globocoxitus* (both species in Australia and Oceania) (Smith *et al.,* 2005; WRBU, 2008). *Culex pipiens*, on the other hand, is practically pan-global (WRBU, 2008). Three species comprise the Trifiliatus subgroup, namely *Cx. restuans* Theobald (Nearctic region), *Cx. torrentium* Martini (Palaearctic region) and *Cx. vagans* Wiedemann (Oriental and Asiatic Regions). Of the members in the Pipiens Group, *Cx. pipiens s.l.* (Pipiens subgroup) and *Cx. torrentium* (Trifiliatus subgroup) are present in the UK (Snow, 1990).

4.1.2 The Pipiens Complex

Culex pipiens s.l. is found in urban and semi-urban areas across eastern and western Europe, the Middle East and North America (Vinogradova & Fomenko, 1968; Snow, 1990; Spielman, 2001; Shaikevich, 2007; Almeida *et al*., 2008; WRBU, 2008). Possibly as a result of its wide geographic occurrence, the species currently has 37 valid synonyms (WRBU, 2008).

Based on differences observed in morphology and host selection, *Culex molestus* Forskål was described as a seperate species from *Cx. pipiens* by Forskål in 1775 (in Harbach, 1984) in Rosetta, Kahira and Alexandria in Egypt. The inadequate original description and the lack of a type specimen meant that no subsequent studies of the type of *Culex molestus* could be made, leading to confusion regarding its specific status. In an early published study, Marshall & Staley (1937) regarded *Cx. molestus* as a seperate species and listed a number of morphological and ecological factors to differentiate these two species. These include average siphonal lengths, the presence of white scales on the legs as well as the length difference between palpal segments and the proboscis in males (Table 4.1). However, in later publications, *Cx. molestus* was considered to be a biotype of *Cx. pipiens* and not a seperate species in the Pipiens Complex (Barr, 1957; Stone *et al*., 1959).

In order to clarify the taxonomic nomeclature and form a solid foundation for future studies on the Pipiens Complex, neotypes were designated for *Cx. molestus* (Harbach *et al*., 1984) and *Cx. pipiens* (Harbach *et al*., 1985). Given the lack of reliable morphological characters between the two species, Harbach *et al*. (1984) proposed that *Cx. molestus* should be regarded as a junior synonym of *Cx. pipiens*; as a 'behavioural/physiological variant' of *Culex pipiens* rather than a separate species. Following these works, *Culex pipiens s.l.* is recognised as comprising two biological forms: *Culex pipiens* f. *pipiens* and *Culex pipiens* f. *molestus*.

Due to the difficulty in identification based on morphology, ecological parameters are most commonly used to differentiate between the two forms of *Cx. pipiens*. *Culex pipiens* f. *pipiens* is reported to be ornithophilic, eurygamous, anautogenous and its larval habitats are above ground, whereas *Culex pipiens* f. *molestus* immatures are found in underground sites

(flooded basements, underground tunnels). *Culex pipiens* f. *molestus* also is autogenous and anthropophilic and the adults are reportedly able to mate in small confined spaces (stenogamous) (Roubaud, 1933; Tate & Vincent, 1936; Mattingly, 1953; Wallace, 1958; Vinogradova & Fomenko, 1968; Bryne & Nichols, 1999). Unfortunately, in comparisons of autogenous and anautogenous populations in Spain (Chevillon *et al.,* 1995), Israel and Egypt (Nudelman *et al.,* 1988), little or no variation of the other behavioural and ecological characteristics (e.g., anthropophily, habitat preference) was observed. In addition, hybridisation of the forms has been reported in populations in Russia (Vinogradova, 1966; Shaikevich, 2007) and in North America (Barr, 1967; Bahnck & Fonseca, 2006), resulting in further ambiguity (Bryne & Nichols, 1999).

Species/Characters	*Culex pipiens* f. *pipiens*	*Culex pipiens* f. *molestus*
Larvae		
• Average value of siphonal index	5.0	<4.3
Adults		
• Common characters	Darker brown	Lighter colouration
	Whitish scales at the tip of the femora and tibia forming conspicuous spots	Spots at the tips of femora and tibia not conspicuous
	Median and lateral patches of dark scales on ventral surface of abdomen	No such scale patches present
• **Male**	Combined length of terminal 4 palpal segments is longer than the overall length of proboscis	Combined length of terminal 4 palpal segments is shorter than that length of proboscis
• **Female**	Pale tergal bands constricted laterally and centrally	Tergal bands are not constricted

Table 4.1 Morphological characters distinguishing *Culex pipiens* f. *pipiens* Linnaeus and *Culex pipiens* f. *molestus* Forskål (after Marshall & Staley, 1937).

4.1.3 Differentiation of *Cx. pipiens* f. *pipiens, Cx. pipiens* f. *molestus* and *Cx. torrentium*

The morphological similarity of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* led to the development of molecular tools to distinguish the two forms. Length variation in the dinucleotide (TG-repeat) microsatellite locus CQ11 allowed for the molecular differentiation between *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* (Bahnck & Fonseca, 2006). A single 180-bp fragment containing 6 TG repeats indicates the presence of *Cx. pipiens* f. *pipiens* while a single 250-bp fragment indicates the presence of *Cx. pipiens* f. *molestus* in which the CQ11 locus is absent. The occurrence of both fragments was interpreted to indicate hybrids of the two forms (Bahnck & Fonseca, 2006). This molecular tool provides a relatively inexpensive identification method as individuals can be scored directly following electrophoresis of the PCR product.

The co-occurrence of the closely related *Cx. torrentium* with *Cx. pipiens s.l.* further complicates correct identification of Pipiens Group members in Europe. This common and often sympatric mosquito differs from *Cx. pipiens s.l.* only in the phallosomic structure on the male hypopygium (Martini, 1925; Mattingly, 1952; Service, 1968a) and the presence of a patch of prealar scales in the females (Jupp, 1979; Harbach *et al*., 1985).

Given the relative difficulty in morphologically differentiating these species in Europe, Shaikevich (2007) developed a two-step RFLP assay based on fixed differences in the mtDNA COI gene between *Cx. pipiens* f. *pipiens*, *Cx. pipiens*, f. *molestus* and *Cx. torrentium* (see Figure 4.1a-c). The enzyme *Hae* III (GG↓CC) cleaves *Culex pipiens* f. *pipiens* at the site indicated in Figure 4.1a, discriminating it from both *Cx. pipiens* f. *molestus* and *Cx. torrentium*. The second digest, using the enzyme *Bc* II (T↓GATCA), cuts *Cx. torrentium* at only one site (79th base, Figure 4.1b) but cuts *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* (Shaikevich, 2007) at two recognition sites (Figure 4.1b & c) at bases 79 (Figure 4.1b) and 485 (Figure 4.1c). Thus facilitating the identification of these species.

Figure 4.1a

Figure 4.1b

Figure 4.1c

Figure 4.1: Alignment of *Cx. pipiens* f. *pipiens*, *Cx. pipiens* f. *molestus* and *Cx. torrentium* using Mesquite (v2.6) showing the three restriction sites in the 710-bp fragment of the COI gene used by Shaikevich (2007)**. Figure 4.1(a)** shows a single A-G polymorphism at base 205 that differentiates *Cx. pipiens* f. *pipiens* from *Cx. pipiens* f. *molestus* and *Cx. torrentium*. This difference is exploited in the first RFLP assay using enzyme *Hae* III (GG↓CC), which cuts the *Cx. pipiens* f. *pipiens* fragment into two, but leaves the *Cx. pipiens* f. *molestus* and *Cx. torrentium* uncut. **Figures 4.1 (b & c)** show the restriction sites of the second enzyme, *Bc* II (T↓GATCA), which cleaves *Cx. torrentium* at only one site (79bp) (Figure 3.3b) while digesting *Cx. pipiens* f. *pipiens and Cx. pipiens* f. *molestus* at two sites (79 and 485bp) (Figure 4.1.b, c).

4.1.4 Vector status of *Culex pipiens s.l.*

Culex pipiens s.l. has been incriminated as a vector of several flaviviruses world-wide: Japanese Encephalitis in Southeast Asia and Australia (Johansen *et al.,* 2002), Ockelbo virus in Sweden (Jaenson *et al.,* 1986b; Lundström *et al.,* 1990b), Rift Valley Fever in Egypt (Meeghan *et al.,* 1980; Turrell *et al.,* 1996), Saint Louis Encephalitis in North America (Meyer *et al.,* 1982; Miller *et al.,* 1996) and West Nile virus (WNv) in Europe and North America (Hayes, 2001; Fonseca *et al.,* 2004; Hamer *et al.,* 2008; see Chapter 1, section 1.4.2). Although (WNv) was first reported in the Rhone delta in 1963, the recent global resurgence of the virus in southern France (Balenghien *et al.,* 2006), Italy (Romi *et al.,* 2004, in horses), Portugal (Estevez *et al.,* 2005; Almeida *et al.,* 2008), Romania (Tsai *et al.,* 1998; Savage *et al.,* 1999), Russia (Lvov *et al.,* 2000; Platonov *et al.,* 2001) and several states in North America (Marfin *et al*., 2001) has rejuvenated research efforts in identifying mosquito vectors and routes of transmission. As well as circumstantial association in WNv endemic areas via patient screening and collections of dead birds (Marfin *et al.,* 2001), the ubiquitous *Culex pipiens s.l.* was incriminated as an efficient vector of WNv following direct isolation of the virus from the mosquito (Romi *et al.,* 2004; Almeida *et al.,* 2008; Hamer *et al.,* 2008), host selection studies (Balenghien *et al.,* 2006; Hamer *et al.,* 2008) and laboratory infection tolerances (Lundström *et al.,* 1990).

In the UK, Buckley *et al*. (2003) provided evidence of WNv, Sindbis virus and Usutu infections in native British birds by screening 353 serum samples for the presence of antibodies to these viruses. Fifty-two (14.7%) of the birds tested were positive for WNv, while two samples each were positive for Usutu and Sindbis, respectively. This was the first indication of the presence of WNv in the British Isles. A later study of sentinel chickens found that 46-day old chicks had neutralising antibodies to WNv, proving the antibodies were not maternally transferred; implying that active transmission of the virus was occurring between endemic birds and mosquitoes in the UK (Buckley *et al*., 2006). The detection of WNv in local birds is of public health concern, especially given the reported human cases of the virus in the USA (Lanicotti *et al*., 1999) and in Europe (Almeida *et al.,* 2008). The source of the virus is most likely the constant influx of birds stopping off in the UK on migratory routes from Africa (Rappole *et al.,* 2000; Higgs *et al.,* 2004; Medlock *et al.,* 2005). Interestingly, the

ornithophilic *Cx. torrentium* is reported as a vector of avian Sindbis virus (Medlock *et al*., 2007), which has been detected in endemic UK birds (Buckley *et al*., 2003). Its role in WNv transmission remains unclear.

Given the established importance of *Cx. pipiens s.l.* and *Cx. torrentium* in arbovirus transmission (Lundström *et al.,* 2000; Medlock *et al.,* 2007), it is essential to be able to accurately identify the exact composition and distribution of these taxa in the UK as the basis for a strategic action plan should these diseases become a problem in the UK in the future.

4.2 Aims

The aims of this study were:

[1] To assess the presence of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* in the UK using the CQ11 microsatellite assay of Bahnck & Fonseca (2006) and

[2] To test the congruence of the results of the CQ11 assay with the mtDNA COI assay of Shaikevich (2007).

4.3 Materials and Methods

4.3.1 Collection of *Culex*

Immature collections were carried out in 34 discrete locations across 4 counties in southern England (Devon, Somerset, Norfolk and Suffolk) and 2 counties of north Wales (Anglesey, Caernarfonshire) in July 2006. Collection methods are detailed in full in Chapter 2 (see section 2.2). All larvae and pupae collected were link-reared through to the adult stage, whereupon adults were card-point mounted (see section 2.2.1.2). These pinned adult specimens and their associated larval and pupal exuviae serve as voucher specimens for this study and are held in the Natural History Museum, London. Adults of these link-reared specimens were identified to species using the morphological keys of Cranston *et al*. (1987) and Snow (1990) and used for subsequent molecular analysis.

4.3.2 Molecular identification

4.3.2.1 CQ11 Microsatellite Assay

The PCR for the amplification of the microsatellite locus CQ11 was carried out using a single leg from 322 specimens that were morphologically identified as *Cx. pipiens s.l.* The PCR was run in a 20µL reaction mix (Table 4.2) using the cycling conditions described by Bahnck & Fonseca (2006). PCR amplified fragments were visually differentiated on a 2% agarose gel, in comparison to a known size standard (HyperLadder IV, BioLine, UK) (Figure 4.2). After Bahnck & Fonseca (2006), a 180-bp fragment was scored as *Culex pipiens* f. *pipiens,* a 250-bp fragment was scored as *Cx. pipiens* f. *molestus* and the presence of both amplicons in a single sample was scored as a putative *Cx. pipiens* f. *pipiens* x f. *molestus* hybrid (Figure 4.2).

Reagents (concentration)	Volume (20µl)	Thermocycler conditions
ddH_20	12.4	1) 95°C- 10mins
10x NH_4 buffer (BioLine)	2.0	
10mg/ml BSA	0.3	2) 94°C- 30secs
10mM dNTPs	0.4	3) 55°C- 30secs
10µM P(CQ11mol)	0.2	4) 72°C- 40secs
10µM P(CQ11F)	0.2	Repeat steps 2-4 for 39 cycles
10µM P(CQ11pip)	0.2	
25mM $MgCl_2$ (BioLine)	1.6	5) 72°C- 5mins
Taq (BioLine)	0.2	
DNA	2.5	

Table 4.2 Composition of the PCR reagents and thermocycler conditions used in the 20µl reaction for the CQ11 microsatellite assay of Bahnck & Fonseca (2006).

Reagents (concentration)	Volume (25µl)	Thermocycler conditions
ddH_2O	14.90	1) 95°C- 5mins
10x NH_4 buffer (BioLine)	2.50	2) 95°C- 30secs
10mM dNTPs	0.50	3) 53.5°C- 45secs
10µM F primer (LCO)	1.25	4) 72°C- 45secs
10µM R primer (HCO)	1.25	Repeat steps 2-4 for 24
50mM $MgCl_2$ (BioLine)	2.50	cycles
Taq (BioLine)	0.10	5) 72°C- 4mins
DNA	2.00	

Table 4.3 PCR reaction mix and thermocycler conditions for amplification of the barcoding region of the COI gene (25µl reaction).

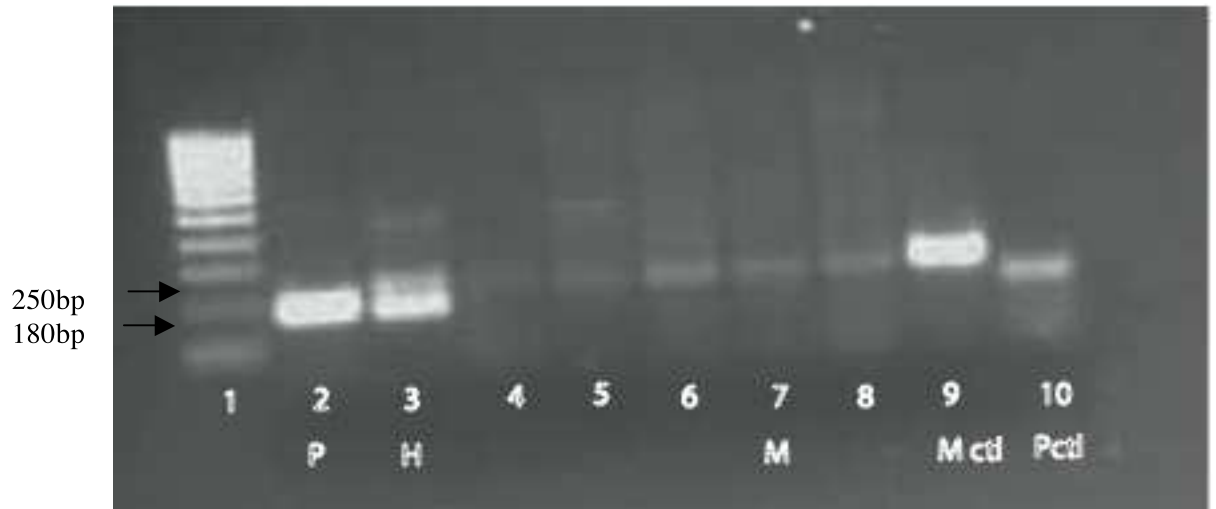

Figure 4.2 Image of the results of the CQ11 microsatellite assay following electrophoresis on a 2% agarose gel. *Culex pipiens f. molestus* (250bp) and *Cx. pipiens* f. *pipiens* (180bp) controls are clearly shown in Lanes 9 and 10 respectively. Lane 1: HyperLadder IV (BioLine), Lane 2: *Culex pipiens* f. *pipiens*, Lane 3: individual with fragments for both *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*, Lanes 4-8: *Culex pipiens* f. *molestus*, Lane 9: *Culex pipiens* f. *molestus* control and Lane 10: *Culex pipiens* f. *pipiens* control.

4.3.2.2 Amplification of mtDNA COI gene fragment

Thirty samples previously identified in Section 4.3.2.1 as *Culex pipiens* f. *pipiens* (n=10), *Cx. pipiens* f. *molestus* (n=14) and *pipiens* x *molestus* hybrids (n=6) were analysed further using the mtDNA COI gene (Table 4.5). Instead of using the COI PCR-RFLP assay of Shaikevich (2007), the COI gene was sequenced and resultant fragments were screened for the *Cx. pipiens* f. *molestus* and *Cx. pipiens* f. *pipiens* diagnostic base change reported by Shaikevich (2007).

A 710-bp region of the mitochondrial Cytochrome Oxidase I (COI) gene (corresponding to the "barcoding" region) that overlapped the restriction sites of the COI RFLP assay (Shaikevich, 2007) was amplified using the primers LCO1490 and HCO2198 (Folmer *et al.,* 1994). Volume and concentration of reagents used in the PCR reaction are given in Table 4.3. PCR products were cleaned in a 200-µl PCR tube using 8µl of positive PCR product and 2µl of a 1:4 dilution of ExoSAP-IT® (GE Healthcare, UK). The mixture was placed in a thermocycler and incubated at 37ºC for 30 minutes and then at 80ºC for a further 20 minutes. Cleaned products were then sent to the Zoological Sequencing Facility at the Natural History Museum for sequencing. All COI sequences were assembled and edited using Sequencher® 4.6 and aligned using ClustalW (Thompson *et al.,* 1997). A maximum parsimony tree was constructed using PAUP 4.0 b10 (Swofford, 2002). Bootstrap values for 100 replicates were calculated using TNT (Goloboff *et al*., 2008) using a heuristic search of 100 replicates of TBR branch swapping. Genetic diversity (pi) within and p-distance between clades (see Results) was calculated using MEGA 4.0 (Tamura *et al.,* 2007) and DnaSP 4.0.2 (Rozas *et al.,* 2003).

As the COI-RFLP assay designed by Shaikevich was intended to distinguish the two forms of *Cx. pipiens s.l.* and *Cx. torrentium*, a further 18 COI sequences were obtained and analysed together with the 30 specimens listed: morphologically verified *Cx. torrentium* specimens (collected in this study, n=10), *Cx. pipiens* f. *molestus* colony individuals from Greece (n=3) (supplied by G. Koliopolous, Benaki Phytopathological Institute, Athens Greece; sequenced by Y. -M. Linton), 1 wild-caught *Cx. pipiens* f. *molestus* from Barking, London (supplied by A. Curtotti, Queen Mary University), GenBank sequences of *Cx. pipiens* f. *pipiens* (accession number AM403476, n=1), *Cx. pipiens* f. *molestus* (AM403492, n=1) and

Cx. torrentium (AM403477, n=1) from Russia (Shaikevich, 2007) and a sequence from colony *Cx. quinquefasciatus* of the Pipiens Group (n=1) (supplied by Prof. A.J. Mordue, University of Aberdeen, Scotland; sequenced by Y.-M. Linton). The closely related *Cx. vagans* (n=4) (sequenced by Y. -M. Linton) belonging to the Trifiliatus subgroup in the Pipiens Group and *Cx. sitiens* (n=1) from Australia (DQ673858) of the Sitiens Group were used as outgroup taxa.

4.4 Results

4.4.1 Collection of *Culex* mosquitoes

Aquatic stages of *Culex* mosquitoes collected only from overground breeding sites in this study (n=427) were morphologically identified as *Cx. pipiens s.l.* (n=322) or *Cx. torrentium* (n=105). *Culex pipiens s.l.* was detected in all counties sampled (Anglesey (n=105) and Caernarfonshire (n=21) in Wales, Devon (n=139), Norfolk (n=27), Somerset (n=24) and Suffolk (n=6) in England). *Culex torrentium* was not detected in Somerset or Suffolk, but was present in the other counties sampled [Anglesey (n=34), Caernarfonshire (n=14), Devon (n=45) and Norfolk (n=12)]. Neither of the other two reported British species of *Culex* (*Cx. europaeus* and *Cx. modestus)* was collected in this study.

