Investigating the influence of hybridisation and larval habitat on the expression of life history traits of *Culex pipiens* in the United Kingdom

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Declaration

I, Laura Ann Jones, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Culex pipiens (Linneaus, 1758) are important arboviral disease vectors consisting of two morphologically indistinguishable forms, *pipiens* (L.) and *molestus* (Forskål, 1775), which differ in behaviours such as host preference and expression of autogeny. Hybrid populations are hypothesised to possess intermediate behaviours that are key drivers of disease transmission. In this thesis, the influence of hybridisation and larval habitat on the expression of phenotypic traits by *Cx. pipiens* were assessed through a combination of field collections and laboratory experiments with colonised mosquitoes.

Extraction of trace DNA from individual pupal exuviae was developed to permit accurate biotype identification of live colony and field *Cx. pipiens*, achieving 100% PCR amplification success within 12-hours of eclosion, facilitating creation of single-biotype colonies for use in subsequent experiments. Single-pair and grouped mating experiments revealed that mating success, larval development time and larval survival varied between homologous and reciprocal crosses of colony *Cx. pipiens* forms, which could influence hybridisation capability under natural conditions. Phenotypic plasticity of *Cx. pipiens* was also demonstrated from six field populations and under varying larval nutritional availability.

Characterisation using CQ11 identified that field-derived aboveground populations are dominated by the *pipiens* form (98.8%) whilst occurrence of *molestus* (0.4%) and hybrids were low (0.8%). Full microsatellite analysis revealed differentiation between UK mosquitoes and autogenous *molestus* samples from Sweden, whilst no discernible grouping according to CQ11 could be determined within UK samples.

Overall, whilst colony and field *Cx. pipiens* demonstrated physiological traits capable of facilitating virus transmission, such as a lack of autogeny and production of viable hybrid progeny, barriers to hybridisation were also identified. Further, low levels of natural hybrid populations suggest biotypes are largely isolated under current conditions. Finally, microsatellite analysis of field and colony individuals questions the reliability of single molecular markers for accurate biotype delineation, demonstrating these are not indicative of phenotype.

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List of Abbreviations

Akaike information criterion	AIC
Base pairs	bp
Biological oxygen demand	BOD_5
Bovine serum albumin	BSA
Centimetre	cm
Cetyltrimethylammonium bromide	CTAB
Cytochrome c oxidase 1	COI
Cytoplasmic incompatibilities	CI
Dissolved oxygen	DO
Deoxyribonucleic acid	DNA
Deoxyribonucleotide triphosphate	dNTPs
Dodecyltrimethylammonium bromide	DTAB
Environmental DNA	eDNA
Ethylenediaminetetraacetic acid	EDTA
Fixation index	F_{ST}
Generalise linear mixed model	GLMM
Grams	g
Litre	L
Magnesium chloride	$MgCl_2$
Male accessory gland	MAG
Microlitre	µl
Microgram	µg
Micromolar	µM
Milligrams	mg
Millilitre	ml
Millimetre	mm
Millimolar	mM
Molar	M
Nanogram	ng
Nucleotide	nt
Ounce	oz
Phosphate buffered saline	PBS
Polymerase chain reaction	PCR
Potential of Hydrogen	pH
Quantitative cycle values	C_q
Relative centrifugal force	rcf
Relative humidity	rH
Revolutions per minute	rpm
Sodium dodecyl sulphate	SDS
Trizma hydrochloride	Tris-HCL
Ultraviolet B radiation	UVB
United Kingdom	UK
Urban heat island	UHI
Usutu virus	USUV
West Nile virus	WNV

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Chapter 1 – Literature review

1. Introduction

1.1 Medical and veterinary importance of mosquitoes

Vector-borne diseases have a global impact on human and animal health. The recent emergence of exotic and re-emergence of previously recorded mosquito-borne arboviruses in Europe has greatly increased their profile. Noteworthy examples include recent incursions of chikungunya virus (CHIKV) in Italy (Angelini *et al.*, 2007) and France (Roiz *et al.*, 2015), as well as autochthonous transmission of dengue fever virus (DENV) in France (Succo *et al.*, 2016) and a widespread outbreak of West Nile virus (WNV) in horses in the Camargue region (Bahuon *et al.*, 2016). Invasive species of mosquitoes have played a key role in the transmission of some of these arboviruses (particularly *Aedes albopictus* (Skuse, 1895) in the case of CHIKV in Italy); others, however, are primarily transmitted by indigenous mosquito populations. Currently, ten arboviruses of medical and veterinary importance, thought to be primarily transmitted by mosquitoes, are circulating in Europe (Table 1.1) (Brugman *et al.*, 2018). A variety of factors govern the ability of a species or population to act as a biological vector of disease including longevity, biting rate, host preference and vector competence which together define the vectorial capacity of a population.

1.2 Mosquitoes and arboviruses in the United Kingdom

Thirty six mosquito species comprising eight genera have been identified in the UK: *Aedes* (4 species), *Anopheles* (6), *Coquillettidia* (1), *Culex* (4), *Culiseta* (7), *Dahliana* (1), *Ochlerotatus* (12) and *Orthopodomyia* (1) (Medlock and Snow, 2008; Medlock and Vaux, 2009; Harbach *et al.*, 2017). While data regarding the distribution and abundance of mosquitoes across different environmental settings are relatively sparse, efforts are being made to improve this through sustained surveillance by Public Health England and dedicated studies of specific environments such as farms and nature reserves (Golding *et al.*, 2012; Golding *et al.*, 2015; Brugman *et al.*, 2017a; Brugman *et al.*, 2017b). These efforts reveal a relatively patchy distribution for most species driven primarily by larval habitat availability and host preference.

Historically, the UK has seldomly experienced outbreaks of mosquito-borne disease transmission of importance to human health. Major outbreaks of both endemic and epidemic malaria occurred until the 1950s (Reiter, 2000; Kuhn *et al.*, 2003), whilst local establishment of *Aedes* facilitated transmission of yellow fever virus in 1865 (Meers, 1986). Furthermore, studies have identified antibodies against

Table 1.1. Mosquito-borne arboviruses currently circulating in Europe, adapted from Brugman *et al.* (2018). Arboviruses primarily transmitted by *Culex pipiens* species are underlined**.**

Virus family	Virus	Vector species	Primary host species
Bunyaviridae	Batai virus	*Anopheles claviger* (Meigen, 1804)*, Anopheles maculipennis* s.l. (Meigen, 1818), *Coquillettidia richiardii* (Ficalbi, 1889)	Pigs, ruminants, horses, and wild birds. Humans can develop influenza-like illness
	Inkoo virus	*Aedes communis* (De Greer, 1776)	Mountain hares. Humans can develop influenza-like illness
	Lednice virus	*Culex modestus* (Ficalbi, 1889)	Birds
	Snowshoe hare virus	*Aedes cinereus* (Meigen, 1818)*, Aedes vexans* (Meigen, 1830)*, Aedes communis, Aedes punctor* (Kirby, 1837)*, Aedes cataphylla* (Dyar, 1916)*, Culiseta inornate* (Williston, 1893)*, Culiseta impatiens* (Waler 1848)	Snowshoe hare, voles, lemmings Non-fatal encephalitis in horses and occasional neurological disease in humans
	Tahyna virus	*Aedes* vexans	Brown hares, hedgehogs, rodents Influenza-like illness in humans with infrequent neurological disease in humans
Flaviviridae	Dengue virus	*Aedes aegypti, Aedes albopictus*	Humans
	Usutu virus	*Culex* spp. including *Culex pipiens*	Birds Humans can be incidental dead-end hosts
	West Nile virus	*Culex pipiens, Culex modestus, Coquillettidia richiardii*	Wild birds Mammals including humans and horses can be incidental dead-end hosts
Togaviridae	Chikungunya virus	*Aedes aegypti, Aedes albopictus*	Humans as primary reservoirs during epidemics. Non-human reservoirs include monkeys, rodents and birds
	Sindbis virus	*Culex pipiens, Culex torrentium, Culiseta morsitans* (Theobald, 1901)*, Coquillettidia richiardii, Aedes communis, Aedes excrucians* (Walker, 1856)*, Aedes cinereus and Anopheles hyrcanus* s.l (pallas, 1771)	Passeriformes Fever, malaise and rare circumstances chronic arthritis in humans

Tahyna virus in rodents (Chastel *et al.*, 1985) along with serological evidence of WNV in wild (Buckley *et al.*, 2003) and sentinel birds (Buckley *et al.*, 2006). Until recently, surveillance of dead avian hosts collected since 2001 has failed to identify circulation of mosquito-borne arboviruses in the avian population (Medlock *et al.*, 2007; Phipps *et al.*, 2008; Brugman *et al.*, 2013; Horton *et al.*, 2013). In August 2020, five Eurasian blackbirds (*Turdus merula*) and one house sparrow (*Passer domesticus*) were submitted to a national surveillance programme aimed at monitoring wildlife diseases within Great Britain with a heavy focus on garden birds (Lawson *et al.*, 2015). Deceased animals were all submitted from a single location in Greater London with individuals showing signs of disease, including dehydration and emaciation prior to death (Folly *et al.*, 2020). Successful isolation of Usutu virus (USUV) was performed on all samples with sequence analysis revealing 100% similarity to USUV African 3.2 lineage, previously isolated from blackbirds in the Netherlands (Cadar *et al.*, 2017). Due to the time between the sample submissions, autochthonous transmission was postulated to have occurred within the local passerine population, most likely vectored by indigenous mosquito species (Folly *et al.*, 2020). Subsequent vector monitoring at the index site identified USUV RNA in local *Culex pipiens* (Linnaeus, 1758) populations (Lawson *et al.*, 2022). Infections of humans with USUV is rare and is predominantly asymptomatic, yet in extreme cases it can result in neurological disease (Pecorari *et al.*, 2009). In response to this finding, the public health risk assessment for USUV in the UK was updated and the risk level increased accordingly by the UK government's Human Animal Infections and Risk surveillance group (HAIRS) (Human Animal Infections and Risk Surveillance Group, 2020).

The emergence and rapid dissemination of WNV across North America in 1999 (Lanciotti *et al.*, 1999) and the emergence and re-emergence of arboviruses in Europe have led to an increasing emphasis on risk assessment of incursion and transmission in the UK (Gould *et al.*, 2006; Gale *et al.*, 2015; Medlock and Leach, 2015). Of the UK mosquito fauna, at least nine species have been implicated in WNV transmission elsewhere (Medlock *et al.*, 2005; Medlock *et al.*, 2007). *Culex pipiens* f. *molestus* (Forskål, 1775) and *Culex modestus* (Ficalbi, 1890) are considered the principal vectors responsible for arbovirus spill over from bird to human populations in Europe and are considered 'bridging vectors' (Balenghien *et al.*, 2008; Munoz *et al.*, 2012; Vaux *et al.*, 2015). A series of reviews have examined the likelihood of arbovirus transmission by the endemic UK mosquito fauna. These have prioritised species by those involved in arbovirus transmission in other countries, their ecology and behaviour (including host preference) and abundance in the UK (Higgs *et al.*, 2004; Medlock *et al.*, 2005; Gould *et al.*, 2006; Medlock *et al.*, 2007; Medlock and Leach, 2015). They have not included explicit estimates of vectorial capacity, primarily due to a lack of species-specific data for UK populations.

Among UK mosquito species, the current evidence for *Cx. pipiens* acting as a potential arbovirus vector is stronger than for any other species. This is due to its proven role as a bridge vector between avian and mammalian hosts (Medlock *et al.*, 2005), known competence for infection with a range of arboviruses (Fortuna *et al.*, 2015; Fros *et al.*, 2015; Vogels *et al.*, 2017a; Lumley *et al.*, 2018) and ability to overwinter in the adult form which is important in sustaining transmission in temperate regions (Spielman and Wong, 1973). *Culex pipiens* is also closely associated with human habitation through exploitation of containers and small water bodies for larval habitat and can persist in urban areas of high human population density (Townroe and Callaghan, 2014). Specifically, *Cx. pipiens* has been shown to be a competent vector of USUV (Fros *et al.*, 2015; Hernandez-Triana *et al.*, 2018), thus, understanding the ecology and behaviour of *Cx. pipiens* forms in the UK and their ability to act as bridging vectors is important for determining the risk posed to human health from the diseases that they can transmit.