4.4.2 CQ11 Microsatellite assay

The 322 *Culex* mosquitoes were analysed using the assay of Bahnck & Fonseca (2006) (Table 4.4). *Culex pipiens* f. *molestus* was detected in all areas sampled but generally in lower numbers than *Cx. pipiens* f. *pipiens* (Table 3.4). Both forms were detected sympatrically in 72.5% sites. Hybrids of *pipiens* x *molestus* comprised 6.8% (n=22) of all *Cx. pipiens s.l.* tested in this study. These twenty-two heterozygous individuals were found in 9 of the 40 sites. This level of hybridization and the detection of overground populations of *Cx. pipiens* f. *molestus* were highly surprising; therefore, verification of these results was sought using the PCR-RFLP method of Shaikevich (2007).

County	*Cx. pipiens* f. *molestus*	*Cx. pipiens* f. *pipiens*	'hybrid'
Anglesey	**49**	**49**	**7**
Caernarfonshire	**5**	**13**	**3**
Devon	**31**	**96**	**12**
Norfolk	**3**	**24**	**-**
Somerset	**5**	**19**	**-**
Suffolk	**2**	**4**	**-**
Total	**95**	**205**	**22**

Table 4.4 Identification of *Cx pipiens s.l*. specimens using the assay of Bahnck & Fonseca (2006); *Cx. pipiens* f. *molestus* (n=95), *Cx. pipiens* f. *pipiens* (n=205), hybrids (n=22).

Morphology	CQ11 (n=30)	COI (n=40)	
		Cx. pipiens f. pipiens	*Cx. torrentium*
Cx. pipiens s.l. (n=30)	10 *Cx. pipiens* f. *pipiens*	7	3
	14 *Cx. pipiens* f. *molestus*	7	7
	6 *pipiens* x *molestus* hybrid	0	6
Cx. torrentium (n=10)		0	10

Table 4.5 A total of 30 specimens (from Table 3.4): *Cx. pipiens* f. *pipiens* (n=10), *Cx. pipiens* f. *molestus* (n=14) and *pipiens* x *molestus* hybrid (n=6) and ten morphologically identified *Cx. torrentium* were sequenced for COI. Upon analysis, the assay of Shaikevich (2007) distinguished 14 *Cx. pipiens* f. *pipiens* individuals, 26 *Cx. torrentium* individuals and no *Cx. pipiens* f. *molestus* or hybrid specimens were detected.

4.4.3 Congruence of CQ11 assay and mtDNA COI sequences

Three separate clades emerged in the tree reconstruction using maximum parsimony: *pipiens/molestus*, *torrentium* and *Cx. vagans* with *Cx. sitiens* as the outgroup (Figure 4.3). The *pipiens/molestus* clade included 14 specimens of purported *Cx. pipiens* f. *pipiens* (n=7) and *Cx. pipiens* f. *molestus* (n=7) samples that were identified using the CQ11 assay of Bahnck & Fonseca (2006). It also included the control specimens of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* from Russia (Shaikevich, 2007), *Cx. pipiens* f. *molestus* from London (n=1) and *Cx. pipiens* f. *molestus* colony specimens from Greece (n=3) (Figure 4.3). Only three unique haplotypes were recovered in the 21 COI sequences, each differing by a single base. Firstly, the sequence of *Cx. quinquefasciatus* from the University of Aberdeen colony showed 100% identity to the 3 *Cx. pipiens* f. *molestus* colony specimens from Greece, which could be due either to laboratory or colony contamination. The control sequence of the Russian *Cx. pipiens* f. *molestus* (AM403492; Shaikevich, 2007) was identical to the one wild-caught specimen from sewage tunnels in London. Finally, an identical COI haplotype was shared between the Russian *Cx. pipiens* f. *pipiens* (AM403476; Shaikevich, 2007) and UK samples identified as *Cx. pipiens* f. *pipiens* (n=7) and *Cx. pipiens* f. *molestus* (n=7).

The *torrentium* clade (n=27) (Figure 4.3) comprised the 10 morphologically verified samples as well as the *Cx. torrentium* of Shaikevich (2007) from Russia (AM403477). However, also in this clade were 16 specimens identified by the CQ11 assay as *Cx. pipiens* f. *pipiens* (n=3), *Cx. pipiens* f. *molestus* (n=7) and all 6 hybrids sequenced. Interestingly, a high level of intraspecific variation was noted in the *torrentium* clade (n=27; 13 haplotypes; Pi=0.0324), while diversity was extremely low *in pipiens-molestus* (n=21; 3 haplotypes; Pi=0.00095). This was despite the wider geographic origin of the specimens (Figure 4.3). *Culex torrentium* and *Cx. pipiens s.l.* COI sequences were 12.48% divergent on average, with 15 polymorphic sites, 6 of which appeared to be clade-specific. A comparison of COI sequences generated herein with those of Shaikevich (2007) showed that despite this intra-specific variation (9 variable sites), *Cx. torrentium* in the UK can be reliably identified using the markers included in the COI-RFLP assay of Shaikevich (2007); however, the purported unique restriction site for *Cx. pipiens* f. *molestus* is only present in one control specimen (from

London) and not in either *Cx. pipiens* f. *molestus* colony material or in the 7 other specimens identified as *Cx. pipiens* f. *molestus* using the CQ11 assay.

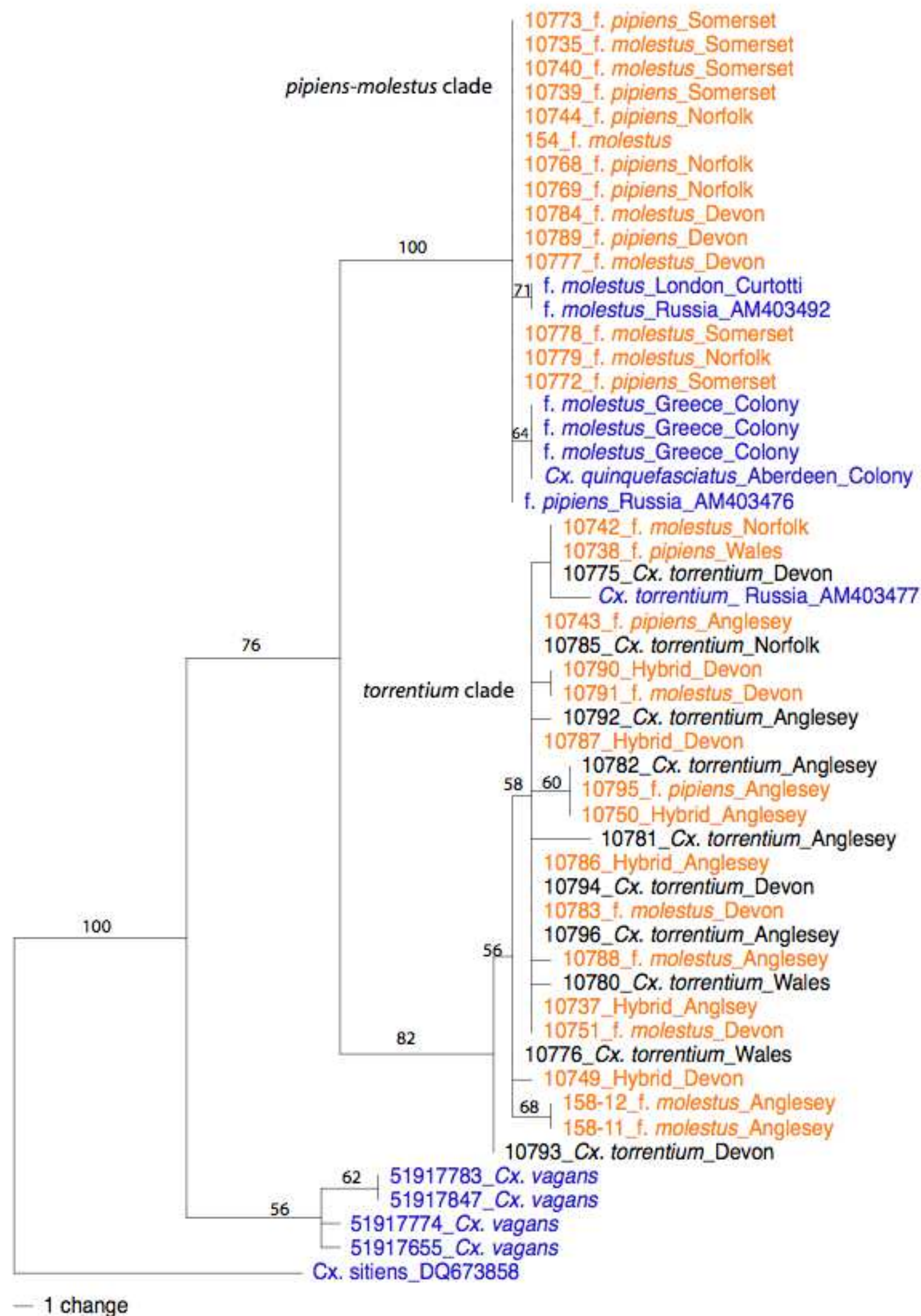

Figure 4.3 Maximum parsimony tree of the mtDNA Cytochrome Oxidase I gene (COI, 710bp) of *Cx. pipiens s.l.* and *Cx. torrentium* specimens (from Table 3.5). Bootstrap values are indicated above branches. Terminal labels show individual DNA numbers, species name, specimen origin or GenBank accession numbers for published sequences. Specimens labelled in orange were morphologically identified as *Cx. pipiens s.l.* and further distinguished as f. *pipiens* (n=10), f. *molestus* (n=14) and hybrids (n=6) using the CQ11 microsatellite assay of Bahnck & Fonseca (2006). Of these, 16 specimens occur within the *torrentium* clade together with morphologically identified *Cx. torrentium* (n=10, marked in Black), *Cx. vagans* and *Cx. sitiens* were included as outgroups (marked in blue), while *Cx. pipiens* f. molestus Greece, *Cx. quinquefasciatus*_Aberdeen both from colonies and f. *molestus*_london collected in sewage tunnels in Barking, London were used as positive controls (marked in blue).

4.5 Discussion

Species complexes are relatively common in the Family Culicidae, particularly in taxa that have large distributions and appear plastic in their ecological requirements (Harbach, 2004), e.g. *Anopheles gambiae* (White, 1985) and *Ochlerotatus caspius* (Schultz *et al*., 1986). The presence of cryptic taxa or the sympatric distribution of closely isomorphic species can make correct species identification problematic. In the UK, the presence of morphologically similar *Cx. torrentium* and *Cx. pipiens s.l.*, combined with the fact that the latter is a species complex (*Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*), makes the identification process challenging. Two recently developed molecular assays differentiating *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* were tested to determine their suitability and accuracy in detecting the presence of in the UK.

From this study, three interesting results were obtained. Firstly, according to the CQ11 assay of Bahnck & Fonseca (2006) *Culex pipiens* f. *pipiens*, *Cx. pipiens* f. *molestus* and hybrids of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* were detected overground in the UK samples. In fact, the presence of *Cx. pipiens* f. *molestus* was found repeatedly, in Devon, Somerset, Norfolk, Suffolk, Anglesey and northern Wales. This paradoxically constitutes the first record of overground presence of *Cx. pipiens* f. molestus in the UK and contradicts the eco-reports of it being found solely in underground habitats (e.g., Cranston *et al*., 1987). One reason for this apparent contradiction could be attributed to the assumption that the two forms are genetically distinct due to a lack of gene flow between overground and underground populations (Bryne & Nichols, 1999; Vinogradova *et al.,* 2007); an assumption on which assays used to discriminate *Cx. pipiens* f. *pipiens* from *Cx. pipiens* f. *molestus* have been designed. However, the presence of overground *Cx. pipiens* f. *molestus* has also been recorded in sympatry with *Cx. pipiens* f. *pipiens* in Portugal (through the presence of autogeny only, Diaz *et al*., 2006) and in Russia (identification by COI only, Vinogradova *et al.,* 2007). These findings imply that *Cx. pipiens* f. *molestus* is not restricted to underground habitats and that these ecological traits may not be as fixed as previously believed.

Secondly, the CQ11 assay identified 3 *Cx. pipiens* f. *pipiens*, 7 *Cx. pipiens* f. *molestus* and 6 hybrids that, based on the COI sequences obtained here, would have been identified as *torrentium* using the RFLP assay of Shaikevich (2007). This result strongly questions the use

of the absence of pre-alar scales as a morphological character for the identification of female *Cx. pipiens s.l.* from *Cx. torrentium*. The phylogenetic tree of COI contained individuals without pre-alar scales (*Cx. pipiens s.l.*) within the *Cx torrentium* clade, further indicating that absence of pre-alar scales is not a reliable diagnostic character. Given that 23% of the identified specimens collected in this study were females and that wild-caught specimens (even those link-reared from immatures as herein) either do not all possess these scales, or they can be easily lost (for example due to rubbing of adult specimens), thus indicating a need for a more robust morphological character that can accurately differentiate females of the two species. Furthermore, the morphological misidentification of *Cx torrentium* as *Cx. pipiens s.l.* caused some confusion in interpreting the results of CQ11 assay as one or both fragments were produced by specimens of this species. This resulted in either a molecular mis-identification of *Cx. torrentium* as *Cx. pipiens* f. *molestus* or the appearance of what appeared to be f. *pipiens* x f. *molestus* hybrids. Despite the fact that closely related mosquito species are known to hybridise (Spielman 1967; Takai & Kanda, 1986; Taylor, 1988; Urbanelli *et al.,* 1997) there is no evidence for this in the UK. Taken together, these findings highlight serious shortfalls in the correct identification of *Cx. torrentium* and *Cx. pipiens s.l.* using either morphology or the CQ11 assay.

Finally, further analysis of the COI data showed that 7 individuals that were identified by the microsatellite assay of Bahnck & Fonseca (2006) as *Cx. pipiens* f. *molestus* were, in fact, *Cx. pipiens* f. *pipiens* according to the COI data. COI sequences obtained from individuals identified by the CQ11 assay were clustered into 2 clades: *pipiens/molestus* and *torrentium*. The single base (A-G, 205 bp) polymorphism was reported to be diagnostic between *Cx. pipiens* f. *pipiens* (A) and *Cx. pipiens* f. *molestus* (G) (Shaikevich, 2007). The G nucleotide (i.e., *Cx. pipiens* f. *molestus*) was observed in underground collections of *Cx. pipiens s.l.* in London as well as in the Russian specimens, both used here. However, this specific G nucleotide was not present in either *Cx. pipiens* f. *molestus* colony material from Aberdeen or from Greece (Figure 3.5). Thus according to the COI-RFLP of Shaikevich (2007), the colony material would have been misidentified as *Cx. pipiens* f. *pipiens*, implying that collections from London and from Russia are "true" *Cx. pipiens* f. *molestus* specimens and that the colony specimens from Greece and Aberdeen are autogenous *Cx. pipiens* f. *pipiens*. This however is contradictory, as autogeny is the single ecological character defining *Cx. pipiens* f. *molestus*. Interestingly, this result raises the possibility that these *Cx. pipiens* f.

molestus specimens are in fact hybrids with a *Cx. pipiens* f. *pipiens* mother (assuming mtDNA was transferred from the female). However, the CQ11 assay was capable of detecting hybrids of the two forms (Bahnck & Fonseca, 2006), visualised by the presence of two fragments (corresponding to each form) on an agarose gel as was seen in the putative hybrids that were, in fact, *Cx. torrentium* (see above). Thus on comparison of the results obtained from these two assays, it is apparent that neither the microsatellite (CQ11) nor the COI-RFLP assay is able to reliably differentiate the *Culex pipiens* complex in the UK.

One plausible reason for the inability for the COI to differentiate *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* consistently could have arisen from the low genetic diversity observed within the *pipiens-molestus* clade. The extremely low COI variation in *Cx. pipiens s.l.*, compared to that noted in *Cx. torrentium*, suggests that differences observed in population level surveys do not hold up across a wider geographic area. In fact, the low level of genetic diversity within the *pipiens-molestus* clade 0.002% (2 bp in 710) brings into question the taxonomic validity of two ecological forms within *Cx. pipiens s.l.* Studies on identification of species based on known COI sequences have reported low level variation of up to 0.25% within species. Interestingly, the presence of this substantial variation observed within and between (from 0.25% within to 8 % between, Hebert *et al.,* 2004) species in the COI gene has resulted in it being used as a "barcode" which has been shown to be highly successful in differentiating mosquito species to date (Cywinska *et al*., 2006; Kumar *et al*., 2007). Variation in COI sequences support the differentiation of *Cx. torrentium* from *Cx pipiens s.l.,* while the low variation observed within the *pipiens-molestus* clade indicates a shared mitochondrial DNA lineage, indicative of a single species.

As mentioned earlier, the single character consistently differentiating the two forms is autogeny (Bryne & Nichols, 1999; Bahnck & Fonseca, 2006; Diaz *et al*., 2006; A. Curtotti, pers. comm.). Given that both molecular assays identified a proportion of overground individuals as *Cx. pipiens* f. *molestus*, both molecular markers may be inefficient in the identification of *Cx. pipiens s.l.* Autogeny is a genotypic trait controlled by two chromosomes in mosquitoes (Spielman, 1957) and its inheritance is reported to be non-Mendelian in nature (Krishnamurthy, 1961). The expression of autogeny in other species of mosquitoes is reported to be flexible in relation to fluctuating environmental pressures. Examples include the presence of carbohydrates for adults (Su & Mulla, 1997) or increased larval nutrition (O'

Meara & Kranjsick, 1970) in *Aedes atropalpus* as well as *Cx. quinquefasciatus* (Olejnick & Gelbic, 2000). Thus expression of autogeny in *Cx. pipiens s.l.* as a result of epigenetic influences should also be considered, as it could imply variability within a single species.

Increased levels of methylated DNA present in *Cx. pipiens* f. *molestus* could explain expressed autogeny in mosquitoes that are found in small confined environments. Methylated DNA is present in 0-3% of an insect genome (Field *et al.*, 2004). It is thought to influence the transcription and expression of specific genes that may otherwise be lost (Mandrioli, 2004). Field *et al.* (1989) showed elevated levels of methylated cytosine in two esterase genes present in insecticide resistant *Myzus persicae* (peach potato aphid) and Hick *et al*. (1996) found that the presence of methylated cytosine decreased with reduced expression of two esterase genes. These studies demonstrate the effect of environmental pressures on gene expression. With such varying levels of autogenic expression, it is proposed that the expression of autogeny could be induced in overground *Cx. pipiens* f. *pipiens* resulting in the seeming underground *Cx. pipiens* f. *molestus*.

In conclusion, there is no molecular evidence presented in this data set to suggest that the two forms of *Cx. pipiens s.l.* can be successfully differentiated based on either the CQ11 Bahnck & Fonseca (2006) assay or COI PCR-RFLP assay (Shaikevich, 2007). Furthermore, the lack of morphological characters, the incongruence of results from molecular assays, the lack of distinct overground and underground forms and the possibility of epigenetically induced expression of autogeny in *Cx. pipiens* f. *molestus* strongly suggest *Cx. pipiens s.l.* is one species. Therefore, all results in this study supports the suggestion of Harbach *et al.* (1984) that *Cx. pipiens* is a single, phenotypically plastic species that is easily adapted to a variety of habitats. Further studies on natural host selection and ecological parameters will be reported for both *Cx. torrentium* and *Cx. pipiens s.l.* in Chapters 4 and 5, respectively.

Chapter 5

Host selection in British mosquitoes

5. Host selection in British mosquitoes

5.1 Introduction

5.1.1 Mosquito-borne diseases in Europe

The emergence of mosquito-borne diseases such as Chikungunya (Rezza *et al*., 2007), the re-emergence of West Nile virus (WNv) into Europe (Hannoun *et al*., 1964; Tsai *et al*., 1998; Hubalek & Halouzka, 1999; Platonov *et al*., 2001; Del Giudice *et al*., 2004; Higgs *et al*., 2004; Esteves *et al*., 2005) and the predicted re-establishment of human malaria in the UK due to climate change (Snow, 1999; Medlock *et al*., 2005), have prompted entomologists to establish the potential of British species as vectors of mosquito-borne disease (Higgs *et al.,* 2004; Medlock *et al.,* 2005; Gould *et al.,* 2006). To date, the isolation of WNv from *An. maculipennis s.l.* in Portugal (Filipe, 1972, in Esteves *et al.,* 2005), *Culex modestus* in southern France (Hannoun *et al*., 1964), *Culex pipiens s.l.* in Romania (Tsai *et al*., 1998; Higgs *et al*., 2004) and *Aedes vexans* in Russia (Fyodorova *et al.*, 2006) have incriminated these species as vectors. In addition to WNv, the isolation of other viruses occurring in northern and eastern Europe has implicated *Aedes vexans, Cx. torrentium and Cx. pipiens* in the transmission of Tahyna (Lundström *et al.,* 2001), Sindbis (Jaenson *et al.,* 1986b) and Usutu and Sindbis (Lundström, 1999) viruses, respectively. All of these species have been recorded in the UK (Snow, 1990) and evidence of local transmission of Sindbis and Usutu, between British mosquitoes and birds has been reported (Buckley *et al*., 2003)

5.1.2 Importance of host selection

Understanding mosquito host selection is essential for the accurate determination of potential bridge and secondary vectors (Lee *et al.,* 2002) and for identifying vertebrate reservoir hosts (Lee *et al.,* 2002; Oshagi *et al.,* 2006). Host selection, as defined by Boreham & Garrett-Jones (1973), is the pattern of feeding observed through the analysis of specific blood meals in a mosquito population within a defined space and time. Host selection has been widely acknowledged as a means of understanding the relationships between hosts and vectors (Janini *et al.,* 1995; Kilpatrick *et al.,* 2007). Host preference, the preferred choice of a host as a

food source for haematophagous insects (Boreham & Garrett-Jones, 1973), can be studied directly by collection of the insects while feeding (Service, 1971) or by using host-specific odour-baited traps (Service, 1969). However, this preference may not be indicative of actual host selection because mosquitoes may feed opportunistically due to lack of available preferred hosts.

A host-seeking female often imbibes a single uninterrupted (unmixed) blood meal, but host irritability or an incomplete meal by the female may cause feeding to be interrupted (Davies, 1990). This can result in multiple meals, where two or more feeds are taken in a single gonotrophic cycle (Boreham & Garrett-Jones, 1973; Boreham, 1975; Romoser *et al*., 1989). Interruption of feeding increases host-vector contact thereby increasing the likelihood of pathogen transmission from the host to the mosquito and onto other hosts (Spielman, 1986; Beach *et al.*, 1985). This capability of a female mosquito to transmit the pathogen amongst different host species increases its potential to be a bridge vector. Thus direct analysis of the mosquito blood meal has been considered the best way to directly assess host selection and multiple feeding under natural conditions (Boakye *et al.,* 1999; Lee, *et al.,* 2002).

5.1.3 Host identification

Host selection of mosquitoes has traditionally been determined through immunological tests, such as precipitin and Enzyme Linked Immunosorbent Assay (ELISA) tests. Both assays rely on the reaction between the host-specific serum from a blood meal and antibodies raised against that serum (Pant *et al.,* 1987). The large amounts of antisera needed means these tests can be expensive and highly labour-intensive (Pant *et al.,* 1987; Lee, *et al.,* 2002). Aside from that, multiple assays are required to determine the specific host source (Burkot & DeFoliart, 1982) which can also compromise the specificity and sensitivity of both assays when heterologous sera or mixed meals are tested (Pant *et al.*, 1987; Beier *et al.,* 1988).

Molecular analysis of vector blood meals was first carried out on the tick, *Ixodes ricinus*, a major European vector of Lyme disease (Kirstein & Gray, 1996). Utilising a Restriction Fragment Length Polymorphism (RFLP) assay, exploiting fixed inter-specific mutations on the rapidly evolving mitochondrial Cytochrome B (CytB) gene, the authors were able to successfully identify the following eleven hosts: common mouse, wood mouse, bank

vole, sheep, red deer, silka deer, cow, rabbit, dog, fox and pheasant. Since then, the CytB gene has been the focus of several assays used to accurately discern vertebrate hosts in insect blood meals (Boakye *et al.,* 1999; Kent & Norris, 2000; Meece *et al.,* 2005; Oshagi *et al.,* 2006). The high representation of vertebrate CytB sequences in Genbank further warrants the use of the CytB, as the sequences can be directly compared to the database for identification of the hosts to the species level.

5.1.4 British mosquitoes

Host selection of nearly all species recorded in the UK is known (Table 5.1). However in relation to vector potential, only the Anopheline mosquitoes are well studied. The transmission of human malaria in the British Isles has been recorded since the early 14th Century as 'ague', 'tertiary' or 'quaternary' fever (Reiter, 2000). Species incriminated include *Anopheles atroparvus* (Curtis & White, 1984) and *An. plumbeus* (Shute, 1954; Curtis & White, 1984; Marchant *et al.,* 1998; Eling *et al*., 2003). The respective roles of *An. messeae* and *An. daciae* are uncertain, but interior malaria transmission in un-forested areas was historically attributed to *An. messeae* (Snow, 1990) and the species has been incriminated in other parts of its range (Detinova, 1953; Bruce-Chwatt & de Zulueta, 1980; Nikolaeva, 1996).

The most comprehensive studies on natural feeding behaviour of non-Anopheline British mosquitoes were carried out on mosquitoes collected in Poole, Dorset (Service, 1968b; 1971) (Table 5.1). Host selection was determined based on direct collection of insects from various hosts and immunological assay of the imbibed blood meal using a precipitin test (Table 5.1). Of the sixteen species whose blood meals were precipitin-tested by Service (1971), five (*Ae. cinereus*, *Oc. caspius*, *Oc. punctor*, *Cx. torrentium* and *Cs. annulata*) were shown to have fed on multiple vertebrate hosts in a single gonotrophic cycle (Table 5.1). In addition, Ramsdale & Snow (1995) reported that 25 of the 33 species of British mosquitoes had been recorded biting man (Table 5.1). Based on this and the status of these species as vectors in Europe and North America, Medlock *et al*. (2005) suggested that principal bridge vectors of WNv in the UK could include *Cx. pipiens* f. *pipiens*, *Cx. torrentium*, *Cs. litorea* and *Cs. morsitans*, while *Ae. cinereus, An. plumbeus, Cs. annulata, Cs. litorea, Cs. morsitans, Cq. richiardii, Oc. cantans, Oc. detritus, Oc. dorsalis* and *Oc. punctor* could also play a role as potential bridge vectors, but to a lesser degree. In a separate study, the isolation of WNv

antibodies from 46-day old chicks Buckley *et al.* (2006), suggests that British mosquitoes are involved in the local transmission of WNv in bird populations in the UK, yet no species can be definitely incriminated. In addition to potential WNv transmission, avian malaria was reported in Bristol Zoo (resulting in the death of 8 penguins), Edinburgh Zoo and in Marwell Zoo, (where 27 penguins succumbed to infection) (BBC news online, 1999). As penguins are non-endemic to the UK, transmission of avian malaria must occur from native birds to penguins

Mosquito species	**Preferred host** (Direct feeding)	**Multiple meals** (Precipitin test)
An. algeriensis	Man[2,3,4]	
An. atroparvus	Man[2,4], Cattle[3], Rabbit[6]	
An. claviger	Cattle[1], Rabbit[1], Mammals[2], Man[4]	
An. daciae	?	
An. messeae	Man[2,4]	
An. plumbeus	Man[2,3,4,5], Cattle[1], Bird[1], Mammals[5]	
Ae. cinereus	Cattle[1,5], Bird[1,5], Mammals[2], Man[4,5]	Cow & Human[1]
Ae. geminus	?	
Ae. vexans	Man[2,4]	
Cs. alaskaensis	Man[2]	
Cs. annulata	Bird[1,2,5], Mammals[2], Man[2,4,5]	Bird & Rabbit[1]
Cs. fumipennis	?	
Cs. litorea	Bird[1,2,5], Cattle[1], Mammal[2], Man[2,4]	
Cs. longiareolata	Bird[1,2]	
Cs. morsitans	Bird[1,2,5], Man[4,5]	
Cs. subochrea	Man[4]	
Cq. richiardii	Bird[1,5,9], Cattle[1,5], Mammals[2], Man[4,5], Amphibians[8]	
Cx. europaeus	Amphibian/Reptiles[2], Man[4]	
Cx. modestus	Man[2]	
Cx. pipiens f. *pipiens*	Bird[1,2,5]	
f. *molestus*	Man[2,4,5], Birds[5]	
Cx. torrentium	Bird[2,5]	Bird & Rabbit[1]
Da. geniculata	Man[2,4]	
Oc. annulipes	Mammals[2,3], Man[4]	
Oc. cantans	Cattle[1,5], Bird[1,5], Man[3,4,5], Mammals[2,3,6]	
Oc. caspius	Man[2,4], Cattle1, Sheep[1], Bird[1]	Bird & Mammal[1]
Oc. communis	Man[2,4]	
Oc. detritus	Cattle[1], Bird[1], Man[2,4]	
Oc. dorsalis	Cattle[1,5], Rabbit[1], Pig[1], Horse[1], Mammals[2,3], Man[3,4]	
Oc. flavescens	Cattle[1,3], Sheep[1,3], Bird[1], Mammals[2,3], Man[4], Horses[3]	
Oc. leucomelas	Man[2]	
Oc. punctor	Man[2,3,4,5], Cattle[1,5], Bird[1,5],	Bird & Mammal[1]
Oc. rusticus	Man[2,3,4]	
Oc. sticticus	Man[2], Mammals[7]	
Or. pulcripalpis	Bird[2]	

Table 5.1 Summary of available data on known host selection and multiple feeds in British mosquitoes ([1]Service, 1971; [2]Snow, 1990; [3]Cranston *et al*., 1987; [4]Ramsdale & Snow, 1995; [5]Medlock *et al*., 2005; [6]Muirhead-Thompson, 1956; [7]Mattingly, 1950; [8]Shute, 1933; [9]Service, 1969).

via endemic mosquitoes. It is uncertain, however, as to which species of endemic mosquitoes are involved in the transmission of avian malaria in the UK.

Given the presence of historical vectors of malaria in the UK and the role that local mosquitoes could play as vectors of emerging diseases such as WNv and malaria makes the introduction and establishment of these diseases a high possibility (Higgs *et al.,* 2004, Medlock *et al*., 2005; Gould *et al.,* 2006). Thus the identification of potential vectors and bridge vectors based on host identification and presence of parasitic infection in field-caught bloodfed females could elucidate the understanding of host-vector interactions in the UK and thereby elucidating the importance that these mosquitoes could have on human and animal health in the future.

5.2 Aims

The aims of this study were:

[1] To identify specific vertebrate hosts of British mosquitoes through the molecular analysis of blood meals in wild-caught mosquitoes collected from five counties in the UK,

[2] To identify candidate species that could act as vectors and bridge vectors of both human and animal diseases in the UK.

5.3 Materials and Methods

5.3.1 Collection and identification of blood-fed females

Resting blood-fed females were collected manually from the walls of animal shelters, derelict buildings, sheds and pillboxes in the English counties of Devon, Somerset and Norfolk (England) and in Anglesey (Wales) in July 2006 and in Kent (England) in August 2006 (Table 5.2) At two of the sampling sites, bloodfed females were also captured using Mosquito Magnet® traps (Liberty Pro) (Table 5.2; Bird World, Anglesey and Pettits Animal Farm, Norfolk). Once collected, blood-fed mosquitoes were visually scored according to their

degree of blood meal digestion as detailed in Figure 5.1(a-d). Gravid and non-bloodfed females were also recorded. Adults were carefully labelled with unique collection numbers and either pinned on card points, or placed in individual Beem® capsules and dried over silica gel (see Chapter 2, section 2.2.1.2 and 2.2.2). Mosquitoes were mostly identified to species using the British identification keys of Cranston *et al*. (1987) and Snow (1990). Members of the *Maculipennis* Complex were identified to species using the ITS2 PCR-RFLP assay designed in Chapter 3 and *Cx. pipiens* were treated as a single species (Chapter 4).

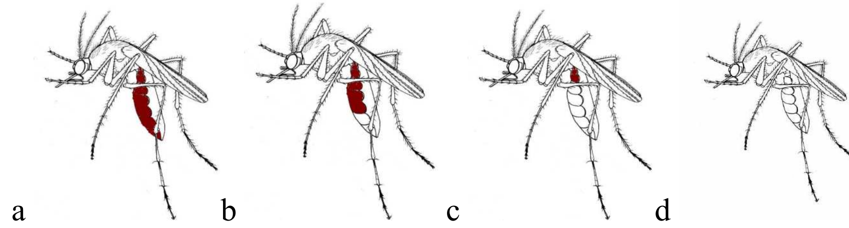

Figure 5.1 Schematic diagram of a female mosquito showing the abdomen (a) fully (3/3) blood fed, (b) 2/3 bloodfed, (c) 1/3 bloodfed and (d) non–bloodfed or gravid. Mosquito outline from http://www.pestworldforkids.org/mosquitoes.html

Exact locality	Date	Collection method	Adult habitat	Exact site Co-ordinates (Deg. Decimal)
Wales: Anglesey, Bird World (nr Newborough)	08-09.07.06	Trap	Inside shed near aviaries	53.1638428'N, 4.3316828'W
England: Devon, Exminster	06.07.06	Resting	Brick Shelter 1	50.6752617'N, 3.4751598'W
Marshes Nature Reserve	06.07.06	Resting	Brick Shelter 2	50.6756321'N, 3.4743221'W
England: Kent, Cliffe Marshes	11.08.06	Resting	Brick shelter	51.4671890'N, 0.4895240'E
	11.08.06	Resting	Sheep Corral	51.4783052'N, 0.4827834'E
England: Norfolk, Pettits Animal	16.07.06	Resting	Goat stables	52.5661404'N, 1.5768701'E
Farm* (nr Reedham)	16.07.06	Resting	Reindeer stables	52.5659609'N, 1.5768554'E
	16.07.06	Resting	Mini horse/donkey stables	52.5659609'N, 1.5768554'E
	16-17.07.06	Trap	Near pond in petting area	52.5661226'N, 1.5774591'E
	17-20.07.06	Trap	Near bird aviaries	52.4656741'N, 1.5704780'E
	20.07.06	Resting	Goat stables	52.5661404'N, 1.5768701'E
	20.07.06	Resting	Rhea stables	52.5659609'N, 1.5768554'E
	20.07.06	Resting	Reindeer stables	52.5659609'N, 1.5768554'E
	20.07.06	Resting	Mini horse/donkey stables	52.5659609'N, 1.5768554'E
England: Somerset, Godney	07.07.06	Resting	Pillbox 1	51.1816487'N, 2.7273050'W
Farm, Godney	07.07.06	Resting	Horse stables	51.1817652'N, 2.7230142'W
	07.07.06	Resting	Pillbox 2	51.1822719'N, 2.7283164'W

Table 5.2 List of habitats, collection dates and co-ordinates (in degree decimal) of sites where all adult blood fed and resting mosquitoes were collected for this study. Resting adults were collected in Devon, Kent, Norfolk and Somerset, while host-seeking adults were collected in Anglesey and Norfolk. *Collections were taken from the low open stables of reindeer and miniature donkeys and ponies in adjacent pens of the Petting Area of Pettits Animal Farm.

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5.3.2 DNA extraction protocols

5.3.2.1 DNA extraction from host serum and dried blood

DNA extracts from serum samples (Sera Laboratories International, West Sussex, England) from cow, dog and horse were used as controls for the optimisation of universal CytB primers and for testing the specificity of designed primers (herein). Proteinase K (20µl) and 200µl of buffer AL (QIAgen®) was added to 200µl of the control animal serum in a 1.5-ml Eppendorf® tube. The sample was mixed by vortexing for 15 seconds and placed in a heat block at 56ºC for 15 minutes. The sample was briefly centrifuged and 200µl of 100% ethanol was added and mixed by vortexing. The mixture was then transferred to a QIAgen® spin column and DNA was extracted using the QIAmp Mini Blood Kit following the manufacturer's instructions for extraction from human blood. Control human DNA was extracted from dried blood smears on filter paper; a 1-cm^2 piece was cut from the filter paper and placed in a 1.5-ml Eppendorf® tube to which 180µl of ATL (QIAgen®) was added. The tube was placed in a rotator and left overnight. DNA was then extracted according to the protocol described above.

5.3.2.2 DNA extractions for mosquito blood meal analysis

Abdomens of engorged females were separated from the head and thorax using a clean pair of forceps and a scalpel and placed individually in a clean 1.5ml Eppendorf® tube. The abdomen was then ground using a battery-operated pestle in 100ml of grinding buffer and DNA extracted according to the phenol-chloroform protocol of Linton *et al.* (2001b). Extracted genomic DNA (from both the mosquito and blood meal) was resuspended in a final volume of 100µl and stored at -20ºC, prior to PCR amplification.

5.3.3 PCR amplification protocols

5.3.3.1 Universal Cytochrome B Oxidase (CytB) amplification

The PCR protocol of Boakye *et al.* (1999) (Table 5.4), employing their universal CytB primers (CytB-F and CytB-R), was optimised using 21 field-caught female mosquitoes as follows: fully bloodfed (n=13), 2/3 bloodfed (n=2), 1/3 bloodfed (n=2), gravid (n=2) and unfed (n=2) from Norfolk and Somerset. PCR fragments of 350bp were obtained, using the PCR conditions listed in Table 5.4. PCR products were cleaned in a 200μl PCR tube using 8μl of positive PCR product and 2μl of a 1:4 dilution of ExoSAP-IT® (GE Healthcare, UK). The mixture was placed in a thermocycler and incubated at 37ºC for 30 minutes and then at 80ºC for a further 20 minutes. Cleaned products were then sent to the Zoological Sequencing Facility in the Natural History Museum for sequencing. Resultant sequences were assembled and edited in Sequencher® version 4.6 (Gene Codes Corporation), aligned with ClustalW (Thompson *et al.,* 1997) and compared to those available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Primer	Primer sequence (5'-3')	DNA fragment (bp)	Optimised primer concentrations and annealing temperatures
Cow-R	GGAATGGGATTTTGTCTACATATGAGG	620	0.3µM, 48ºC
Deer-R	GGTGTATGATCCGTAGTATAGGCC	250	0.3µM, 51ºC
Dog-R	CAGTTCCGATATAAGGGATGGCAGAG	450	0.5µM, 51ºC
Goat-R	TATGAATGCTGTGGCCATTGTCGCGAGC	350	0.3µM, 51ºC
Horse-R	GGAGAGGATTAGGGCTAATACGCCG	800	0.3µM, 48ºC
Human-R	TCGAGTGATGTGGGCGATTGA	200	0.3µM, 51ºC
CytB-R	CCCTCAGAATGATATTTGTCCTCA	350	0.2µM, 60ºC
Bird-F	TACAAAAAAATAGGCCCCGAAGG	650	0.2µM, 51ºC

Table 5.3 Host-specific primers designed in this study to complement the CytB-F primer (5´-CCATCCAACATCTCAGCATGATGAA-3´ of Boakye *et al.* (1999), showing expected fragment sizes and optimal primer concentrations and annealing temperatures. Bird-F (designed by Y.-M. Linton) was designed to pair with the CytB-R primer of Boakye *et al.* (1999).

Reagents	Volumes (25µl) reaction	Thermocycler conditions
ddH_2O	18.15	1) 95°C for 3.5mins
10x NH_4 buffer	2.50	2) 95°Cfor 50 secs
10mM dNTPs	0.50	3) 51°C for 50secs
10µM CytB-F	0.50	4) 72°C for 40secs
10µM host-R	0.50	Steps 2-4 repeated for 34 cycles
50mM $MgCl_2$	0.75	5) 72°C for 5mins
Taq	0.10	
DNA	2.00	

Table 5.4 Optimised PCR master mix and thermocycling conditions for the amplification of vertebrate CytB gene, using host-specific primers designed in this study [after Boakye *et al.* (1999)]. Reagents including 10x NH_4 buffer, 50mM $MgCl_2$ and Taq were from BioLine®.

5.3.3.2 Host-specific primer design and PCR optimisation

Seven potential vertebrate host groups were determined, based on their prevalence near collections sites in summer 2006, as follows: birds, cattle, deer (including reindeer), dogs, goats, horses and man. Where multiple sequences were available from GenBank, pairwise intra-group p-distances were calculated in PAUP* 4.0 b10 (Swofford, 2002) and the two most divergent sequences were then manually re-aligned using Sequencher® version 4.6. Sequences used to develop the host-specific forward primers (Table 5.3): *Bos taurus* (Cow; AB074963, AF490529), *Capreolus capreolus* (European Roe Deer: Y14951), *Cervus elaphus* (Red Deer: AJ000022), *Dama dama* (Red Water Deer: X56290I); *Muntiacus reevesi* (Muntjac Deer: EF035447), *Rangifer tarandus* (Reindeer: NC007703); *Canis familiaris* (Dog: NG002008); *Canis lupus* (Wolf: DQ480500); *Capra hicrus* (Goat: EU130780, AF217254); *Equus caballus* (Horse: NC001640, EF597512) and *Homo sapiens* (Man: NC001807, EU935442).

All species-specific reverse primers designed herein were developed to be paired with the universal forward primer of Boakye *et al*. (1999), producing amplified fragments of sufficiently differing sizes that each host species could be visually determined directly from an agarose gel. Expected product sizes are shown in Table 5.3. For birds, a bird-specific primer obtained from Y.-M. Linton (pers. comm.) was designed to work with the universal reverse primer of Boakye *et al.* (1999) and yielded a 600bp fragment, including a short fragment (50bp) of the ND5 region.

Primers were checked for complementing annealing temperature and the capability to form primer-dimers using an online oligo-analyser (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx). Optimal annealing temperatures (48-52ºC) and primer concentrations (1-5μM) for each primer pair (Table 5.3) were determined using the control host DNA as well as the relevant the universal CytB primer (section 5.3.3.1), in a temperature and primer concentration gradient PCR reaction. Controls for cow, dog and horse were obtained from serum, human controls from blood smears and controls for bird, deer and goat were obtained from blood meals sequenced for the optimisation of Universal CytB primers (See section 5.4.1).