1.3 The *Culex pipiens* complex

Culex pipiens was first described in 1758 and has subsequently been recognised as a global species complex comprising *Cx. pipiens, Culex quinquefasciatus* (Say, 1823), *Culex australicus* (Dobrotworsky and Drummond, 1953) and *Culex globocoxitus* (Dobrotworsky, 1953) (Harbach, 2012). Within the complex, *Cx. pipiens* and *Cx. quinquefasciatus* are distributed across temperate and tropical/sub-tropical regions respectively (Figure 1.1), while the distribution of *Cx. australicus* and *Cx. globocoxitus* are restricted to Australia. Additionally, the *pipiens* complex includes a subspecies, *Culex pipiens pallens*, distributed in Japan and the far east of Eurasia, plus two physiological forms of *Cx. pipiens*. The taxonomy of the *Cx. pipiens* complex remains uncertain due to morphological similarity of its members, despite apparent disparity between their ecological, physiological, and behavioural traits (Fonseca *et al.*, 2004). These traits influence the vectorial capacity of populations worldwide and play a major part in determining their role in the transmission of pathogens.

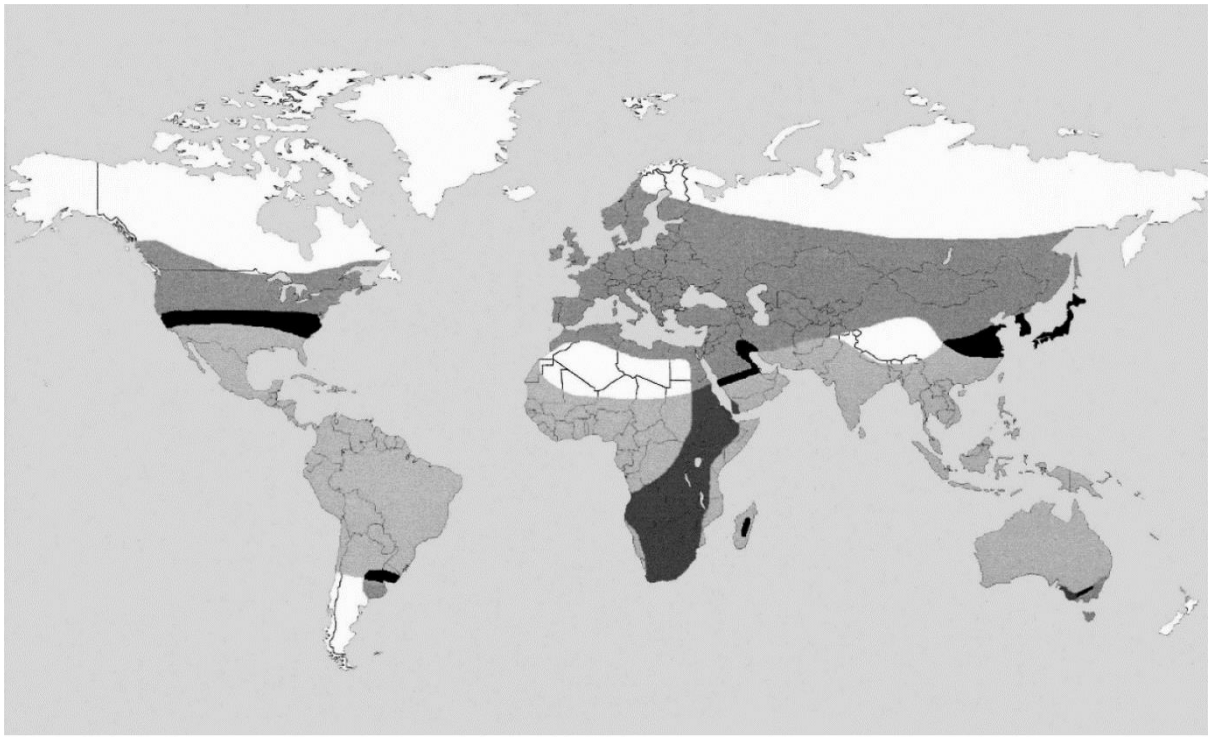

Figure 1.1. Shaded areas indicate known distributions of *Culex quinquefasciatus* (light grey), *Culex pipiens* (medium grey), areas of overlapping distributions (dark grey) and zones of overlap and introgression (black). White areas indicate an absence of data for *Culex pipiens* distribution. Source: Harbach (2012)

Two morphologically indistinguishable forms of the *Cx. pipiens* species have been identified, *pipiens* (L.) and *molestus*. The taxonomic status of these different forms of *Cx. pipiens* are still unresolved, with current literature utilising multiple terms to reference them including biotypes and ecoforms. Herein, the term 'biotype' is used when referencing the *Cx. pipiens* forms.

To date, studies have failed to detect any constant morphological features able to accurately differentiate the biotypes. In 1890, Ficalbi first noted differences between the biotypes (Harbach *et al.*, 1984), observing that adults of the *molestus* form were lighter in colouration and lacked prominent pale spots at the apices of the femora and hind-tibia (for morphological reference see Figure 1.2). Moreover, in 1896, differences in ratios of the length of the maxillary palpi to the proboscis were noted (Christophers, 1951). Following this, numerous authors reported additional egg (Christophers, 1945), larval (Marshall and Staley, 1935b) and adult (Marshall and Staley, 1937; Jobling, 1938) characters believed to be diagnostic for the differentiation of the *Cx. pipiens* forms. Some authors suggested that the shape of the basal pale bands of the abdominal terga of the *pipiens* form differed from that of *molestus* (Marshall, 1938; Marshall, 1944) whilst other studies have reported that the tergal bands are of no diagnostic value (Jobling, 1938).