Specificity of host primers was also tested against both target and unintended hosts using controls listed above. The production of bands for the target host and the absence of bands in all other host controls indicated primer specificity. To ensure accurate amplification of target host, 12 positive PCR products from control samples [bird (n=1), deer (n=1), dog (n=2), goat (n=3), horse (n=2) & man (n=3)] were cleaned and directly sequenced (see section 4.3.3.1) in both directions. Further to that, 20 positive PCR products from field caught bloodfed mosquitoes [bird (n=9), deer (n=1), dog (n=3) and goat (n=7)] were cleaned and directly sequenced (see section 4.3.3.1) in both directions, to ascertain accurate identification of hosts on bloodfed specimens using host specific primers. Resultant sequences were compared to those in GenBank using BLAST to confirm the identity of the host.

5.3.3.3 Determination of host selection

Single PCR reactions (see section 5.4.2) for all but the cow-specific primers (due to the lack of specificity of this primer), were carried out for each of the above primer pairs on the DNA extracted from 280 mosquito abdomens to determine natural host selection. PCR was performed under conditions listed in Table 5.4. Fragments were visualised following electrophoresis of 4μl of PCR reaction on a 1.5% agarose gel at 70V containing 1% ethidium bromide. Fragment sizes were measured against Hyperladder IV standards (BioLine®)**.** In cases where the host-specific primers did not yield any PCR product, the DNA was re-amplified using the universal CytB primers of Boakye *et al.,* (1999) (Table 5.3) and subsequently sequenced.

In order to ensure the accuracy of the host-specific primers when applied to blood meals from field-caught adults, PCR products were sequenced from 20 bloodfed mosquitoes that had been shown to feed on birds (n=9), deer (n=1), dogs (n=3) and goats (n=7) (for protocol see section 5.3.3.1). Results were compared with reference sequences in GenBank using BLAST. Sequences were edited and aligned as above and a phylogenetic tree was constructed using a maximum likelihood search under a GTR model of nucleotide substitution with empirical base frequencies. Node support was calculated with nonparametric bootstrap (100 reps). Analyses were performed using PhyMLv2.4.4 (Guindon & Gascuel, 2003).

5.4 Results

Blood-fed female mosquito specimens from seven species were collected mainly as resting adults (n=668) and incidentally as host seeking females (n=119). Of the resting adults collected hosts from 280 specimens were identified (Table 5.5).

5.4.1 Optimisation of Universal CytB primers

DNA sequences of PCR products obtained in the optimisation of the universal CytB primers were recovered from all 21 field-caught mosquitoes [bloodfed (17), unfed (2) & gravid (2)]. Following blast searches with the resultant DNA sequences, four host species were detected in 12 of the 17 bloodfed individuals (70.6%), as follows: *Capra hircus* (goat: *An. maculipennis s.l.,* n=2), *Homo sapiens* (man: *Cq. richiardii*, n=1), *Rangifer tarandus* (reindeer: *An. daciae*, n=5; *An. messeae,* n=3) and *Pterocnemia pennata* (Darwin's Rhea: *An. daciae,* n=1). The 9 remaining DNA sequences produced from gravid (n=2), unfed (n=2) and bloodfed (n=5) mosquitoes showed 99-100% similarity to *Armigeres subalbatus*, indicating that the universal primers also amplified mosquito DNA. Subsequently vertebrate host-specific primers were designed and used in widespread screening for host selection in this study.

County (n=280)	Adult habitat	Species	Bloodfed females Proportion bloodfed (n=280)
Anglesey (n=1)	Shed	*Cx. pipiens*	2/3 (1)
Devon (n=58)	Brick Shelter 1	*An. messeae*	1/3 (7), 2/3 (4)
	Brick Shelter 2	*An. messeae*	1/3 (25), 2/3 (19), 3/3 (2)
		Maculipennis Gp	1/3 (1)
Kent (n=36)	Brick Shelter	*An. atroparvus*	3/3 (2)
		Maculipennis Gp	2/3 (1)
	Derelict building used as sheep corral	*An. atroparvus*	1/3 (25)
		An. daciae	1/3 (6)
		Maculipennis Gp	1/3 (2)
Norfolk (n=148)	Goat stables	*An. atroparvus*	1/3 (1), 2/3 (5), 3/3 (1)
		An. daciae	1/3 (5), 2/3 (36), 3/3 (12)
		Maculipennis Gp	1/3 (3), 2/3 (10), 3/3 (4)
		An. messeae	2/3 (1)
		Cs. spp	3/3 (1)
	Near pond in Petting area	*Cq. richiardii*	3/3 (1)
	Rhea stables	*An. daciae*	3/3 (2)
		An. messeae	3/3 (1)
	Reindeer stables	*An. atroparvus*	2/3 (1)
		An. daciae	2/3 (23), 3/3 (1)
		Maculipennis Gp	2/3 (2)
		An. messeae	2/3 (1)
		An. spp	2/3 (3)
	Mini horse/donkey stables	*An. daciae*	2/3 (1), 3/3 (27)
		Maculipennis Gp	2/3 (1), 3/3 (2)
		An. messeae	3/3 (3)
Somerset (n=37)	Pillbox 1	*An. daciae*	1/3 (4), Gravid (2), Non-BF (1)
		Maculipennis Gp	2/3 (1)
		An. messeae	1/3 (1)
		Cx. pipiens	1/3 (1), 3/3 (2)
		Cx. torrentium	1/3 (1)
		Cs. subochrea	2/3 (2)
	Horse stables	*An. daciae*	1/3 (7), 3/3 (1)
		Maculipennis Gp	1/3 (2)
		An. messeae	1/3 (1)
		Cq. richiardii	Non-BF (1)
	Pillbox 2	*An. daciae*	1/3 (6), 3/3 (3)
		Cs. spp	Non-BF(1)

Table 5.5 List of 280 bloodfed (BF) specimens used to analyse host selection and determine natural parasitic infection. Abdomens of bloodfed mosquitoes were scored visually (Figure 4.1a-d) as follows: 1/3 bloodfed, 2/3 bloodfed, 3/3 bloodfed, gravid and non-bloodfed (non-BF). Gravid and non-bloodfed specimens were included as negative controls.

5.4.2 Optimisation of host specific primers

Of the 7 host-specific primers, all but the cow primer, were shown to amplify the target host CytB gene when tested on known controls: bird (n=1), human (n=3), horse (n=2), goat (n=3, dog (n=2) & reindeer (n=1). Due to the lack of specificity, the designed cow primer was not used in subsequent analysis. No cross-contamination was otherwise noted. Following optimisation, host-specific primers were then used for screening the 280 wild-caught specimens.

Out of the 280 field caught bloodfed mosquitoes analysed, twenty specimens [bird (n=9), deer (n=1), dog (n=3) and goat (n=7)] were randomly sequenced to ascertain the accurate identification of hosts using the designed host specific primers.

Of these, nine specimens positive with the bird-specific primer, four (all *An. daciae*) had 100% sequence similarity to *P. pennata* [Figure 4.2 (Darwin's rhea) from Pettits Animal Farm, Norfolk] and one *Cx. torrentium* from Somerset shared 100% similarity to *Fringilla coelebs* (Figure 5.2b) From the remaining four specimens (*An. messeae*, n=1 and *An. daciae*, n=3) sequence data from a 50bp mitochondrial ND5 fragment was recovered that was similar to that of *Lanius meridionalis* (grey shrike) (AM494443). Interestingly, this species of bird is neither endemic to the UK nor present in the environs of the Pettits Animal Farm in Norfolk and was only detected in these individuals. The blood meal of one *An. daciae* collected at the same site showed 95% similarity to *C. elaphus* (Red Deer, Figure 5.2b).

Of the seven DNA sequences generated from blood meals amplified using the goat-specific primer, six of showed 100% similarity to *C. hircus* (Figure 5.2b) (all *An. daciae*). However, the remaining single sequence generated from *An. daciae* with the goat-specific primer and the blood meal of all three *Cx. pipien*s specimens amplified using the dog primers shared 100% similarity to the mosquito *Armigeres subalbatus* (Figure 5.2a), despite producing good clean sequences. This suggests that in some cases, the dog primer and the goat primer also amplifying mosquito DNA.

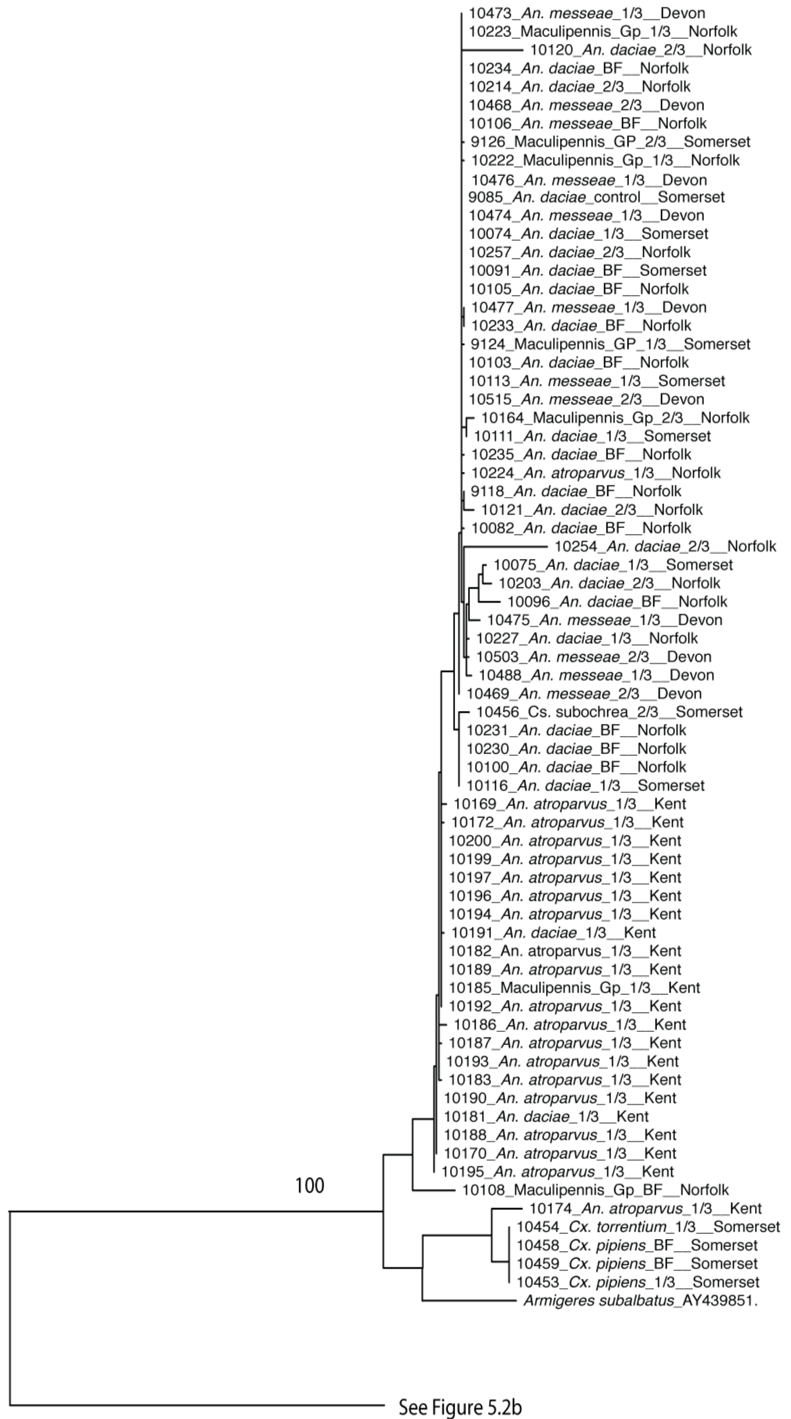

Figure 5.2a

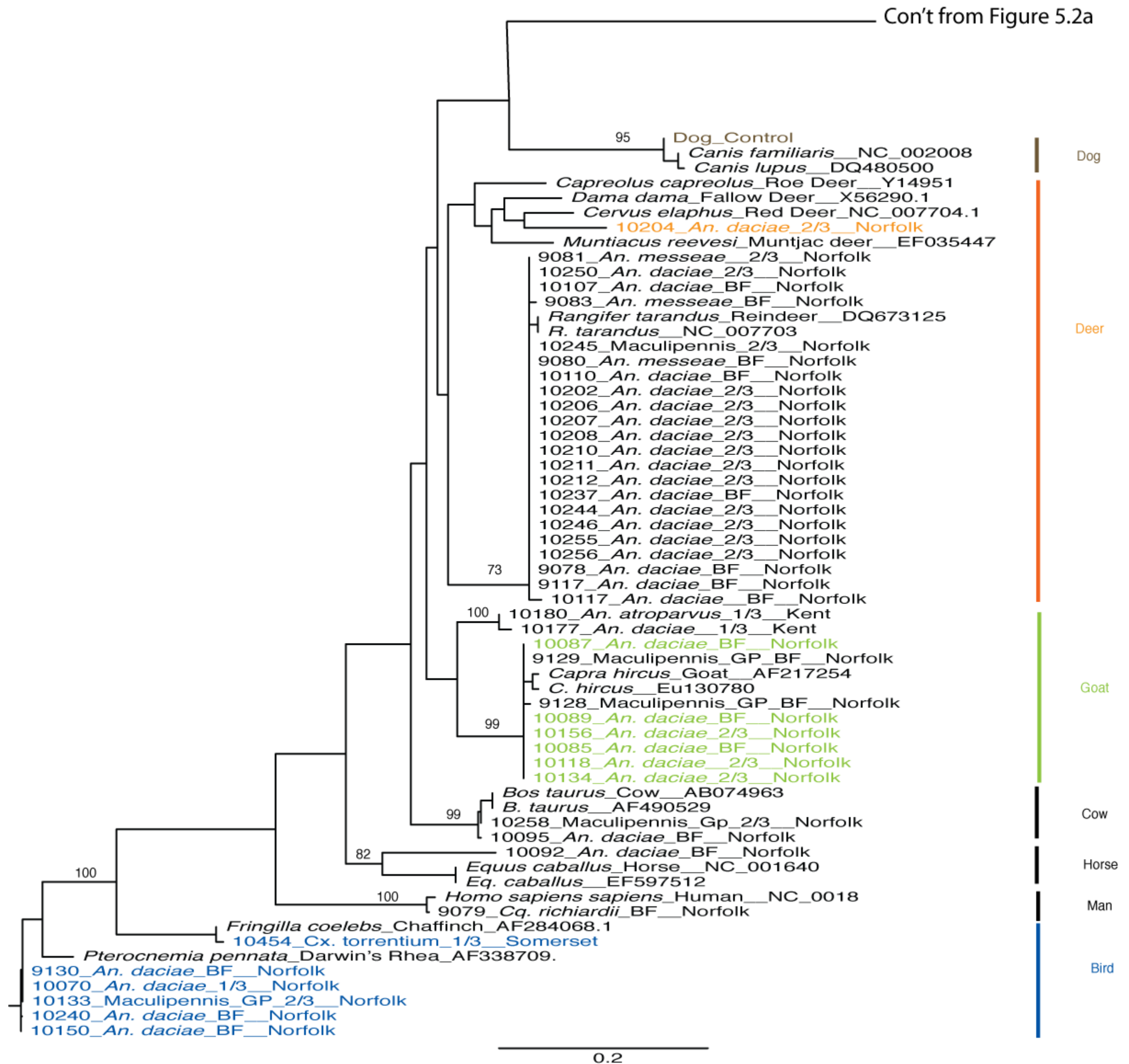

Figure 5.2b

Figure 5.2 A Maximum likelihood tree, with bootstrap values of nodes, of mtDNA Cytochrome B oxidase gene sequences. Terminal labels show DNA Number_species_Common name_degree Bloodfed_location or_Genbank accession numbers. Altogether there are 8 distinct clades: Dog (n=3, Genbank sequences=2), Deer (n=22, Genbank sequences = 6), Goat (n=10, Genbank sequences = 2), Cow (n=2, Genbank sequences= 2), Horse (n=1, Genbank sequences =2), Man (n=1, Genbank sequences=1), Bird (n=6, Genbank sequences =2), Mosquito (n=70, Genbank sequences =1).

Figure 5.2a shows bloodfed mosquito specimens from which no vertebrate hosts could be identified using host-specific primers. All specimens shown here were sequenced using Universal CytB primers and the closest match to the sequences obtained was *Armigeres subalbatus* (mosquito).

Figure 5.2b shows the hosts identified from bloodfed mosquito specimens. Coloured labels indicate samples sequenced with host specific primer: Orange-Deer (n=1), Green-Goat (n=3), Blue- Bird (n=5), brown- Dog (n=3), Black labels indicate samples sequences with Universal CytB primers (Boakye *et al.,* 1999) when host specific primers did not work.

5.4.3 Host Selection

Of the 280 mosquitoes tested (including 5 negative controls), host selection was successfully determined for 166 (60.4%) specimens comprising seven UK mosquito species (Table 5.6). Blood meals were successfully amplified from mosquitoes 1/3 engorged through to fully engorged, of which 20 were sequenced to ascertain the accurate identification of hosts (Figure 5.2b). Hosts were identified from 134 specimens using the host-specific primers for bird, dog, deer, goat horse and man. Of the remaining specimens for which host-specific primers did not produce a result, universal CytB primers were used and sequences obtained. From this, host CytB sequences were generated from 32 individuals (Figure 5.2b) were identified while the remaining 109 specimens (39%) amplified mosquito DNA only. Interestingly, 4% of the all DNA fragments sequenced using the universal primer produced were ambiguous, showing double-peaks that were due either to the presence of amplified DNA from the mosquito itself, or possibly from an additional host. The majority of the specimens (n=141) were found to have had blood meals from a single host, whereas mixed blood meals were detected in 25 specimens (15%) (Table 5.6).

Locality	Species sampled (n=166)	Unmixed meals (n=141)							Mixed meals (n=25)
		Bird (17)	Man (18)	Deer (43)	Goat (58)	Horse (2)	Cow (1)	Dog (2)	
Bird World, Anglesey	*Cx. pipiens* (1)	1	0	0	0	0	0	0	
Exminster Marshes, Devon	*An. messeae* (40)	7	0	1	28	0	0	0	Bird/Goat (4)
	An. maculipennis s.l. (1)	0	0	0	1	0	0	0	
Cliffe Marshes, Kent	*An. atroparvus* (5)	0	0	4	1	0	0	0	
	An. daciae (4)	0	0	3	1	0	0	0	
	An. maculipennis s.l (3)	0	0	0	1	0	0	0	Man/Deer (1), Bird/Deer (1)
Pettits Animal Farm Norfolk	*An. atroparvus* (5)	0	1	0	3	0	0	0	Man/Goat (1)
	An. daciae (69)	4	11	29	14	2	1	0	Deer/Goat (2), Man/Goat (3), Man/Deer (2), Bird/Man/Dog (1)
	An. messeae (6)	0	1	3	0	0	0	0	
	An. maculipennis s.l. (17)	2	4	2	5	0	0	1	Bird/Goat (2) Bird/Deer (1), Man/Goat (1), Man/Deer (1)
	Cq. richiardii (1)	0	1	0	0	0	0	0	
Godney, Somerset	*An. daciae* (9)	3	0	0	4	0	0	0	Man/Bird (1), Bird/Deer (1)
	Cx. torrentium (1)	0	0	0	0	0	0	0	Bird/Dog (1)
	Cx. pipiens (3)	0	0	0	0	0	0	1	Man/Dog (1), Deer/Dog/Goat(1)
	Cs. subochrea (1)	0	0	1	0	0	0	0	

Table 5.6 Host selection of British mosquitoes (n=166) successfully analysed in this study using species diagnostic primers designed herein and CytB DNA sequences.

Mosquito species	Preferred host (Direct feeding)	Multiple meals (CytB PCR assay)
An. algeriensis	Man[2,3,4]	
An. atroparvus	Man[2,4], Cattle[3], Rabbit[6], **Deer**	
An. claviger	Cattle[1], Rabbit[1], Mammals[2], Man[4]	
An. Daciae	**Man, Bird, Deer, Goat, Horse, Dog**	**Bird & Man, Man & Goat, Man & Deer**
An. messeae	Man[2,4], **Bird, Deer, Goat**	**Bird & Goat**
An. plumbeus	Man[2,3,4,5], Cattle[1], Bird[1], Mammals[5]	
Ae. cinereus	Cattle[1,5], Bird[1,5], Mammals[2], Man[4,5]	Cow & Human[1]
Ae. geminus	?	
Ae. vexans	Man[2,4]	
Cs. alaskaensis	Man[2]	
Cs. annulata	Bird[1,2,5], Mammals[2], Man[2,4,5]	Bird & Rabbit[1]
Cs. fumipennis	?	
Cs. litorea	Bird[1,2,5], Cattle[1], Mammal[2], Man[2,4]	
Cs. longiareolata	Bird[1,2]	
Cs. morsitans	Bird[1,2,5], Man[4,5]	
Cs. subochrea	Man[4], **Deer**	
Cq. richiardii	Bird[1,5,9], Cattle[1,5], Mammals[2], Man[4,5], Amphibians[8,]	
Cx. europaeus	Amphibian/Reptiles[2], Man[4]	
Cx. modestus	Man[2]	
Cx. pipiens f. *pipiens*	Bird[1,2,5], Man, **Dog**	**Man & Dog**
Cx. torrentium	Bird[2,5]	Bird & Rabbit[1]
Da. geniculata	Man[2,4]	
Oc. annulipes	Mammals[2,3], Man[4]	
Oc. cantans	Cattle[1,5], Bird[1,5], Man[3,4,5], Mammals[2,3,6]	
Oc. caspius	Man[2,4], Cattle1, Sheep[1], Bird[1]	Bird & Mammal[1]
Oc. communis	Man[2,4]	
Oc. detritus	Cattle[1], Bird[1], Man[2,4]	
Oc. dorsalis	Cattle[1,5], Rabbit[1], Pig[1], Horse[1], Mammals[2,3], Man[3,4]	
Oc. flavescens	Cattle[1,3], Sheep[1,3], Bird[1], Mammals[2,3], Man[4], Horses[3]	
Oc. leucomelas	Man[2]	
Oc. punctor	Man[2,3,4,5], Cattle[1,5], Bird[1,5],	Bird & Mammal[1]
Oc. rusticus	Man[2,3,4]	
Oc. sticticus	Man[2], Mammals[7]	
Or. pulcripalpis	Bird[2]	

Table 5.7 Summary of available data as shown on page 109, Bold text shows the additional hosts detected in this study section 5.4.3.