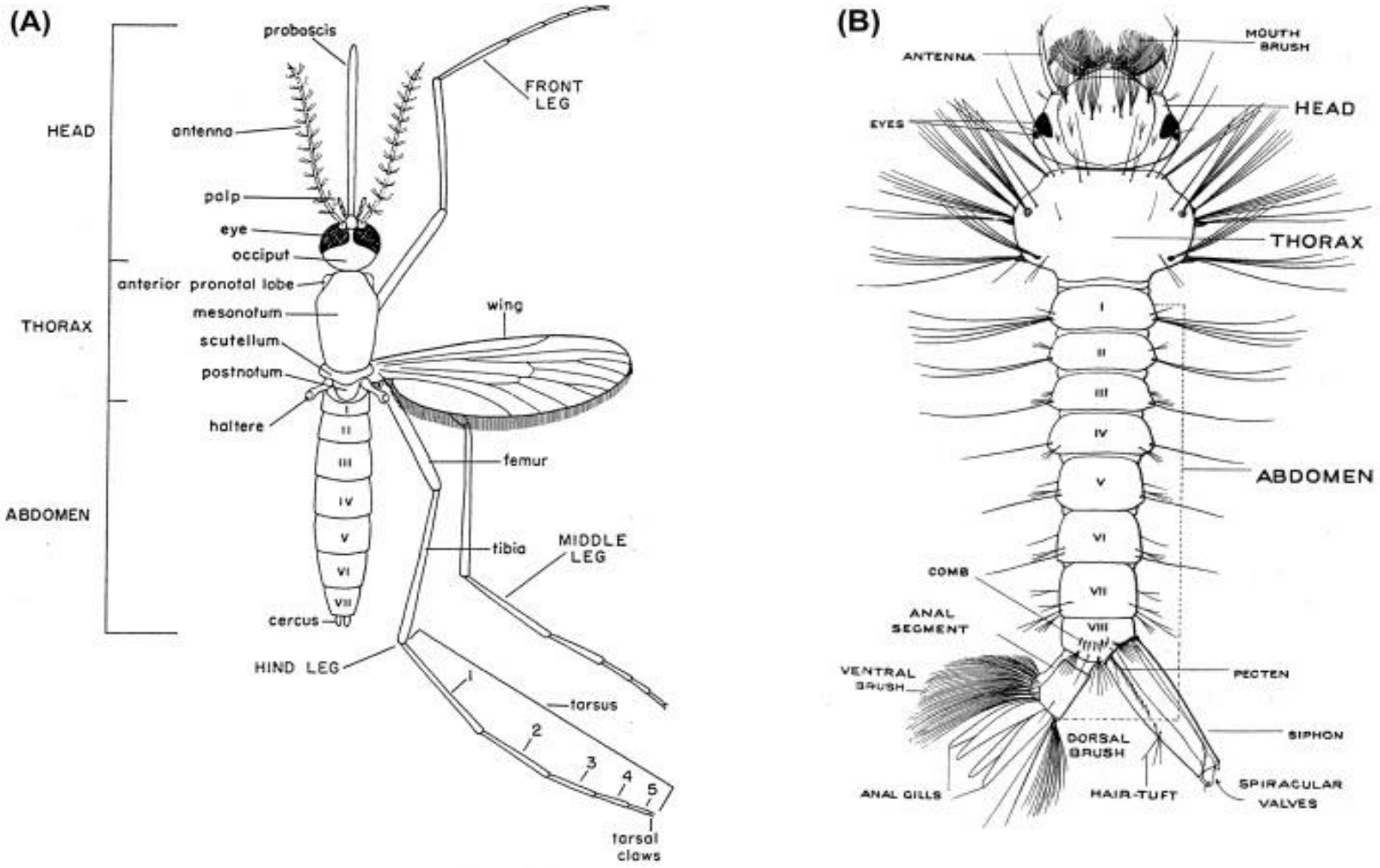

Figure 1.2. Dorsal view of the generalised external anatomy of an adult mosquito (A); Dorsal view of external anatomy of Culicine mosquito larvae (B) Source: Foster and Walker (2019)

Variation in the number of setae on the nine tergum have also been described, yet these differences are too inconsistent to be utilised as a diagnostic tool (Jobling, 1938). Further studies found that geographic and ecological variation also influenced morphology of the forms, while the discovery of natural hybrid populations with intermediate features of the two parental forms added further complexity to the matter (Vinogradova, 2003).

With a lack of morphologically distinguishing features, identification of the *Cx. pipiens* forms has relied on the expression of distinct phenotypic characteristics. Ecological and physiological differentiation of the *Cx. pipiens* biotypes is well established and widely implemented with traits related to each form thought to confer a selective advantage for their respective ecological niches (Brugman *et al.*, 2018). The *pipiens* biotype is typically anautogenous (requiring a bloodmeal for egg development), ornithophilic (feeding primarily on birds), eurygamous (mating in flight) and heterodynamic (entering a winter diapause) (Harbach *et al.*, 1985). In contrast, the *molestus* biotype has been reported to be autogenous (can produce an initial egg batch without the need for a bloodmeal), mammophilic (preferentially feeding on mammals), stenogamous (can mate in confined spaces) and homodynamic (active all year round) (Harbach *et al.*, 1984).

Vector competence is the ability of a vector to acquire, maintain and transmit a pathogen (Kenny and Brault, 2004) and is one of many factors which combined, determines the vectorial capacity of a vector species. The differential traits displayed by the *Cx. pipiens* biotypes, as well as their hybrids, influences their ability to transmit disease. As an example, the ability of the *molestus* form to produce an initial egg batch without a bloodmeal is considered an adaptive trait facilitating their survival in underground environments where bloodmeal host availability may be limited. However, the expression of autogeny can reduce the vectorial capacity of the *molestus* form, especially in populations with obligatory autogeny. This delays the age at which adult females are able to acquire an infectious bloodmeal (Kassim *et al.*, 2012b), thus reducing the chances of onwards transmission. Moreover, the preferential feeding on avian hosts by the *pipiens* form coupled with their high abundance during summer, close habitat association with host species, and proven vector competence makes, *Cx. pipiens* f. *pipiens* an important contributor to WNV transmission, facilitating amplification of the disease within the avian reservoir.

However, plasticity of these traits within the two forms, such as the identification of autogenous egg production in the *pipiens* biotype (Gomes *et al.*, 2009; Beji *et al.*, 2017), has led to uncertainty when using the presence or absence of phenotypic traits to accurately differentiate the biotypes. As such, several molecular techniques have been developed for their delineation. Subsequent differentiation methodologies have largely focused on either PCR or sequencing of target genes with many relying on

a single locus to distinguish between the two forms. One of these assays was designed to amplify a single nucleotide substitution from G to A at the 3rd position of the 68th codon of the cytochrome c oxidase subunit I (COI) gene (Shaikevich, 2007). Although this assay appears to be diagnostic in some populations, such as in Russia (Shaikevich, 2007; Vinogradova and Shaikevich, 2007), many studies have been unable to replicate this in native populations including the UK (Danabalan *et al.*, 2012; Manley *et al.*, 2015).