5.5 Discussion

The study of host selection was carried out in this study as a means towards understanding the dynamics of disease transmission with specific reference to the recent resurgence and introduction of diseases such as WNv and Chikungunya beyond their range. Potential vector and bridge vector species in the UK have been identified based on previously published data for Europe and North America (Higgs *et al.,* 2004; Medlock *et al.,* 2005; see also Table 5.1) however, host selection and the vector potential of mosquito species in the UK have not been specifically tested in relation to these diseases. Herein host selection was determined, through the analysis of the mitochondrial CytB gene in blood meals of seven species of British mosquitoes (see Table 5.7) collected in (Devon, Norfolk, Somerset and the Isle of Anglesey): *An. atroparvus, An. daciae*, *An. messeae*, *Cx. pipiens*, *Cx. torrentium*, *Cs. subochrea* and *Cq. richiardii*. Seven host groups (bird, cow, dog, deer, goat, horse and man) were amplified from bloodfed female mosquitoes using host-specific primers designed herein for deer, dog, goat, horse and man as well as Universal CytB primers (Boakye *et al.,* 1999).

The host selection of *An. daciae* is reported here for the first time. Both host-specific primers and CytB sequencing showed the host choices of *An. daciae* to be very indiscriminate. Six vertebrate hosts (bird, man, cow, deer, goat and horse) were determined from 84 individuals of *An. daciae* collected across 3 counties. That *An. daciae* is opportunistic in its feeding behaviour is exemplified in Norfolk where 71 bloodfed females, collected within the environs of the petting stables in Pettits Animal Farm, were shown to have fed on all six hosts present. Interestingly, *An.messeae* and *An. atroparvus* were far less diverse in their choice of hosts, despite being closely related to *An. daciae*. The selection of hosts for both *An. messeae* and *An. atroparvus* in Pettits Animal farm, where all three species were collected in sympatry, comprised of Deer, Goat and Man. Given that neither the number of hosts nor the accessibility to these hosts were a limiting factor in this particular sampling site, these results indicate that both *An. messeae* and *An. atroparvus* tend to be selectively zoophilic than *An. daciae*. The seeming indiscriminate feeding behaviour by *An. daciae* could be attributed to the response of *An. daciae* to various chemical cues from different host species including CO_2 and fatty acids (Knols *et al.,* 1997; Costantini *et al.,* 1998). Alternatively it could also indicate an inability of *An. daciae* to discern the presence of more

than one host within a small area (Thomas 1951 cited in Port & Boreham, 1980) as observed in Pettits Animal Farm.

Interestingly, compared to *An. messeae* and *An. atroparvus*, 7 *An. daciae* females had fed on more than one host, an indication of an interruption in the bloodmeal. In fact, 15% of bloodfed mosquitoes screened had fed on multiple hosts. The prevalence of multiple bloodmeals has been reported in various studies conducted on biting and feeding behaviour of mosquitoes (Zimmerman *et al.,* 2006; Michael *et al.,* 2001; Gordon *et al.,* 1991; Boreham & Lenahan, 1979). This detection of multiple hosts in a single bloodmeal is of particular interest in the transmission of arbo-diseases. A host-seeking female often imbibes a single uninterrupted (unmixed) bloodmeal, however, host irritability, incomplete feeding (Davies, 1990) or even a possible infection (viral or parasitic) of the vector (Rosomer *et al.,* 1989) can lead to interrupted feeding. This then results in a female taking multiple meals, i.e., two or more feeds in a single gonotrophic cycle (Boreham & Garrett-Jones, 1973; Boreham, 1975; Rosomer *et al.,* 1989). Interruption of feeding increases host-vector contact, thereby increasing the likelihood of pathogen transmission from the host to the mosquito and to other hosts. Thus the selection of multiple hosts such as birds (which serve as reservoirs for arbo diseases such as WNv and Sindbis) and man (through which arbodiseases such as malaria can be circulated among the population) observed in *An. daciae* in Norfolk as well as in Somerset presents it as a potential bridge vector of the West Nile virus.

Of equal interest is the feeding by *An. daciae* on exotic species such as *R. tarandus* (reindeer) and *P. pennata* (Darwin's Rhea), which further illustrates the potential role that it could play as a bridge vector. The reindeer has been identified as a reservoir host for the enzootic Tahyna and Inkoo viruses (Brummer-Korvenkontio, 1973; L'vov *et al.,* 1989) that commonly occur in Northern Europe (Ramsdale & Gunn, 2005). This unusual host choice by *An. daciae* (as well as *An. messeae* and *Cs. subochrea*) and that of local deer and man (*An. daciae* & *An. messeae,* herein; *Cs. subochrea* (Ramsdale & Snow, 1995) (Table 4.8), suggests that the importation of infected reindeer from other countries where these diseases are endemic, could pose a health risk to both human and wild deer populations in the UK. Screening measures should be undertaken to ensure animals are free of these diseases prior to importation.

Prior to modernisation of houses, *An. messeae* was found to rest indoors and was thought to be solely anthropophilic (Snow, 1990; Ramsdale & Gunn, 2005). *Anopheles atroparvus* was previously recorded to be voraciously anthropophilic and as such was circumstantially incriminated as a vector of malaria in the UK post WWI (Shute & Maryon, 1974; reviewed in Cranston *et al*., 1987 & Rees & Snow, 1990). However, since the eradication of malaria from the UK (Dobson, 1989), *An. atroparvus* has been recorded to be largely zoophilic (Cranston *et al*., 1987; Snow, 1990). Given the host selection reported herein, it is likely that *An. atroparvus* and *An. messeae*, as well as *An. daciae* could also serve as vector of Tahyna & Sindbis viruses and that *An. messeae* could play a role in the maintenance of arboviruses such as WNv within bird populations. The documented role of *An. atroparvus* in malaria transmission in the UK and the recent findings of two specimens feeding on a human host does not eliminate this species as a potential vector of human as malaria, as malaria is no longer endemic in the UK.

Host selection of *Cx pipiens* was unclear, in part because of the uncertainty of its taxonomic status (Chapter 3, section 3.1). In the UK, prior to our proposal of synonymy between *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*, a distinction in feeding behaviour between the two forms was recorded: *Cx. pipiens* f. *pipiens* was ornithophilic and *Cx. pipiens* f. *molestus* was anthropophilic (Chapter 3, section 3.1). In the US (Apperson *et al.,* 2002; Patrican *et al.,* 2007) and southern France (Balenghien *et al*., 2006), *Cx. pipiens* has been recorded to feed on mammals and birds whereas in Sweden *Culex pipiens* is recorded to be primarily ornithophilic. Results presented herein show *Cx*. *pipiens* (in its current proposed synonymy; Chapter 3, Section 3.5) to be capable of feeding on both bird (Anglesey) and man (Somerset), albeit in two different localities. Its role as an incriminated vector of diseases, such as WNv in the US (Marfin *et al*., 2001) and Europe (Platonov *et al.,* 2001; Estevez *et al.,* 2005; Romi *et al.,* 2004), Japanese Encephalitis in Asia (Johansen *et al.,* 2002) as well as Rift Valley Fever in Africa (Turrell *et al.,* 1996) and its current non distinctive feeding behaviour does implicate it as major candidate for the transmission of enzoonotic diseases in the UK.

Only a single bloodfed *Cx. torrentium* female was captured in this study and as such little can be said about its vector potential. Host preference exhibited by *Cx. torrentium* has reported to be solely ornithophilic (Snow, 1990; Medlock *et al*., 2005) and the only bloodfed *Cx. torrentium* (DNA 10454) collected here (in a pillbox in Godney, Somerset) was found to have had a multiple meal on a chaffinch and dog, thus the latter comprises a new host record

for *Cx. torrentium* in the UK. Interestingly, chaffinches have been thought to be a reservoir host for the Sindbis virus (J. Lundström pers comm.) of which *Cx. torrentium* is an incriminated vector (Lundström *et al.,* 1990a, b). In the UK, although transmission of Sindbis virus is thought to occur (Buckley *et al.,* 2003), no evidence has been shown linking *Cx. torrentium* as a vector. Considering all factors, *Cx. torrentium* could definitely play a role in maintaining the virus in the local bird population, thus enabling the transmission by another mosquito species, such as *An. messeae* or *An. daciae* which feed on birds, but also have a wider host selection.

The single *Cq. richiardii* bloodfed specimen analysed showed a preference for man. Previously, host preference of *Cq. richiardii* showed an affinity for mammals (Service, 1971; Snow, 1990), man (Ramsdale & Snow, 1995; Medlock *et al*., 2005) and birds (Snow, 1990). This diversity was also observed by Balenghien *et al.* (2006) in southern France, where specimens of *Cq. richiardii* were collected in both horse and bird baited traps and in human landing catches. Based on this diversity and the collection of specimens from near a West Nile virus case (Higgs *et al.,* 2004), *Cq. richiardii* was considered to be a potential vector of WNv in the UK (Medlock *et al*., 2005; Higgs *et al*., 2004). However, its role as a potential vector, based on a single specimen, cannot be inferred in this study.

In general, host specific primers designed in this study could successfully identify six out of the seven target hosts however, there was still a need for sequencing in order to verify PCR products and to avoid the misidentification of hosts by preferential amplification of mosquito DNA. The use of universal CytB primers of Boakye *et al*, (1999) on field-caught samples showed these primers to identify arthropod blood meal and to also amplify mosquito DNA. Thus five host specific reverse and one host specific forward primer were designed to ensure amplification of target hosts. While host specific primers successfully amplified target host when control specimens were used, insect DNA was still preferentially amplified in 67% of individuals sequenced (See sections 4.4.2, 4.4.3). The seemingly 'selective' amplification of insect over host DNA could be attributed to the preservation of bloodfed mosquitoes. The preservation method of drying bloodfed mosquitoes does not allow for the extraction of vertebrate DNA only, thus enabling the competing amplification of mosquito DNA as shown in section 4.4.3. This problem could be overcome by the freeze killing of field caught bloodfed mosquitoes, dissecting the abdomen and smearing the blood meal on a piece of filter

paper, which is then stored in a container with silica (Weitz *et al.,* 1956). Thus reducing the presence of insect DNA from the sample altogether.

The inability to consistently identify a host in bloodfed mosquitoes (both fully bloodfed and partially bloodfed) coupled with the lack of clear unambiguous sequences (4%) could lead to inaccurate identification of hosts, as illustrated by the detection of *L. meridionalis* in two individuals. This warrants the need for the further development of robust and sensitive molecular methods. One option would be to clone all fragments obtained with the use of universal CytB primers on fully bloodfed female mosquitoes. Cloned fragments could then be sequenced to determine host species selected which could in turn be used as a template on which more efficient primers could be designed. Alternatively, the use of Real-Time (RT) PCR has been optimised for the identification of mosquito blood meals (van den Hurk *et al.,* 2007). The use of host-specific primers and probes allows for the quantification of DNA during the exponential amplification of the template (Heid *et al.,* 1996), thereby determining the amount of host DNA present in a single mixed or unmixed blood meal. The primers designed herein could be used to in the design of a real-time PCR. Though RT-PCR eliminates the need for the visual identification of DNA fragments, it still requires very specific primers and verification by sequencing, as demonstrated here.

However, despite these shortcomings, this study was able to provide molecular evidence to the initial identification of potential vectors and bridge vectors in the UK, as well as identifying means through which arbo viruses such as WNv or even Sindbis could be transmitted at low levels (Buckley *et al.,* 2003) in the UK. Unfortunately, the incrimination/implication of a vector cannot be done solely on the identification of host species using a mosquito blood meal. As demonstrated by Spielman (1986) and Beach *et al.* (1985), the release of saliva, particularly apyrase, during probing and feeding, can result in the release of the pathogen without taking a blood meal. Thus in addition to host selection, the isolation or detection of pathogen (either viral or parasitic) within the salivary glands of the mosquito will serve to incriminate species as vectors. Future work should focus on incrimination and determination of the role of British mosquitoes as primary and secondary vectors of disease, through viral and parasitic isolation and the collection of vectors in areas afflicted with viral or parasitic infection (Defoliart *et al.,* 1987; Janini *et al.,* 1995; Dutta *et al.,* 1997). This knowledge would provide a better understanding of vector competency in

British mosquitoes, a tailored monitoring and surveillance system as well as the implementation of a targeted vector control programme.

Chapter 6

Occurrence and habitat preference of mosquitoes in southern England and northern Wales

6 Occurrence and Habitat Preference of Mosquitoes in southern England and northern Wales

6.1 Current occurence of British mosquitoes

As part of a wider study into malaria in England and Wales, Nutall *et al.* (1901) produced the first guide to the distribution of *Anopheles* mosquitoes, but did not differentiate between species. Lang (1918) reported occurrence of *An. claviger* (as *An. bifurcatus*), *An. maculipennis s.l.* and *An. plumbeus* in England and Wales and later Ashworth (1927) presented distribution maps for Scotland. Various species were reported in the works of Marshall (1938) and a review of the distribution of the mosquitoes of Ireland was added much later by Ashe *et al*. (1991). The first detailed distribution maps of UK mosquitoes were provided by Rees & Snow (1990, 1992, 1994, 1996) by genera: *Anopheles* (Rees & Snow, 1990; also reviewed by Snow, 1998), *Culex* (Rees & Snow, 1992), *Coquillettidia*, *Culiseta* and *Orthopodomyia* (Rees & Snow, 1994), *Aedes* and *Dahliana* (as *Aedes*, *Aediomorphus* and *Finlaya*) (Rees & Snow, 1995) and *Ochlerotatus* (as *Aedes*, subgenus *Ochlerotatus*) (Rees & Snow, 1996). Information gathered for these maps were obtained from the British Mosquito Recording Scheme and on the older records (Lang, 1918; 1920; Marshall, 1938) and, although the maps are highly useful, included no detailed information on component species in cryptic complexes, such as *An. maculipennis s.l*. In light of new molecular techniques to differentiate cryptic species (Proft *et al*., 1999; Linton *et al*., 2002a; Linton *et al*., 2005; herein Chapter 3), updated specific maps can and must now be provided, as this information is imperative in risk assessment and vector control strategies for mosquito-borne diseases in the UK in the future.

6.2 Aims

The objectives of this study were:

[1] To document the species of mosquitoes collected in 7 counties in southern England and northern Wales, using integrated systematic methods for accurate species identification and

[2] To accurately characterise the preferred mosquito larval habitats and adult resting sites.

6.3 Materials and Methods

6.3.1 Collection of larvae and pupae

Larval and pupal stages were collected from natural (stream pools, stream margins, ponds) and artificial habitats (buckets, water troughs) using the dipping method outlined in Chapter 2 Section 2.2.1

Collected specimens were then individually reared to the adult stage. Emerged adults and associated larval and pupal skins were stored and labelled as detailed in Chapter 2, section 2.2.1.2.

6.3.2 Collection of resting and host seeking Adults

Both resting and host seeking adults were collected from brick shelters, war bunkers and animal shelters using manual aspirators and the Mosquito Magnet Trap Liberty Pro respectively (Chapter 2 Fig 2.2.2). Collected adult specimens were then processed according to the method detailed in Chapter 2 Section 2.2.2.

6.4 Results

Of the adult collections, 861 adults (resting, n=728; host seeking, n=133) were identified to species (Table 6.1). While 564 specimens were identified from the aquatic collections. In total of 13 species were collected: *An. algeriensis*, *An. atroparvus*, *An. claviger*, *An. daciae, An. messeae*, *Cs. annulata, Cs. subochrea, Cq. richiardii*, *Cx. pipiens*, *Cx. torrentium*, *Da. geniculata, Oc. detritus and Oc. leucomelas* (Table 6.1).

Human landing collections were not undertaken, but the single representative of *Da. geniculata* collected in this study was captured landing on (and presumably trying to bite) the author at dusk in the Bridestowe caravan park in Bridestowe, Devon.

Species	Collection type	Anglesey (n=233)	Caernarfonshire (n=35)	Devon (n=340)	Kent (n=108)	Norfolk (n=565)	Somerset (n=133)	Suffolk (n=12)
An. algeriensis	Aquatic	12	--	--	--	--	--	--
An. atroparvus	Resting	--	--	--	97	14	--	--
An. claviger	Aquatic	22	--	39	--	1	--	--
	Host seeking	--	--	5	--	4	--	--
An. daciae	Aquatic	1	--		--	2	--	3
	Resting	--	--	--	6	373	86	--
An. messeae	Aquatic	--	--	2	--	--	--	3
	Resting	--	--	97	5	13	7	--
Cq. richiardii	Resting	--	--	--	--	3	1	--
	Host Seeking	--	--	--	--	115	--	--
Cx. pipiens	Aquatic	105	21	139	--	27	24	6
	Resting	1	--	8	--	--	8	--
	Host Seeking			1		1		
Cx. torrentium	Aquatic	34	14	45	--	12	--	--
	Resting	--	--	--	--	--	1	--
Cs. annulata	Aquatic	41	--	3	--	--	--	--
	Resting	2	--	1	--	--	3	--
	Host Seeking	2						
Cs. subochrea	Aquatic	7	--	--	--	--	--	--
Cs. subochrea	Resting	--	--	--	--	--	3	--
Da. geniculata	Human Landing	--	--	1	--	--	--	--
Oc. detritus	Host Seeking	4	--	--	--	--	--	--
Oc. leucomelas	Host Seeking	2	--	--	--	--	--	--

Table 6.1 Numbers of larval and adults collected per species in each county, by collection type. Immatures (n=222), resting (n=3) and 8 host-seeking individuals were collected using the Mosquito Magnet® trap in Anglesey. Human landing (n=1), larvae/pupae (n=208) resting (n=106) and host seeking female (n=6) collections were made in Devon. Only Resting (n=108) adults were identified in Kent, Larvae/pupae (n=42), resting (n=403) and host-seeking (n=220) adult collections were made in Norfolk. Only Larvae/pupae (n=24) and resting (n=109) collections were made in Somerset. Larvae/pupae (n=12) were collected in Suffolk and in Wales (n=35).

6.4.1 Characterisation of aquatic stages

Of the 60 individual aquatic localities sampled, twenty-four mixed species collections were found, with single species (possibly isofamilies) being detected in the remainder. Even in mixed species collections, one species tended to be overwhelmingly dominant, with the others most frequently represented by only a few specimens.

The majority of immature collections came from Devon, comprising 43% of all specimens collected, followed by Anglesey (n=222). Of all immatures collected, 60% (n=956) belonged to genus *Culex*, which were found in all counties except Kent (Figure 6.1).

In general, immatures of *Culex* spp. could be found in artificial habitats (84%) such as discarded bath tubs, boats and buckets, generally lacking any aquatic vegetation although some specimens were collected in natural habitats such as stream pools, groundpools and ditches containing floating and emergent vegetation (Figure 6.1). *Anopheles* larvae, however, were found in 8.9% of all larval collections showing a strong preference for natural habitats, such as ground pools, stream margins and ditches (Figure 6.2), in which moderate amounts of floating and emergent vegetation were present (Figure 6.1). However a few individuals were collected in artificial habitats, including: a tyre in Anglesey (n=2), a fire bucket in Bridestowe Caravan Park and in a water trough in Caernarfonshire, Wales (Figure 6.2). Aquatic stages of the genus *Culiseta* were found only in two counties (Table 6.2) in natural habitats including stream margins and stream pools, with 0.3% of all *Culiseta* collected also being detected in moored boats with collections of rainwater. Interestingly, buckets were found to be the most productive artificial habitat, yielding 17% of all immature habitats sampled, while ditches and stream pools were the most productive natural habitats (10% each) (Figure 6.3).

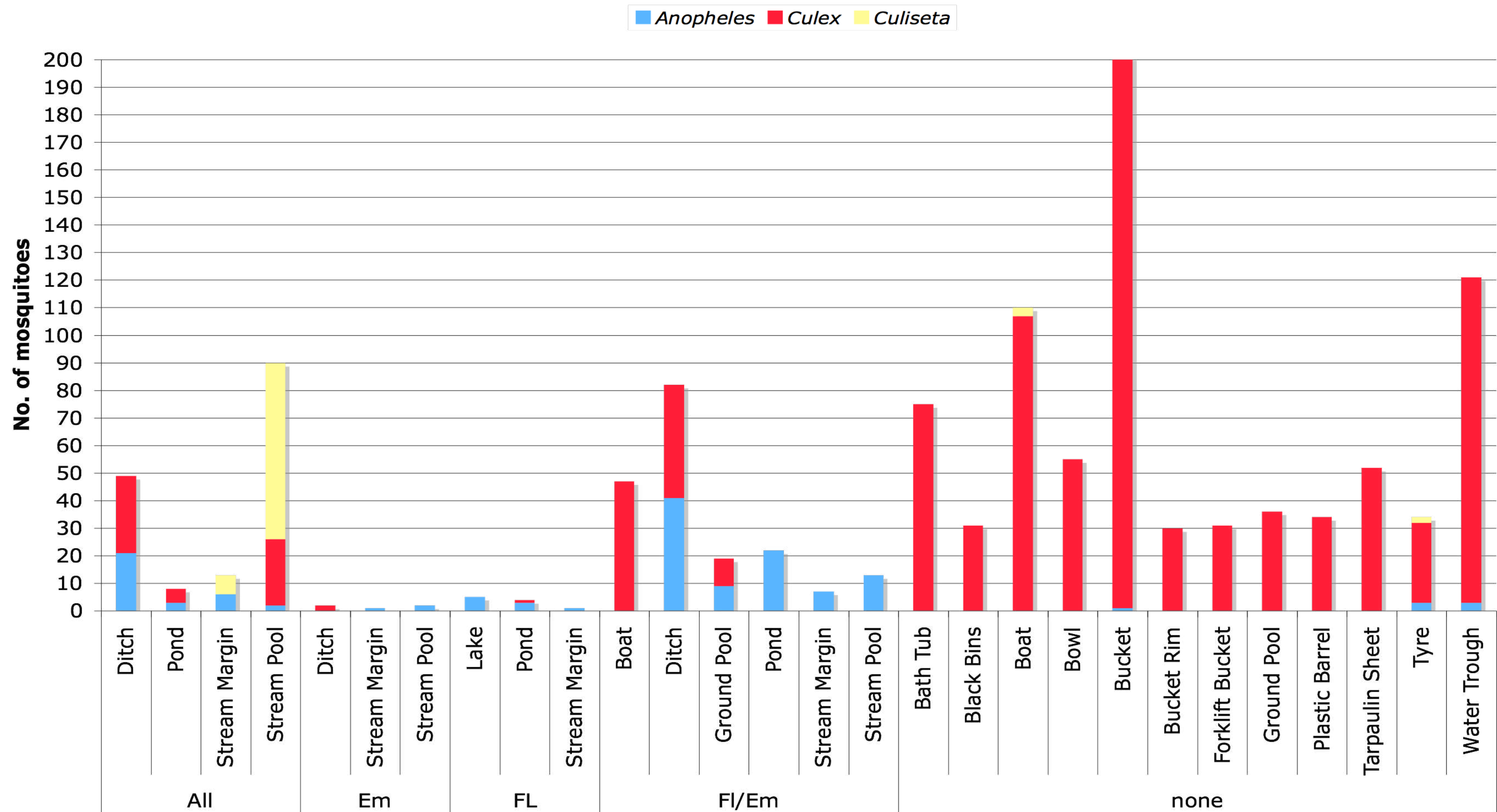

Figure 6.1 Graph showing the relative proportions of immature stages collected by genera in relation to the amount and type of vegetation found in the larval habitats. (Em = emergent vegetation; Fl = floating vegetation; All = floating and emergent vegetation).

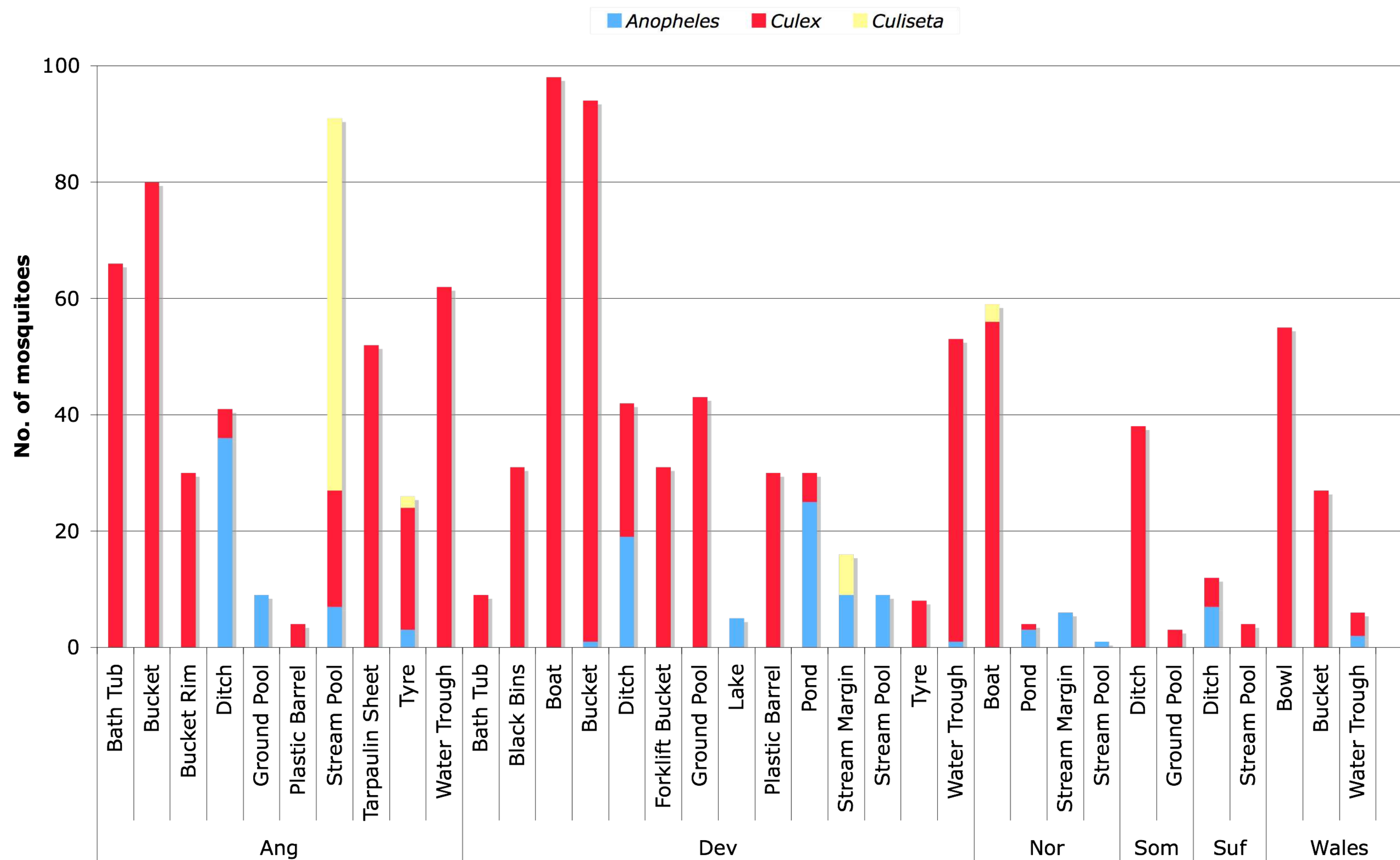

Figure 6.2 Graph showing association of three mosquito genera (*Anopheles, Culex and Culiseta*) with different larval habitats in five regions of England: Devon (Dev), Norfolk (Nor), Somerset (Som), Suffolk (Suf) and two regions of northern Wales (Anglesey (Ang) and Caernarfonshire (Wales).

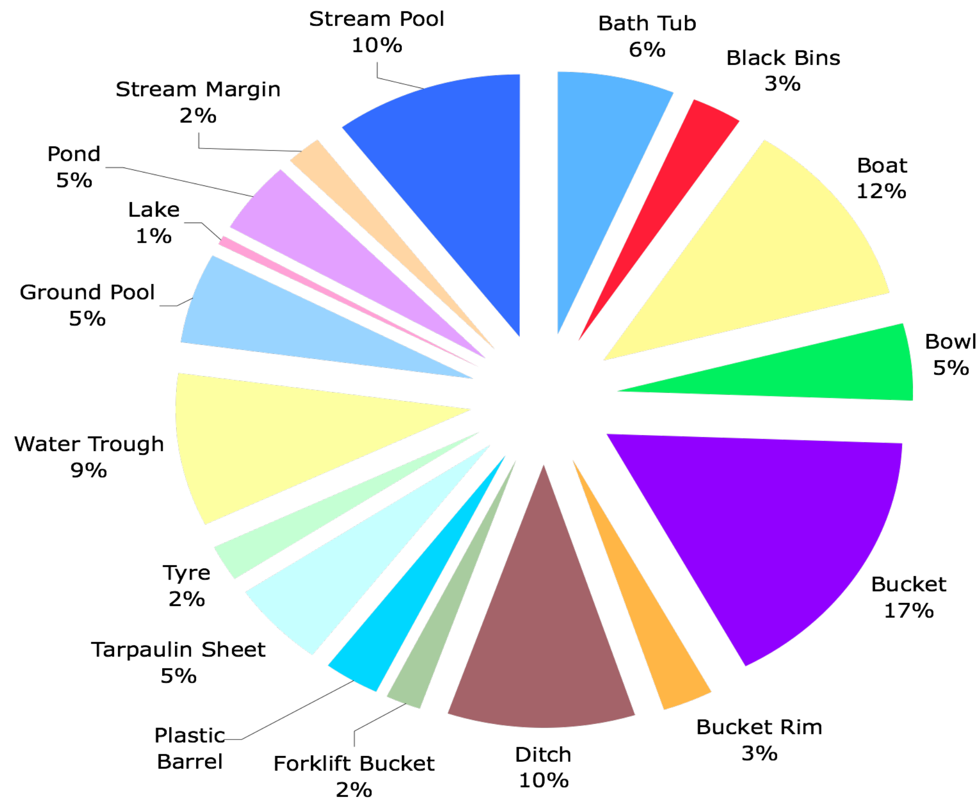

Figure 6.3 Relative proportions of mosquito immatures collected in July 2006 by habitat type. Natural habitats (denoted with 'N') yielded only 33% of total mosquitoes collected (n=530) and included stream margins, stream pools, ponds, lakes, ground pools and ditches. A total of 1072 specimens were collected in man-made habitats. Artificial habitats included water collections in forklift buckets, tarpaulin sheets, tyres, boats, buckets and troughs.

6.4.2 Collected British mosquitoes detailed by genera

6.4.2.1 Genus *Anopheles*

Five species of *Anopheles* were collected in this study: *An. algeriensis*, *An. claviger* and three species in the Maculipennis Group – *An. atroparvus*, *An. daciae* and *An. messeae*. Specimens of the Maculipennis group were identified using the ITS2 PCR_RFLP assay designed in Chapter 2. Despite searches in treeholes, where available, no *Anopheles plumbeus* were collected in this study.

6.4.2.1.1 *Anopheles algeriensis*

Anopheles algeriensis (n=12) was collected as immatures in three sites: two ditches (Larvae (L=3), Pupa (P=4)) and one ground pool (L=3, P=2), all with abundant vegetation, in the calcarious marshland habitat of Cors Goch Nature Reserve in Anglesey. No adults were collected in this study (Figure 6.4).

6.4.2.1.2 *Anopheles atroparvus*

Five adult *An. atroparvus* (four non-bloodfed females, one male) were collected from a brick shelter on Salt Lane in the Cliffe Marshes in Kent were collected together with *An. messeae* and an unidentified *Culiseta*. In another bunker, used mainly as a sheep corral, also located in the Cliffe marshes, *An. atroparvus* (n=92; 26 bloodfed females, 62 non-bloodfed females and four males) was collected in sympatry with *An. daciae* and *An. messeae*. Fourteen adult *An. atroparvus* were collected resting in goat houses (n= 8) and reindeer stable (n= 6) in Pettits Animal Farm in Norfolk, sympatrically resting with *An. daciae* and *An. messeae*. All but one of the females collected were bloodfed. No immature stages of *An. atroparvus* were collected in this study (Figure 6.4).

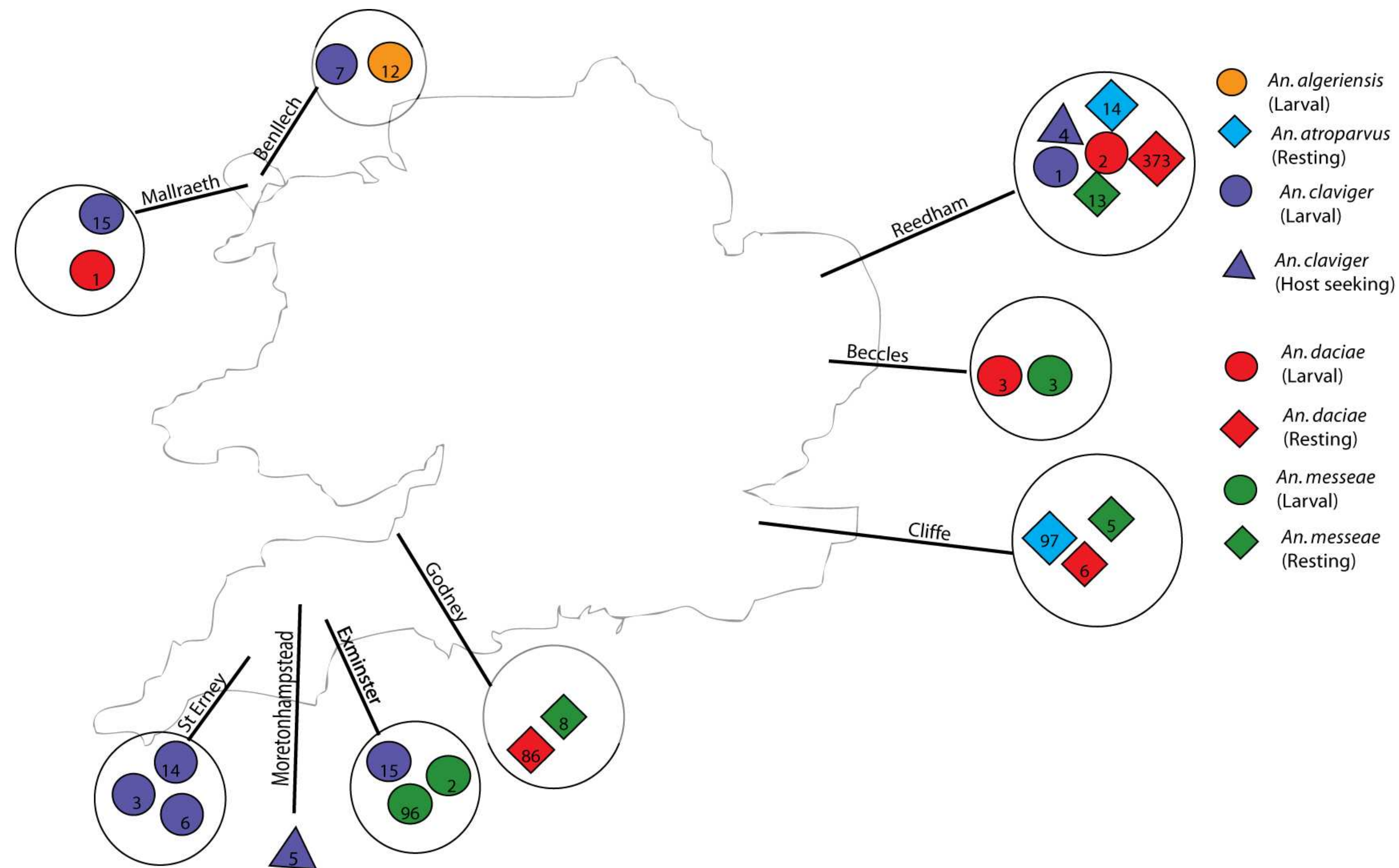

Figure 6.4 Occurrence map of 5 species of British *Anopheles* mosquitoes collected in southern England and northern Wales in July and August 2006. Symbols contained within a circle indicate several collections in the locality indicated. Numbers written in the symbols indicate the total number of specimens collected. Co-ordinates for all collections are available in Chapter 2 Table 2.2. ***Anopheles algeriensis*** was collected as larvae in Anglesey; ***An. atroparvus*** as resting adults in Norfolk and Kent; ***An. claviger*** collected as larvae in Anglesey, Devon and Norfolk and as host seeking adults in Devon and Norfolk; ***An. daciae*** collected as larvae in Anglesey, Norfolk and Suffolk and as resting adults in Somerset, Kent and Norfolk; and ***An. messeae*** collected as larvae in Devon and Suffolk; as resting adults in Somerset, Kent and Norfolk.

6.4.2.1.3 *Anopheles claviger*

In Devon, *An. claviger* was detected in three sites: in a stream near Yelland farm (L=1, P=2), in a woodland pool near Boating World (L=12, P=2) and a stream pool (L=3, P=3) in Simmon's Park. It was also collected in three sites in the Exminster Nature Reserve, Devon; in a ditch (L=5, P=4) and in two stream margins (L=5, P=1). One individual was collected in a stream margin on Horsey Road, in Reedham, Norfolk. Aquatic stages were found in three sites in Anglesey: a single *An. claviger* pupa was collected in a stream pool near a horse farm, and larvae and pupae were collected in a stream near Mallraeth Marsh (L=8, P=7) and in a ditch near a cottage in Benllech (L=4, P=2). *Anopheles claviger* was most often found as pure immature collections, with the exceptions of only three collections. The species was collected sympatrically with *An. messeae* and *Cs. annulata* (in a stream margin in Exminster marshes, Devon), with *An. daciae* (in a stream margin in Norfolk and with *Cx. torrentium* (in a ditch in Benllech, Anglesey).

Five host seeking adults were collected near horse stables in the Miniature Pony Centre in Moretonhampstead, Devon and four in Pettits animal farm in Reedham, Norfolk, where miniature donkeys, miniature horses, reindeer, Darwin's Rhea and goats were present (Figure 6.4).

6.4.2.1.4 *Anopheles daciae*

A single *An. daciae* pupa was collected in Anglesey, in a stream pool behind a horse farm. In Norfolk, two larvae were detected in a stream margin along Horsey Road. Larvae (n=3) of *An. daciae* were also found in two ditches in the Carlton Marsh Nature Reserve in Suffolk, in sympatry with *An. messeae*.

All resting collections made in Norfolk came from animal stables in Pettits Animal Farm: in goat houses (n=197), miniature horse and donkey stables (n=45), reindeer stables (n=124) and a Rhea stable (n=7). Of these, 72.6% (n=271) were bloodfed (see Chapter 5). Sympatry with *An. messeae* was observed in all the animal stables sampled. In addition, all three species of the Maculipennis Group were collected in both goat and reindeer stables in Norfolk. Eighty-six adults were collected from three localities in Godney, Somerset: a horse

stable (n=7) and two pillboxes (n=79). *Anopheles daciae* adults collected in the horse stable also was found resting with *An. messeae* and *Cq. richiardii*. In the first pillbox in Godney village, *An. daciae* was found resting in sympatry with *An. messeae*, *Cx. pipiens*, *Cx. torrentium*, *Cs. annulata* and *Cs. subochrea* and with *An. messeae*, *Cx. pipiens* and *Cs. annulata* in the second pillbox (Figure 6.4).

6.4.2.1.5 *Anopheles messeae*

Anopheles messeae larvae were found in pure populations in two stream margins in Exminster Nature Reserve, Devon. It was also collected in two ditches in the Carlton Marshes Nature Reserve, in Suffolk, together with *An. daciae*.

In the Cliffe Marshes in Kent, *An. messeae* was collected in sympatry with *An. daciae* and *An. atroparvus* in an abandoned brick shelter and together with *An. atroparvus* in a bunker on Salt Lane (n=1). *Anopheles messeae* was also found in Norfolk in goat stables (n=1), miniature horse and donkey stables (n=4), reindeer stable (n=7), in sympatry with *An. daciae* as well as *An. atroparvus* and in a Rhea stable (n=1). Ten individuals were found to have had a blood meal (see Chapter 5). Two females were resting in a horse stable in Somerset, one in a pillbox on near Godney Farm and five individuals were resting in a pillbox on Garslade farm (Figure 6.4).

6.4.2.2 Genus *Culex*

6.4.2.2.1 *Culex pipiens*

In Anglesey, fifteen localities were positive for *Cx. pipiens* (13 artificial and 2 natural sites) in Benllech (n=36), Brynteg (n=20), Llangferfechan (n=12), Newborough (n=6), Penraeth (n=26) and Mallraeth (n=5) (Table 5.3). In Devon, *Cx. pipiens* was detected in 14 localities (11 artificial, 3 natural) in Belstone (n=28), Bridestowe (n= 27), Cheriton Bishop CP (n=32), Moretonhampstead (n=40), St Erney (n=1), Trevollard (n=9) and Dartmoor forest (n=2). *Culex pipiens* were found in two separate boats in Norfolk in Ranworth Wildlife Centre (n=11) and in the margins of a small stream in West Somerton (n=16). All three habitats sampled in Somerset were natural: a ground pool on Garslade farm (n= 3) and two ditches (n= 13, n= 8). The same was found in Suffolk, where all aquatic stages of *Cx. pipiens* were collected in natural habitats in and around Beccles Marsh. *Cx. pipiens* (L=8, P=13) was also detected near horse stables near Betws-y-Coed in Caernarfonshire, in artificial habitats including horse troughs and barbeque bowls.