Of late, biotype delineation has focused on the amplification of the CQ11 microsatellite locus (Bahnck and Fonseca, 2006), usually preceded by a multiplex PCR to differentiate between *Cx. pipiens* and its sibling species *Culex torrentium* (Martini 1925) where their ranges overlap (Smith and Fonseca, 2004). For the CQ11 assay primers are designed to exploit polymorphisms between the two forms within the flanking region of the CQ11 microsatellite locus which produces differentially sized amplicons for the two forms, a difference that can be observed following gel electrophoresis. As morphological differentiation between *Cx. pipiens* and *Cx. torrentium* either relies on differences in structure of the male genitalia or the infrequent presence of preallar scales in the female *Cx. torrentium* mosquitoes, distinction by PCR is usually favoured. This is conducted through the amplification of the acetylcholinesterase 2 (ACE-2) gene which is used to assign members of the *Culex* family to species level by exploiting polymorphisms in the nuclear intron between the species, producing PCR products of differing sizes (Smith and Fonseca, 2004). Failing to differentiate these species prior to biotype identification by CQ11 can result in cross-amplification of primers with *Cx. torrentium* DNA, resulting in overestimation of the presence of hybrid forms (Danabalan *et al.*, 2012). Adaptation of the CQ11 assay to a probe-based detection real-time PCR has also been performed to incorporate the ACE-2 assay for simultaneous assignment to species and biotype level whilst also improving sensitivity and streamlining *Cx. pipiens* identification (Rudolf *et al.*, 2013).

Due to the variability in some assays many authors have cautioned against the use of a single marker to differentiate the biotypes and instead advocate a multilocus approach. A recent paper assessing the reliability of biotype identification in the UK (Danabalan *et al.*, 2012) reported disparity between results from the CQ11 and COI assays, a finding that has been noted by other research groups (Di Luca *et al.*, 2016). Additionally, full microsatellite analysis using multiple markers, has demonstrated the underestimation of hybrid specimens under continued conditions of hybridisation and backcrossing when compared with results detected when using a single genetic marker, for example when using the CQ11 microsatellite locus in isolation (Gomes *et al.*, 2009). Despite this, of the existing molecular markers (Smith *et al.*, 2005a; Bahnck and Fonseca, 2006; Shaikevich and Zakharov, 2010), the CQ11 is

considered the most accurate locus for the differentiation of the *Cx. pipiens* biotypes (Gomes *et al.*, 2009) and is the most widely implemented.

More recently, a new method of differentiating the forms by using high resolution melting (HRM) analysis was suggested to detect single nucleotide polymorphisms (SNPs), genetic variation at a single position within the genetic sequence, in the ACE-2 gene (Kang and Sim, 2013). A further two target genes (Kim *et al.*, 2018a), CPIJ005487 (tublin gamma 1 chain) and CPIJ001674 (exocyst complex component 2), were proposed to contain SNPs which are diagnostic for *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* for mosquitoes derived from America. However, a study in Sweden failed to replicate the distinction between the *Cx. pipiens* forms when testing CPIJ001674 (Luande *et al.*, 2020).

1.4 Origin of the *Culex pipiens* forms

There have been two alternative hypotheses proposed to explain the ecological and physiological disparities between *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*. The first hypothesis suggests that differences arose because of repeated and independent adaptations to underground environments causing a rapid shift in physiological and behavioural traits (Byrne and Nichols, 1999). Under this scenario, *Cx. pipiens* f. *molestus* is considered an eco-physiological variant of *Cx. pipiens* f. *pipiens*, where populations of *Cx. pipiens* f. *molestus* would be most closely related to local *pipiens* forms.

Early behavioural and morphological observations suggested that North American *molestus* were more similar to local *pipiens* populations compared with European *molestus* (Richards, 1941). This hypothesis was formulated from observations of morphological differences in the respiratory siphon between autogenous strains identified in North America compared with England as well as the propensity for anautogenous North American strains for human blood (Richards, 1941). In agreement with this, genetic analysis employing microsatellites and restriction fragment length polymorphisms, concluded that North American *molestus* samples from New York and Chicago were each more genetically similar to local *pipiens* populations than they were either to each other or to their European counterparts (Shaikevich, 2007; Kothera *et al.*, 2010; Kothera *et al.*, 2012). Finally, allozyme analysis of specimens collected from the London Underground hypothesised that local *pipiens* species were the source of *molestus* populations found within the underground system (Byrne and Nichols, 1999).

An alternative hypothesis suggests that *Cx. pipiens* f. *molestus* is a separate entity to *Cx. pipiens* f. *pipiens*, which is hypothesised to have originated in Egypt (Fonseca *et al.*, 2004). This hypothesis proposes that physiological and ecological strategies employed by the *molestus* biotype could have arisen through exposure to warmer climates. These evolutionary strategies proved beneficial to

survival in underground environments, such as tunnels and basements, allowing the geographic spread of *Cx. pipiens* f. *molestus*. This scenario suggests that *Cx. pipiens* f. *molestus* arose from a single speciation event and is therefore considered as a separate evolutionary entity. Studies utilising microsatellite analysis have demonstrated that *molestus* specimens from Europe, America, and Jordan are genetically closer to one another than to local *pipiens* populations (Bahnck and Fonseca, 2006; Strickman and Fonseca, 2012), alluding to a common origin for these *molestus* samples. Moreover, recent assessment of genomic structure and differentiation of *Cx. pipiens* from different continents using whole genome analysis supported the hypothesis that *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* represent separate evolutionary units with monophyletic origin (Yurchenko *et al.*, 2020).

1.5 Distribution and hybridisation of the *Culex pipiens* forms

The *Cx. pipiens* species has a near ubiquitous distribution across its range. Despite this, broadening of our understanding of local and regional distributions of the biotypes has only recently been achieved, aided by the increased application of molecular methods for species delineation. However, understanding of the relationship between biotype genetics and their expressed phenotypes is still lacking (Manley *et al.*, 2015).

Studies have previously shown clear separation between the biotypes based on their ecological characteristics, occupying distinct habitats (Becker *et al.*, 1999; Byrne and Nichols, 1999; Vinogradova and Shaikevich, 2007; Huang *et al.*, 2008). Initial evidence indicated the *pipiens* form was predominantly associated with natural and artificial aboveground habitats across urban and rural areas, whilst the *molestus* form was confined to urban underground environments (Vinogradova, 2000). This habitat separation was believed to serve as the primary barrier to hybridisation between the forms, particularly in northern Europe (Byrne and Nichols, 1999; Fonseca *et al.*, 2004). More recently, a greater plasticity of *Cx. pipiens* biotypes to a wide range of ecological conditions was demonstrated in America (Fonseca *et al.*, 2004), Algeria (Benallal *et al.*, 2015; Korba *et al.*, 2016), Morocco (Amraoui *et al.*, 2012), Tunisia (Beji *et al.*, 2017), and several European countries including Italy (Di Luca *et al.*, 2016), Portugal (Gomes *et al.*, 2009; Osorio *et al.*, 2012) and the Netherlands (Reusken *et al.*, 2010) with co-occurrence of both biotypes and hybrids in different breeding sites reported.