One adult *Cx. pipiens* was collected resting in a shed in Bird World, Anglesey, in sympatry with *An. maculipennis s.l.* and *Cs. annulata*. All seven individuals in Devon were collected resting inside a horse stable in Eastland Horse Farm; none was blood fed. A single host-seeking female were collected using the Mosquito Magnet Trap ®, near a chicken coop in the Miniature Pony Centre in the New Forest and one resting adult was collected in sympatry with *An. messeae* and *Cs. annulata* in a brick shelter in Exminster Nature Reserve, also Devon. *Culex pipiens* was collected as resting adults in two pillboxes in Godney, Somerset (n=8) in sympatry with *An. daciae*, *An. messeae* and *Cs. subochrea* (Figure 6.5).

6.4.2.2.2 *Culex torrentium*

Culex torrentium was collected in 11 habitats (9 artificial and 2 natural) in four localities in Anglesey: Benllech (L=9, P=3), Brynteg (L=3, P=5), Llangferfechan (L=4, P=7) and Penraeth (P= 3). Ten habitats (8 artificial and 2 natural) in six localities sampled in Devon were also found to support *Cx. torrentium*: Belstone (L=3, P=9), Bridestowe (L=15, P=8), Cheriton Bishop (L=1, P=2), Moretonhampstead (L=1, P=2) and Dartmoor Forest (P= 3) and

St. Erney (P=1). In Norfolk, a total of twelve individuals (L=1, P=11) were collected in a moored boat in Ranworth Wildlife Centre. In Caernarfonshire, *Cx. torrentium* was found in three artificial habitats in Ty Coch Riding Stables (L=11, P=3) near Betws-y-Coed. A single bloodfed adult was collected resting in a pillbox in Godney village in Somerset (Figure 6.5). *Culex pipiens* and *Cx. torrentium* were collected in sympatry in 68.6% (n=24) of all localities sampled: Anglesey (n=10), Devon (n=10), Norfolk (n=1) and Wales (n=3).

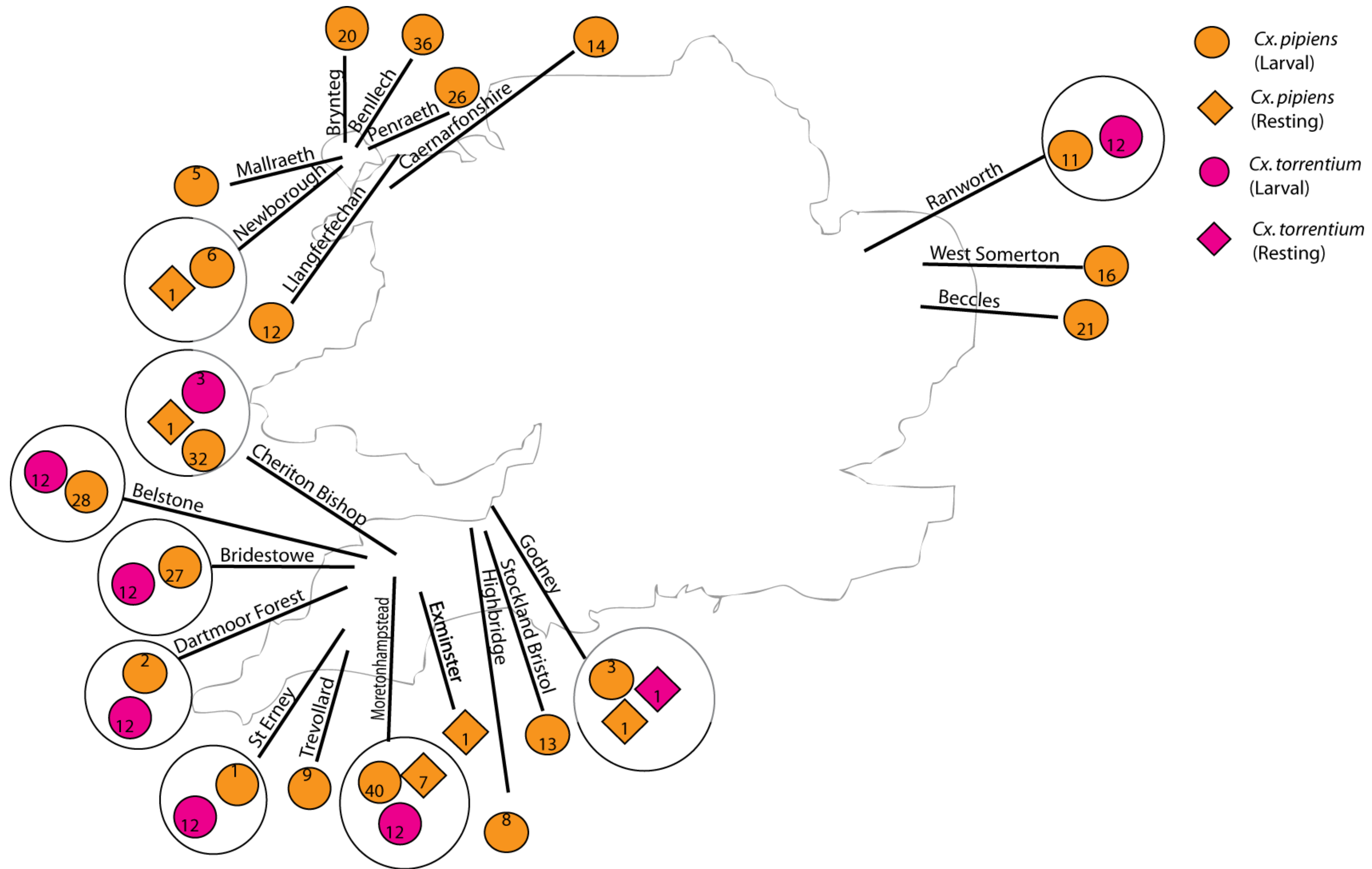

Figure 6.5 Presence of *Culex* mosquitoes collected in northern Wales and in southern England in this study. Symbols contained within a larger circle indicate several collections in the specified locality. Numbers written in the symbols indicate the total number of specimens collected. Co-ordinates for all collections are available in Chapter 2 Table 2.2. ***Culex pipiens*** was collected as larvae in Anglesey, Devon, Norfolk, Somerset, Suffolk and in northern Wales and as resting adults in Anglesey and Somerset; as host seeking adults in Devon. ***Culex torrentium*** collected as larvae in Devon, Norfolk, England, in Anglesey and Caernarfonshire in Wales and as resting adults in Somerset.

6.4.2.3 Genus *Culiseta*

Immature stages of the genus *Culiseta* were relatively uncommon, with only ninety-six individuals collected in this study. Of these, only sixty were identified to species Table 6.1. Both *Culiseta* species were collected in sympatry as larvae in a stream pool in Cronllech Manor Farm, Anglesey and as resting adults in Somerset.

6.4.2.3.1 *Culiseta annulata*

Forty individuals (L=5, P=35) of *Cs. annulata* were found in a stream pool in Cronllech Manor Farm in Anglesey together with immature *An. claviger*, *Cx. pipiens* and *Cs. subochrea.* A single collection of *Cs. annulata* was collected in an abandoned tyre in the Mallraeth Marshes in Anglesey together with *Cx. pipiens* and a single undetermined *Anopheles*. *Culiseta annulata* was also collected in a stream margin in Exminster Nature Reserve, Devon together with *An. claviger*. Resting adults were collected in three localities (Table 6.1); none of the females were bloodfed. Two individuals were collected in shed in Bird World on Anglesey together with *An. maculipennis s.l.* and *Culex spp*. (Figure 6.6)

6.4.2.3.2 *Culiseta subochrea*

Culiseta subochrea (n=7) was collected in a stream pool in Cronllech Manor Farm. This was the only larval collection from which *Cs. subochrea* was identified. Resting adult females of *Cs. subochrea* (n=3) were collected in a pillbox in Godney village Somerset; two of these were blood fed (Figure 6.6).

Figure 6.6 Occurrence map of *Culiseta* mosquitoes collected in southern England and northern Wales in July and August 2006. Symbols contained in a larger circle indicate several collections within the specified locality. Numbers written in the symbols indicate the total number of specimens collected. Co-ordinates for all collections are available in Chapter 2 Table 2.2. ***Culiseta annulata*** collected as larvae in Anglesey; as resting adults in Devon and Somerset and ***Cs. subochrea*** collected as larvae in Anglesey; as resting adults in Somerset.

6.4.2.4 Genus *Coquillettidia*

Host seeking females were collected in Pettits Animal Farm in Norfolk (Table 6.1), where further specimens were collected resting in the miniature horse and donkey stables (n=1) and in goat stables (n=2). A single resting adult female was collected in a horse stable on Garslade farm, Godney, Somerset (Figure 6.7).

6.4.2.5 Genus *Dahliana*

A single *Da. geniculata* female was manually collected from the knee of the author (presumably looking for a host) in Bridestowe Caravan Park, Devon (Figure 6.7). Despite searching in tree holes, where possible, no larval stages, or resting adults, were collected.

6.4.2.6 Genus *Ochlerotatus*

Both *Oc. detritus* and *Oc. leucomelas* (Figure 6.7) were collected as host seeking females (Table 6.1), in the environs of Bird World in Anglesey. Sampling of aquatic stages within the vicinity was fruitless, despite extensive sampling of nearby water sources.

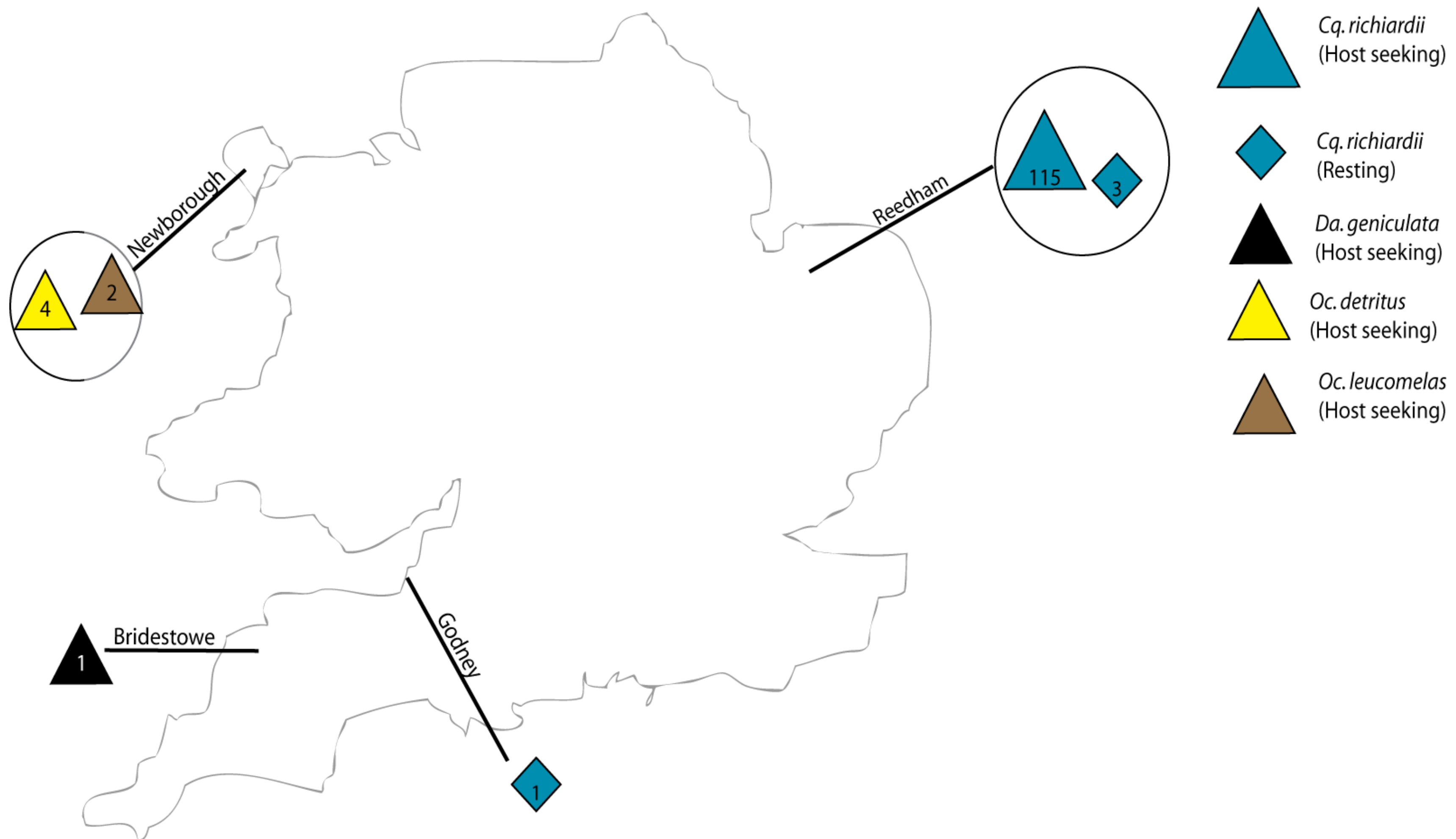

Figure 6.7 Occurrence map of *Coquillettidia*, *Dahliana* and *Ochlerotatus* mosquitoes collected in southern England and northern Wales in July 2006. Symbols contained in a larger circle indicate several collections within the specified locality. Numbers written in the symbols indicate the total number of specimens collected. Co-ordinates for all collections are available in Chapter 2 Table 2.2. ***Coquillettidia richiardii*** was collected as resting adults in Somerset and as host seeking adults in Norfolk. ***Dahliana geniculata*** was collected as a host-seeking adult in Devon. ***Ochlerotatus detritus*** and ***Oc. leucomelas*** were collected only as host seeking adults in Anglesey.

6.5 Discussion

This study, although somewhat comprehensive across seven counties in southern England and Wales, comprises only a "snap shot" of the composition and relative abundance of mosquitoes in the UK, as it reflects only mosquitoes present in July (Anglesey, Caernarfonshire, Devon, Norfolk, Somerset & Suffolk) and August (Kent) of 2006. Of the 33 documented British mosquito species, only 13 species, belonging to six genera (*Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Dahliana* and *Ochlerotatus*), were collected in this study as follows: *An. algeriensis*, *An. atroparvus*, *An. claviger*, *An. daciae*, *An. messeae*, *Cq. richiardii*, *Cs. annulata*, *Cs. subochrea*, *Cx. pipiens*, *Cx. torrentium*, *Da. geniculata*, *Oc. detritus* and *Oc. leucomelas*. All these species have previously been reported in the UK (Cranston *et al*., 1987; Snow, 1990; Linton *et al.,* 2002a; Linton *et al*., 2005). Adults from twenty-two species have been documented (Cranston *et al*., 1987; Snow, 1990; Snow & Medlock, 2008) from May to September. Of these, 13 were not collected in this study: *Ae. cinereus*, *Ae. geminus*, *Ae. vexans*, *An. plumbeus*, *Cs. morsitans*, *Oc. annulipes*, *Oc. cantans*, *Oc. caspius*, *Oc. dorsalis*, *Oc. flavescens*, *Oc. punctor*, *Oc. rusticus* and *Or. pulcripalpis*.

The absence of species such as *Oc. flavescens* could be attributed to its recorded patchy/sparse distribution (Rees & Snow, 1996). This would also explain the absence of species such as *Oc. communis* (reported four times, Snow *et al.*, 1998), *Oc. sticticus* (last reported in 1938, Rees & Snow, 1996), *Cx. territans*, *Cx. modestus* (last reported in Hayling Island in 1945, Snow *et al.,* 1998), *Culiseta longiareolata* (recorded on three occasions, Rees & Snow, 1994), *Cs. litorea* (last reported in 1955 in Surrey, Rees & Snow, 1994) and *Cs. alaskaensis* (Linton *et al*., 2005 confirmed one specimen of this from Kent). The lack of recent records suggests that these species are either elusive or may no longer occur in England. While *Ae. cinereus*, *An. plumbeus*, *Cs. morsitans*, *Oc. annulipes*, *Oc. caspius*, *Oc. dorsalis*, *Oc. punctor* and *Oc. rusticus* were not collected in this study, recent records of these species were made in Epping Forest (Hutchinson *et al*., 2007; Snow & Medlock, 2008), in the Isle of Sheppey (Hutchinson *et al*., 2007) and in Wicken Fen (Hutchinson *et al*., 2007). Given that *Aedes* and *Ochlerotatus* species overwinter as eggs (Snow, 1990), the dry summer of 2006 could account for the lack of suitable aquatic habitats thus delaying the emergence of adults.

In England, a total of ten species were collected from Devon (*An. claviger*, *An. messeae*, *Cx. pipiens*, *Cx. torrentium, Cs. annulata* and *Dahliana geniculata*), Kent (*An. atroparvus*, *An. daciae*, *An. messeae, Cs. annulata*), Norfolk (*An. atroparvus*, *An. claviger*, *An. daciae*, *An. messeae*, *Cq. richiardii*, *Cx. pipiens* and *Cx. torrentium*) Somerset (*An. daciae*, *An. messeae*, *Cq. richiardii*, *Cs. annulata*, *Cs. subochrea*, *Cx. pipiens* and *Cx. torrentium*) and Suffolk (*An. daciae*, *An. messeae* and *Cx. pipiens*). The presence of *An. atroparvus* in Norfolk, *An. messeae* in Devon, *An. daciae* in Kent, Norfolk, Somerset and Suffolk, *Cs. subochrea* in Somerset and *Cx. torrentium* in Somerset comprise new distribution records in England.

The Welsh island of Anglesey proved highly speciose, with 9 of the 13 taxa discovered in the whole survey: *An. algeriensis*, *An. claviger*, *An. daciae*, *Cx. pipiens*, *Cx. torrentium*, *Cs. annulata*, *Cs. subochrea*, *Oc. detritus* and *Oc. leucomelas*. Only two species were collected in Caernarfonshire, Wales: *Cx. pipiens* and *Cx. torrentium*. Given the lack of detailed mosquito surveys in Wales (Morgan, 1978; Rees & Rees, 1989), this study contributes to the reported diversity in this region of UK in particular. The collection of *An. daciae*, *Cs. subochrea* and *Oc. leucomelas*, in Anglesey, as well as *Cx. torrentium* in Caernarfonshire and Anglesey comprise new country and distribution records.

Most surprisingly, this study showed that *Anopheles daciae* was one of the most widespread and locally dominant species in this study, despite its relatively recent discovery (Linton *et al*., 2005). Prior to this study, little data were available on either the ecology of immatures and adults. The large collections of *An. maculipennis s.l.* herein provided sufficient material for the development and optimisation of an ITS2 PCR-RFLP assay (see Chapter 3) that discriminates between the three members of the Maculipennis Group occurring in England. *Anopheles daciae* was identified in the counties of Devon, Norfolk, Suffolk, Somerset (reconfirming its presence) and in Anglesey, Wales (Chapter 3). The Welsh record of *An. daciae* near Mallraeth is the most northerly documentation of *An. daciae* thus far. Resting adults were found in sympatry with *An. messeae* as well as *An. atroparvus*, whilst immatures were found in sympatry with *An. messeae* in Suffolk. This sympatric larval occurrence was also documented the type series in Romania, where *An. daciae* adults were found with *An. atroparvus* (Nicolescu *et al*., 2004). That *An. daciae*, the newest member of the British mosquito fauna is actually one of the most common and numerous species in the UK collections, serves to highlight how little we understand of the current demographics of local mosquito species in the UK.

Also found in this study was three relatively uncommon species: *An. algeriensis*, *Cs. subochrea* and *Oc. leucomelas*. *Anopheles algeriensis*, a predominantly Mediterranean species, was recorded in Norfolk by Edwards (1932) and later reconfirmed some 20 years later by Hart (1954). The presence of *Anopheles algeriensis* in the Cors Gogh Nature Reserve in Anglesey, herein, confirms earlier reports of Morgan (1987) and Rees & Rees (1989), although, no samples were detected in Norfolk in this survey.

Culiseta subochrea was previously reported to occur only in southeast England (Marshall, 1938; Rees & Snow, 1994; Snow *et al.,* 1998). In the present study, larvae were collected in Anglesey in sympatry with *Cs. annulata* (as also reported by Cranston *et al*., 1987) and resting adults were collected in Somerset. It has more recently been reported in Epping Forest, Essex (Snow & Medlock, 2008). Both collections make these the most recent records of this species in the literature since 1968 (Cranston *et al*., 1987).

Only two host-seeking adults of *Ochlerotatus leucomelas* were collected in this study, from Bird World in Anglesey. The only other occurrence records for this species, in the UK, were made by Carr (1919, in Marshall, 1938) near Nottingham and by Martini (1920, in Cranston *et al*., 1987) in Dartford, Kent. This collection in Anglesey reported here constitutes the most current occurrence in the UK and also a new distribution record in Anglesey for *Oc. leucomelas*. Although manual collections of resting adults were carried out in Bird World, no specimens of *Oc. leucomelas* were found indoors, which suggests that it may rest outdoors on vegetation.