Natural hybridisation between the two biotypes has been reported from several European countries with hybridisation rates up to 25.7% from aboveground habitats in Spain (Bravo-Barriga *et al.*, 2017). In America, genetic analysis revealed that over 40% of specimens were of hybrid ancestry (Fonseca, 2004). Additionally, both hybrid and non-hybrid forms were successfully reared under laboratory

conditions and in at least one case sustained all three forms in a single line (Manley *et al.*, 2015). Geographic disparity in relative abundance of each of the forms and their hybridisation rates were previously reported, with the proportion of *pipiens* form relative to *molestus* decreasing from northern to southern latitudes (Vogels *et al.*, 2016). Hybridisation is hypothesised to modify host preference, the rate of autogeny in a population, the capacity to forgo diapause as well as vector competence which can all have downstream effects on virus transmission dynamics (Vogels *et al.*, 2017b). Genetic analysis of *Cx. pipiens* populations from Portugal demonstrated that gene flow between the forms predominantly occurred from the *molestus* to *pipiens* biotype (Gomes *et al.*, 2009). This pattern of asymmetric introgression between the forms has been shown to facilitate alteration in host preference (Fritz *et al.*, 2015) and autogenous egg production (Strickman and Fonseca, 2012), which could be an important factor when considering the risk posed to the human population.

Culex pipiens play a crucial role in transmission of three arboviruses of medical and veterinary importance currently circulating in Europe, due to their ability to bridge disease into a susceptible mammalian population. Therefore, understanding factors that may influence the levels of hybridisation observed within a population is important when considering risk of disease transmission. Despite this, research regarding the factors limiting hybridisation between the biotypes is lacking, with variable levels of hybridisation reported from different populations suggesting the existence of multiple barriers to gene flow.

1.6 *Culex pipiens* in the United Kingdom

Previous studies have investigated the distribution of the *Cx. pipiens* biotypes across the UK and their rate of hybridisation, yet understanding of the differential distribution and inbreeding rates between *Cx. pipiens* biotypes in the UK is still lacking. Sympatry of both *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* has been reported from aboveground larval habitats in the south of England and Wales (Danabalan *et al.*, 2012) according to the CQ11 microsatellite marker, with hybrid individuals also identified in a subset of sites, although larval habitat type was not clear. However, this study relied on morphological separation of *Cx. pipiens* and *Cx. torrentium* rather than molecular characterisation using the ACE-2 assay. Upon further investigation a subset of individuals identified as hybrid specimens by the CQ11 assay were found to be *Cx. torrentium* following DNA sequencing of the COI barcoding gene, thus hybridisation rates reported during this study should be interpreted with caution. Moreover, samples taken from a container habitat located in an allotment area in Surrey identified larval *Cx. pipiens* f. *pipiens* and hybrid specimens in sympatry when tested using the ACE-2 and CQ11 microsatellite marker (Manley *et al.*, 2015). Although the *molestus* form was absent during

initial field screening in which only five individuals were tested, following colony rearing for 20 generations all three forms were detected within the population when a larger sample size was tested. In contrast, studies investigating mosquito abundance and species composition on rural farms failed to identify *Cx. pipiens* f. *molestus* or hybrids whilst *Cx. pipiens* f. *pipiens* were abundant according to CQ11 biotype delineation (Brugman *et al.*, 2017a).

Furthermore, little is known about the species across different habitat types especially in urban environments. Although research has demonstrated *Cx. pipiens* larvae are abundant in container habitats in urban gardens from Reading, delineation of the forms was not performed (Townroe and Callaghan, 2014). Dedicated studies aimed at understanding the distribution of *Cx. pipiens* f. *molestus* have been conducted across London with a heavy focus on underground populations (Byrne and Nichols, 1999; Curtotti, 2009). The original study focused on genetic variation at twenty allozyme loci of *Cx. pipiens* populations in London (Byrne and Nichols, 1999). This study suggested that belowground populations from eight tube stations were relatively homogenous and more closely related to each other than to local aboveground individuals, supporting the conclusion that underground populations are reproductively isolated and share a single origin. The research also hypothesised that these underground populations evolved *in situ* due to the identification of all but one of the allozyme alleles specific to belowground populations also being identified in aboveground samples. Although this pattern supports the hypothesis, it is unlikely that this would have occurred over only a couple of centuries since the underground was constructed (Haba and McBride, 2022). Subsequent genomic analysis has also questioned this, concluding that belowground mosquitoes from diverse geographic locations including UK, Kyrgyzstan, Belarus, USA and Germany form a single genetic cluster, suggesting that most *molestus* populations likely share a common origin (Yurchenko *et al.*, 2020).

In contrast, a further study assessing the distribution of *Cx. pipiens* f. *molestus* across east London failed to identify larval habitats containing *molestus* individuals, with the notable exception of a sewage treatment works, rather all populations were dominated by the *pipiens* biotype (Curtotti, 2009). Interestingly, this study included a survey of drains, a larval habitat frequently thought to be utilised by the *molestus* biotype. Yet all samples displayed genetic signature of *Cx. pipiens* f. *pipiens* according to characterisation by the CQ11 microsatellite locus and displayed physiological characters associated with the *pipiens* form, such as anautogeny and eurygamous mating when assessed in the laboratory. This study also found no evidence of hybridisation between the two forms, suggesting that the forms were completely separate (Curtotti, 2009). Much of the work assessing *Cx. pipiens* populations in the UK has focused on larval sampling, yet the two forms are thought to utilise different

larval habitats. Therefore, it is possible that choice of larval sampling site may have biased previous results.

1.7 Urbanisation and the potential risk of arboviral emergence

In the UK, an estimated 83% of the human population reside in urban settings (Smith *et al.*, 2005b) with the population of London currently around 9 million people. London has many green spaces providing ideal breeding sites for a range of mosquito species. Thus, the proximity of mosquitoes to high-density human populations may be cause for concern should local species prove competent vectors of human pathogens. Organisms inhabiting urban areas encounter a multiplicity of challenges to their survival, such as air and water pollution, noise, artificial light, and a lack of suitable breeding habitats. Many studies have documented the negative effects of polluted water on mosquito development, while noise in an urban environment has been proposed to interfere with conspecific mate recognition in mosquitoes, which is primarily driven by wing beat frequency (Gibson and Russell, 2006; Robert, 2009). In contrast, some mosquito species of the genera *Aedes, Anopheles* and *Culex* have considerable environmental tolerances and have benefitted from anthropological environmental change brought about by urbanisation (Derraik, 2005).

Within the UK, *Culex* mosquitoes are known to dominate urban populations, breeding in both containers and small ponds (Townroe and Callaghan, 2014; Manley *et al.*, 2015). While biting rates are thought to be limited in these areas, occasional public health reports of nuisance biting do occur (Medlock *et al.*, 2012). A key research question, however, is the degree to which the biology of *Cx. pipiens* biotypes and hybrids can contribute to the spread of arboviruses in endemic circulation between birds and also between birds and humans in urban habitats. This is likely to be a major determinant of the severity and sustainability of future outbreaks.

Artificial lights at night (ALAN), such as streetlights, interfere with natural daily activity patterns such as host seeking and blood-feeding, prolonging these behaviours in *Aedes aegypti* (Linnaeus, 1762) (Chadee and Martinez, 2000). In contrast, exposure of female *Cx. pipiens* f. *molestus* to ALAN maintained under a normal light:dark cycle showed reduced activity compared with control groups under ALAN simulated conditions, with increased activity observed during the dark phase (Honnen *et al.*, 2019). Furthermore, alteration in gene expression in *Cx. pipiens* in response to ALAN has also been reported, with the most significant changes noted in genes associated with immunity and gametogenesis (Honnen *et al.*, 2016). Although transcriptome analysis of *Cx. pipiens* f. *molestus* has demonstrated a sex-specific response to ALAN with a stronger response seen in males (Honnen *et al.*, 2016), further research has demonstrated that female *Cx. pipiens* mosquitoes exposed to ALAN were

more active later at night (Honnen *et al.*, 2019) and can avert diapause (Fyie *et al.*, 2021), prolonging the mosquito biting activity. Research regarding the effect of ALAN in *Cx. pipiens* is still in its infancy therefore assessments of how this may influence vector competence in this species is lacking. Any alteration to vector competence in response to ALAN that may enhance virus transmission would increase disease risk to urban residents. In addition, the factors influencing vectorial capacity in urban populations of vectors, may also have an effect on virus transmission in hosts. For example, ALAN has been shown to prolong the infectious period of the house sparrow, an urban dwelling avian reservoir host of WNV (Kernbach *et al.*, 2019). This study demonstrated that sparrows exposed to ALAN maintained transmissible viral titres for two days longer than controls whilst experiencing no greater WNV-induced mortality.

Recent studies in the UK have identified an urban heat island effect (UHI) with increased water and air temperatures of 1.3 and 0.9^{o}C respectively, in urban compared with rural gardens (Townroe and Callaghan, 2014). Increased temperatures experienced due to UHI effect may have implications for mosquito physiology. As an example, the expression of autogeny has been shown to be influenced by a range of environmental factors experienced at both the larval and adult stage including temperature. The delay in the initial bloodmeal resulting from the development of autogenous eggs, reduces the probability of potential arbovirus transmission by increasing the age at which adults develop an infective bloodmeal compared with anautogenous populations or species (Reisen *et al.*, 1983). Therefore, it is an important contributor to a species vectorial capacity.

Exposure to temperatures of 28^{o}C was found to increase the number of autogenous eggs laid 50-fold in *Ae. aegypti* mosquitoes compared with 22^{o}C (Ariani *et al.*, 2015). Likewise, autogeny in *Culex tarsalis* (Coquillett, 1896) was demonstrated to peak at 32^{o}C and was suppressed at temperatures below 21^{o}C (Brust, 1991). Additionally, *Culex salinarius* (Coquillett, 1904) demonstrated a conversion between obligatory and facultative autogeny when exposed to higher and lower temperatures respectively (Tveten and Meola, 1988). In *Cx. pipiens*, the number of autogenous egg rafts produced by females has also been shown to be positively correlated with average temperatures experience by larval stages in the 7 days prior to emergence (Strickman and Fonseca, 2012). Moreover, vector competence of the forms has been shown to be differentially affected by temperature, with WNV transmission rate positively correlated with temperature for *Cx. pipiens* f. *pipiens* and hybrids (Vogels *et al.*, 2017a).

Larval nutrition and stress also affect autogenous egg production, with increases in protein content of larval diets resulting in greater numbers of autogenous eggs laid by *Ae. aegypti* and *Cx. pipiens* f. *molestus* (Reisen *et al.*, 1984; Telang and Wells, 2004; Telang *et al.*, 2006; Kassim *et al.*, 2012b; Ariani *et al.*, 2015). Studies have demonstrated negative effects of larval crowding and stress on autogenic

expression accompanied by high rates of larval mortality (Kassim *et al.*, 2012b). Increased larval nutrition and greater larval densities are factors that have been associated with increased urbanisation and, therefore, could influence virus transmission by altering fitness of mosquitoes.

Successful transmission of diseases vectored by *Culex* to human populations is reliant on the complex interplay between physiological and behavioural factors of the vector as well as environmental conditions experienced during the larval and adult life stages. Specifically, urban areas can provide a range of challenges to mosquito survival and may regulate life history traits such as development period and adult fitness. The ornithophilic nature of *Cx. pipiens* f. *pipiens* can facilitate the establishment, amplification, and maintenance of disease in the avian reservoir upon introduction to a new area. The ability of this species to exploit a range of manmade container habitats, especially in urban areas, increases proximity with potential human hosts. Elevated temperatures due to UHI effects (Townroe and Callaghan, 2014) can facilitate colonisation of these habitats as well as influence developmental characteristics of mosquitoes which can increase mosquito population density, thereby enhancing the rate of vector-host interactions. Additionally, vector competence of *Cx. pipiens* for WNV and USUV have been experimentally demonstrated (Fros *et al.*, 2015; Vogels *et al.*, 2017a) with the proven role of the species as vectors of these diseases demonstrated in Europe and America. Presence of *Cx. pipiens* f. *molestus* or species hybrids, or high levels of plasticity in host preference of *Cx. pipiens* f. *pipiens*, can subsequently facilitate bridging of viruses from the avian reservoir to the human population.