In general it was found that aquatic stages of *Anopheles* and the majority of *Culiseta* larvae were most closely associated with natural environments, such as pools, streams and ditches, with floating (e.g. algae) and/or emergent (e.g. reeds, grasses) vegetation. In contrast, *Culex* mosquitoes were found to favour artificial habitats (e.g. buckets, bath tubs, water troughs). This observation was also reported by Marshall (1938), Rees and Snow (1990), Rees and Snow (1992) and Rees and` Snow (1994). However, contrary to reports of Cranston *et al.* (1987), who documented the presence of *An. claviger* in both brackish as well as freshwater, *An. claviger* was only found in freshwater habitats, in this study, such as pools, ditches and streams. In addition, 7 specimens of *Anopheles* sp. were collected in a tyre (Anglesey) or water troughs (Anglesey, Devon). Given that these specimens were preserved in 80% ethanol,

possible misidentification of the genus could have occurred, thus accounting for the ecological anomaly. Interestingly, *Cx. torrentium*, is reported to "exhibit a marked preference" for artificial habitats (Cranston *et al*., 1987; Snow, 1990) and it was thought that with the increase in suitable contained-habitats *Cx. torrentium* could be replacing *Cx. pipiens* (Mattingly, 1967). In this study, *Cx. torrentium* was commonly collected in artificial habitats with the exception of two sites, where it was collected in a ditch (Anglesey) and a ground pool (Devon). However, when collected in sympatry with *Cx. pipiens*, *Cx. torrentium* was less numerous.

Resting adult collections carried out in animal shelters, bunkers and pillboxes, recovered 8 species, of which members of the Maculipennis Complex were by far the most abundant. Seemingly localised abundance of *An. atroparvus* in Kent, *An. daciae* in Norfolk and Somerset and *An. messeae* in Devon was observed, suggesting that this method of capturing adult *Anopheles*, which also allow studies of natural infection and blood meal analysis (see Chapter 5), is effective for malarial surveillance in the UK. However collecting indoor resting adults in this manner alone excludes the collection of species like *An. algeriensis, Cq. richiardii*, *Cs. morsitans*, *Oc. cantans*, *Oc. caspius* and *Oc. rusticus* which prefer to rest in vegetation outdoors (Cranston *et al.,* 1997; Snow, 1990), thus presenting a bias in the sampling strategy and reducing the diversity of adult species collected in each sampling site.

One solution to overcome this sampling bias was to place the Mosquito Magnet Trap® in areas where resting adults were collected as well immature collections made. With this trap, 5 species were collected as host-seeking adults in addition to either immature or resting adult collections: *An. claviger*, *An. maculipennis s.l.*, *Cq. richiardii*, *Cs. annulata*, *Cx. pipiens*. Only two species of *Ochlerotatus* (*Oc. detritus* and *Oc. leucomelas*) were collected solely as host seeking adults in Anglesey despite attempts at collecting resting adults and immatures. This illustrates the importance of using different sampling methods to ascertain the occurrence of species in an area. The use of this trap as a tool for monitoring species diversity was also reported by Hutchinson *et al.* (2007) who compared mosquito collections using a CDC Light Trap with those of the Mosquito Magnet Trap ® in England (Chadwell Heath, Epping Forest, Isle of Sheppey and Wicken Fen). They found that the Mosquito Magnet® attracted a wider diversity of mosquito species such as *An. claviger*, *Cq. richiardii*, *Oc. annulipes*, *Oc, cantans*, *Oc. caspius*, *Oc. geniculatus*, *Oc. punctor* and *Oc. rusticus* compared to the CDC light traps. The combination of carbon dioxide and 1-Octen-3-ol (Octenol) in the Mosquito Magnet® trap was found to be attractive to host-seeking mosquito species (Takken & Kline, 1989; Becker *et*

al., 1995). Although, diversity of species recorded is affected by the number of Mosquito Magnet Traps ® used and localities in which they are placed (Brown *et al.,* 2008). Thus, in wider arbovirus surveillance studies the use of: more than one Mosquito Magnet Trap ® in an area, sweep net and automated suction pootering of vegetation would perhaps be more effective in capturing various species for vector incrimination of other mosquito-borne diseases.

Although this field study only documents British mosquitoes across a limited geographic area, the ecology of both adults and aquatic stages of the 13 species collected have been characterised and recent knowledge of host preference determined. Data from this study have been accessioned into Mosquito Map (www.wrbu.org) and Mosquito Watch (http://www.cieh.org/policy/npap_uk_sightings.html) to serve as baseline data for future studies. With the resurgence of emerging (Chikungunya) and re-emerging (West Nile fever, Dengue fever, malaria) diseases in and around Europe, knowledge of local mosquito species is essential for the identification and incrimination of mosquito vectors. The need for further extensive field surveys across the season, using the combined collections of the Mosquito Magnet Trap®, resting indoor and outdoor adult resting collections and immature samplings, combined with revisiting former collection localities of rare taxa and establishing new ones is apparent.

Chapter 7

General Discussion

7 General Discussion

7.1 Discussion

Mosquitoes in the UK have been studied intermittently since the early 20th century, driven primarily by the need to understand vector-host relationships and the dynamics of malaria transmission (Nutall, 1901; Lang, 1918, 1920). Following this early work, information on local mosquito fauna increased and detailed publications on life cycle and morphology (Edwards, 1932), oviposition and host selection (Service, 1969; 1971) and identification keys (Cranston *et al*., 1987; Snow, 1990) were made available. Unfortunately, since eradication of malaria from the UK after WWII (Dobson, 1989), there has been a paucity of mosquito studies, leading to a significant gap in knowledge of the current ecology and distribution of British mosquitoes. This in turn makes it difficult to monitor, even predict, the introduction of enzoonotic diseases such as Chikungunya and West Nile virus into the UK. The importance of knowing the species present in a given region as well as the ability of these species to transmit emerging/re-emerging diseases, created the need to reassess the vector potential of mosquitoes endemic to the UK. With this intention, the overall aim of this thesis was to document and characterise the ecology (adult and larval habitats and host preference) of British mosquitoes so as to facilitate the identification of potential vector species.

Out of the 33 species recorded in the UK, 13 were collected in this study. Taking together ecological studies conducted by Snow & Medlock (2008) and Hutchinson *et al.* (2007), the continued presence of 24 species is currently documented in southern England. Species that remain elusive include *Oc. communis*, *Oc. sticticus*, *Cx. territans*, *Cx. modestus*, *Culiseta longiareolata*, *Cs. litorea* and *Cs. alaskaensis* suggesting either their possible absence or localised presence in the UK (Cranston *et al*., 1987). The two commonly collected species, *An. daciae* and *Cx. pipiens*, both belong to complex groups (Maculipennis Group and Pipiens Group respectively) and the accurate identification of members in a complex species is particularly important for studies on vector competency. Out of the 11 recognised members of the Palaearctic Maculipennis Group, 3 are considered to be efficient vectors of malaria (Chapter 2, section 2.1.3), of which *An. atroparvus* is one.

The presence of *An. atroparvus* (Snow *et al.,* 1998) has been documented in the UK, together with *An. messeae* and *An. daciae* (Linton *et al.,* 2002a; Linton *et al.,* 2005). Yet prior to DNA sequencing of the nuclear ITS2 region (Linton *et al*., 2002a; Linton *et al*., 2005), which led to the development of the ITS2-PCR assay (Chapter 3, section 3.3.4), adult females of the Maculipennis Complex could not be reliably differentiated on morphology alone. In fact, recent publications have referred to both these taxa as "*An. messeae*" with the acknowledgement that *An. daciae* may well be included (Hutchinson *et al*., 2007; Snow & Medlock, 2008), or *An. maculipennis s.l.* The ITS2-PCR RFLP assay designed herein (Chapter 3) successfully distinguished all three species of the Maculipennis Group occurring in the UK. Despite only a 5-bp difference in the ITS2 region between *An. daciae* and *A. messeae*, the RFLP was able to accurately identify the two sibling species in all localities sampled in the UK. The most surprising result obtained from this assay was the prevalence of *An. daciae*, comprising 63.7% of the adult collections. *Anopheles daciae* was initially detected in 5 individuals collected Somerset in 2001 (Linton *et al*., 2005) and it was the latest addition to the British faunal list. Collections made in this study showed *An. daciae* to be present in not only in Somerset but in Norfolk, Kent, Suffolk and Anglesey as well. Interestingly, the presence of *An. daciae* in the UK corroborates the hypothesis of Edwards (1936), who suggested the presence of a third member of the Maculipennis Group in the UK. However it is more likely that the third member could have been *An. daciae* and not *An. maculipennis s.s.* as originally proposed.

While molecular markers such as ITS2 and COI are able to aid the differentiation of members of a species complex, this was not the case for the two forms of the Pipiens Complex studied here: *Culex pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*. Both forms, reported to occur in the UK, are morphologically and genetically indistinct with only differences in host and ecological preferences (Chapter 4, Section 4.1.2) to serve as a guide in discriminating between the two. Two assays, recently developed to differentiate the two forms, both failed here to consistently separate *Cx. pipiens* f. *pipiens* from *Cx. pipiens* f. *molestus* in the UK (Chapter 4, Section 4.4.3). In fact, the microsatellite assay (CQ11, Bahnck & Fonseca, 2006), detected *Cx. pipiens* f. *molestus* as well as hybrids of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* occurring aboveground (Chapter 4, Section 4.4.2), neither of which have been documented in the UK. Subsequent sequencing of a subset of the CQ11-identified individuals allowed for a comparison with the COI-RFLP assay of Shaikevich (2007); designed to differentiate both forms of the Pipiens Complex as well as *Cx. torrentium*. Only *Cx. pipiens* f.

pipiens and *Cx. torrentium* were detected with COI. Interestingly, 16 individuals identified by the CQ11 assay as either *Cx. pipiens* f. *molestus*, *Cx. pipiens* f. *pipiens* or hybrids were *Cx. torrentium* according to COI (Chapter 4, Figure 4.5). Dspite morphologically identifying all specimens prior to using both assays, the morphological misidentification of *Cx. torrentium* as *Cx. pipiens s.l.* resulted in the inaccurate detection of *Cx. pipiens* f. *molestus* and of hybrids, thus highlighting the unreliability of using the absence of pre-alar scales as a means to identify females of *Cx. pipiens s.l.* from *Cx. torrentium* (Section 4.5). In addition, the COI-RFLP assay had also identified *Cx. pipiens* f. *molestus* colony material as *Cx. pipiens* f. *pipiens* suggesting that either the colony was not *Cx. pipiens* f. *molestus* or that the polymorphisms on which the COI-RFLP, as well as the microsatellite assay, was designed cannot be used outside its geographical range. Further analysis of the mitochondrial marker showed a low variability between *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* (Section 4.4.3, Figure 4.5), suggesting that the two forms of *Culex pipiens* may not be taxonomically viable.

The stark difference observed in the analysis of the Maculipennis Group and *Cx. pipiens* complex, emphasizes the need for a multi-characteristic support on the taxonomic differentiation of species prior to the development of molecular assays (DeSalle *et al.*, 2005); for e.g. either by both morphology and genetic characteristics, or geographical and morphological characteristics. The differentiation of two putative forms of *Cx. pipiens* (*Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*) was supported primarily on the presence/absence of autogeny (ecology), purported differences in host selection (ecology) and morphology. In turn, independently designed molecular assays (genetic), using both microsatellite (CQII) and mitochondrial COI markers, alluded to the accurate identification of the two forms. Thus according to the proposition of DeSalle *et al.* (2005), there were three characteristics supporting the two forms of *Cx. pipiens*. Inconsistent results produced by both assays (Chapter 4, section 4.4.3) coupled with the indistinct morphological characteristics (Harbach *et al.,* 1985) and variability of expressed autogeny in other mosquito species suggested, instead, that *Cx. pipiens* was a single species (Chapter 4, section 4.5). On the other hand, *An. daciae*, a recently recorded species of the Maculipennis Group in the UK, was first discriminated from *An. messeae* based on egg morphology as well as fixed polymorphisms on both nuclear ITS2 and COI markers (Nicolescu *et al*., 2004). The PCR-ITS2 assay developed in this study consistently and accurately differentiated *An. daciae* and *An. messeae* across their range including the UK, (Chapter 3, section 3.4.2), Romania, as well as in Poland and

Bulgaria (Linton, unpublished). Thus the presence of both morphological and genetic characteristics supports the species of the Maculipennis Group in the UK.

The final aspect of this study dealt with the host selection of field-caught bloodfed mosquitoes. The selection of hosts by a mosquito is an important determinant of the potential role played by the mosquito species in disease transmission. Up until now, no current data were available on the host selection of British mosquitoes, and therefore it is difficult to identify potential vectors and potential bridge vectors in the UK. Using individually designed primers for a fragment of mitochondrial CytB, mosquito bloodmeals were screened for six vertebrate hosts: Bird, Dog, Deer, Goat, Man and Horse.

The most interesting result here was the indiscriminate feeding behaviour observed in *An. daciae*. Its selection of non-endemic species such as the Reindeer and Darwin's Rhea (both found in Pettits Animal Farm, Norfolk) and selection of more than one host in the same reproductive cycle is suggestive of *An. daciae* as a potential bridge vector. Having been found to feed on both birds and man opens the possibility for it to be a transmitter of Sindbis or even WNv, as antibodies for both have been detected in chicks in the UK (Buckley *et al.,* 2003). The occurrence of *An. daciae* in Norfolk and Kent (chapter 3) and its observed selection of man (chapter 5), combined with the historical incidence of malaria in these counties (chapter 3, section 3.1) could imply its potential to transmit human diseases such as malaria. Aside from *An. daciae*, *An. atroparvus*, *An. messeae* were also found to have fed on more than one host in the same reproductive cycle. However, in comparison to the diverse selection of hosts by *An. daciae*, both *An. atroparvus* and *An. messeae*, seemed to be zoophilic in nature feeding mainly on deer. Interestingly, *An. atroparvus* has been implicated in malaria transmission in the UK, however it is highly likely, given its zoophilic preference shown in this study, that its purported anthropophily could have been due to either a shift in the indoor resting place or the high presence of human hosts. Nevertheless, based on previous reports for *An. atroparvus* and current host data for *An. daciae* both species could serve as vectors of human borne arboviruses in the UK.

Interestingly, *Cx. pipiens*, in its current synonymy (chapter 4 Section 4.5), was found to have fed on bird and man. Although this result was obtained from two different individuals in two localities, it shows the potential of *Cx. pipiens* to feed on both hosts. *Culex pipiens* is a known vector of WNv in Europe and North America (Chapter 3 section 3.1.4). Higgs *et al.*

(2004) and Medlock *et al*. (2005) have discussed the potential role that *Cx. pipiens* and other species could play as vectors in the UK. The identification of hosts, particularly avian and human hosts, from bloodfed *Cx. pipiens* reported in this thesis (Chapter 4) provides molecular evidence for this hypothesis. *Culex torrentium*, on the other hand, is reported to be an efficient vector of arboviral disease such as Sindbis (Lundström, 1999), although it is not considered a vector in the UK. Nonetheless its reported and observed (Chapter 5, Figure 5.2 single *Cx. torrentium* specimen fed on a chaffinch) affinity to birds could indicate a role in the low-level transmission of Sindbis observed in UK chicks (Buckley *et al.,* 2003). In the event of the virus establishing itself in Britain, *Cx. torrentium* would then serve as a potential vector of the arbovirus.

However, it must be said that hosts identified from bloodfed females collected in this study is not definitive; females could be opportunistic or selective in the choice of hosts based on the prevalence and variety of host species present in a given area (Constantini *et al.,* 1998). This was demonstrated by the diverse feeding selection of *An. daciae* and the converse for both *An. messeae* and *An. atroparvus* specimens sampled in Pettits Animal Farm Norfolk as well as the feeding preference of *Cx. pipiens*. Thus the ecological data presented here provides a starting point for the incrimination of vectors in the UK, the accurate identification of species and identification hosts including multiple hosts within a bloodmeal. The isolation of a pathogen in the mosquito and host is paramount to the determination and incrimination of vectors.

7.2 Future work

While the data gathered in this study represent a snap shot approach and is by no means exhaustive, this current understanding (prior to an outbreak) provides a much-needed baseline upon which future outbreaks of animal and human diseases could be predicted (Spielman, 1994). Transmission of disease is dependent on several factors: presence of vectors and hosts, climatic conditions and the herd immunity of a population. Based on this and the host selection study presented in this thesis, the introduction of enzoonotic diseases into the UK is tangible. The geographic position of the British Isles, on the fringes of Europe and on the northerly migration route from Africa to Greenland, puts it at risk to the potential introduction of West Nile virus, Sindbis, malaria and Chikungunya via commercial travel (Whitfield *et al.*, 1984; Curtis & White, 1984; Rezza *et al.*, 2007), migratory patterns (Mackenzie *et al.*, 2004), animal movement (Purse *et al*., 2005; Mellor, 2008) as well as

climate change. Global temperatures are expected to increase by at least 6°C by the end of the 21st century (Meteorological Office UK, 2008). This predicted effect of global warming on disease incidence is reported to cause a concomitant increase in humidity and altered rainfall that could be conducive for the development of both vectors and pathogens outside its natural range (Khasnis *et al.*, 2005; Haines *et al.,* 2006).

For example, *Aedes albopictus* (*Stegomyia albopicta*) also known as the 'Asian Tiger mosquito' is considered to be a highly invasive species in Europe (Gratz, 2004). Indigenous to Southeast Asia (Medlock *et al.*, 2006), it is quickly establishing itself in many parts of Europe such as Spain (Erijta *et al.*, 2005), The Netherlands (Takumi *et al.*, 2008) and Italy (Romi *et al.*, 2001, reviewed in Gratz, 2004). The ability of the eggs to withstand cold temperatures up to -10°C, makes it very adaptive to temperate climates and thus capable of establishing itself in the UK, should it be introduced (Medlock *et al.*, 2006). Also an increase in environmental temperatures can be conducive for the transmission of malaria in the UK. Considering the annual introduction of imported cases of *P. falciparum* malaria (1,548 cases in 2007) into the UK (DEFRA, 2008), the presence of known vectors *An. atroparvus, An. plumbeus*, which have been shown to be susceptible to *P. falciparum* infections and potential (*An. daciae*) vectors does allow for this possible occurrence.

However, the role of British mosquitoes as vectors should be further established through a multi-factorial approach involving local zoos and farms. As demonstrated in Chapter 5 and by Hutchinson *et al.* (2007), the Mosquito Magnet Trap ® is a useful tool in monitoring mosquito species in a particular area over a period of time, especially as it was shown to attract species not collected by other means in this study (*Oc. detritus* & *Oc. leucomelas,* Chapter 5, section 5.6.2.5). This use of the Mosquito Magnet® in areas such as animal farms [Pettits Animal Farm (Norfolk), Bird World (Isle of Anglesey), Miniature Pony Centre (Devon)] or in domestic farms [such as a Eastlake horse farm (Devon), Cronllech Manor (Anglesey) and Godney Farm (Somerset)], as well as in local zoos [for e.g. Paignton and Jersey Zoos involved in the conservation of non-endemic animals such as Grey Duikers and Madagascan Teal respectively, through the use of studbooks (BIAZA, 2009)] could prove useful in determining interaction of mosquitoes and potential hosts and transmission cycles. These smaller eco-habitats will then facilitate the identification of hosts, using the primers developed in Chapter 4 (Section 4.3.3.2) within the vector blood meal and establish the presence of both viral and parasitic infections in both that host and the vector. This would thus

efficiently determine the species of mosquitoes involved in active transmission of arbo-pathogens. It would also tie in with the current effort by the National Expert Panel on New and Emerging Infections (NEPNEI) (Department of Health, 2008) to identify and assess the threat of potential infectious diseases such as malaria, WNv through vector and host surveillance.

All data generated in this study have been submitted to MosquitoMap (www.mosquitomap.org). This freely accessible site stores individual collection records of mosquitoes, complete with georeferenced locality data, method of identification, details of collectors, identifiers, voucher specimen housing and additional fields for detailed ecological and parasite infection data. This database serves to produce risk maps of malaria worldwide using the mosquito species knowledge, environment and human population size using its inbuilt malaria risk assessment software MAL-AREA. This ensures that all data generated in this study are widely accessible and provides a permanent legacy for this research.

Whilst the incidence and threat of introduction of these diseases in the UK are thought to be low, knowledge of local mosquito fauna and vector host interactions should be ascertained and continued surveillance of mosquitoes carried out. In addition, the impact of factors, such as bird migration and climate change on disease transmission in the UK as well as the immunity of the population to mosquito-borne infections, needs to be analysed as interdependent factors. The studies carried out in this thesis contribute towards the current understanding of these ecological processes through the establishment of working laboratory protocols and an ecological database. However, the interpretation of collected ecological results should be done conservatively; as the old medical adage goes “Common things occur commonly, uncommon things don’t. If you hear the sound of hoofbeats think horses, not zebras.”

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