1.8 Overview of PhD aims, objectives and hypotheses

The aim of this thesis is to elucidate the influence of hybridisation and larval habitat on the plasticity of life history traits of the *Culex pipiens* forms through a series of experiments utilising field and colonised mosquitoes. This will help in understanding the risk posed by *Cx. pipiens* as a primary bridge vector between human and bird populations. Specific objectives and hypotheses for each chapter are presented below.

Outline of the thesis

Chapter 2 investigates the development, optimisation, and validation of a non-invasive DNA extraction method with the objective of non-lethal biotype delineation from pupal exuviae collected from both laboratory and field reared *Cx. pipiens*. To determine time restraints on the utility of the assay and its versatility across different methods, DNA yield and PCR amplification success at varying time points post-eclosion as well as for different processing methods were tested. Finally, this method

was implemented with the objective of establishing 'pure' colony lines of individual *Cx. pipiens* biotypes which were successfully reared for ten generations, with the *pipiens* and *molestus* lines retaining their pure genetic signature, according to the CQ11 microsatellite. Development of this method and creation of these colony lines facilitated work carried out in subsequent chapters. Hypotheses for chapter 2 are listed below:

1. Pupal exuviae can be successfully used to differentiate the biotypes of *Cx. pipiens* under colony conditions and can be used to generate 'pure' lines from a mixed population.
2. DNA yield and PCR amplification success will be negatively influenced by time post-eclosion, defining a time sensitive limitation of the assay. Processing method will also be shown to influence DNA yield and PCR amplification success, with methods requiring prior extraction of DNA being superior to direct PCR assays.
3. Pupal exuviae collected under field conditions will have limited utility for individual extraction but pooled samples can provide a useful tool for initial non-invasive population screening.

Chapter 3 presents a series of experiments to determine the effect of hybridisation on life history traits between the *Cx. pipiens* biotypes. This chapter includes an assessment of mating success between homologous and reciprocal crosses of the forms under both single-pair and grouped mating experiments and demonstrates the influence of these different crosses on larval development parameters. To determine whether female *Cx. pipiens* will preferentially mate with conspecific males an assessment of mating choice was undertaken utilising molecular methods to characterise sperm stored in the female spermathecae. Hypotheses for chapter 3 are listed below:

1. Mating success of females will be influenced by male pairing with homologous crosses achieving a higher mating frequency than reciprocal crosses. Experimental conditions will also influence mating success in the *pipiens* biotype, with individuals performing significantly better under grouped compared with single-pair mating.
2. Reciprocal crosses will suffer reduced fitness resulting in decreased larval survival and adult fitness compared with the homologous crosses.
3. When provided with a choice of male pairing, the frequency of conspecific mating will be higher than with non-conspecifics in all female *Cx. pipiens* biotypes.

Chapter 4 details a field study across six larval habitats to determine the influence of environmental factors experienced during larval development on adult reproductive traits. To determine the effect of larval nutrition on plasticity of life history traits field and colonised *Cx. pipiens* were reared under varying levels of larval nutrition. Finally, to determine the genetic structure of UK *Cx. pipiens*

microsatellite analysis of field derived specimens as well as samples from pure colony lines were compared with *Cx. pipiens* f. *molestus* control samples from Sweden. Hypotheses for chapter 4 are listed below:

1. Larval density of *Cx. pipiens* larvae will increase with increasing urbanisation of habitats with larval habitats in more urban areas recording greater water temperatures.
2. Larval habitat and environmental conditions experienced during larval development will influence female reproductive traits including fecundity.
3. Larval diet quantity will significantly influence life history traits of *Cx. pipiens* with increased fitness of mosquitoes reared on high protein diets. Despite similar trends, important differences will be noted in response to larval diet variation from field and colonised mosquitoes as well as species differences between the biotypes.
4. Genetic analysis of field and colony *Cx. pipiens* will reveal low hybridisation rates with UK *molestus* samples demonstrating closer ancestry to UK *pipiens* compared with *Cx. pipiens* f. *molestus* from Sweden.

Chapter 5 integrates the results of this thesis into a broader context and considers how these factors may influence vectorial capacity of populations. Suggestions for further work are also provided.

Chapter 2 – Investigating the utility of *Culex pipiens* pupal exuviae as a non-invasive method of biotype differentiation

2.1 Introduction

The females of the *Culex pipiens* (Linnaeus, 1758) species are morphologically indistinguishable from other members of the *Culex pipiens* complex, with differentiation of species reliant on molecular methods (Becker *et al.*, 2012). The *Cx. pipiens* species comprises two morphologically indistinguishable biotypes, *Cx. pipiens* f. *pipiens* (L.) and *Cx. pipiens* f. *molestus* (Forskål, 1775). Since their identification, several morphological features have been suggested as diagnostic in their separation, however, this is not well established. Rather, delineation of the forms is performed by amplification of a segment of the CQ11 microsatellite (Bahnck and Fonseca, 2006; Rudolf *et al.*, 2013).

With a lack of morphologically distinguishing features, identification of the *Cx. pipiens* forms relied on the expression of distinct phenotypic characteristics. Ecological and physiological differentiation of the *Cx. pipiens* biotypes is well established and widely implemented (Brugman *et al.*, 2018). The presence of behavioural traits such as autogeny as well as ecological characteristics such as habitat associations have been used to differentiate the two forms (Byrne and Nichols, 1999). Differences in habitat associations were initially thought to be consistent with the *pipiens* form associated with aboveground, natural and human-associated artificial habitats across rural and urban areas whilst the *molestus* biotype was predominantly confined to underground environments (Becker *et al.*, 1999; Fonseca *et al.*, 2004).

In northern Europe in particular, habitat separation was thought to serve as a barrier to hybridisation between the forms (Byrne and Nichols, 1999). More recent evidence, however, suggests this habitat separation is far less rigid than previously thought with genetic analysis indicating inter-breeding of sympatric populations across Europe from both above- and belowground habitats including Germany (Rudolf *et al.*, 2013), Portugal (Osorio *et al.*, 2014), the UK (Manley *et al.*, 2015), the Netherlands (Reusken *et al.*, 2010; Vogels *et al.*, 2015), Austria (Zittra *et al.*, 2016) and Italy (Di Luca *et al.*, 2016). This lack of clarity has led to an increase in the application of molecular methods for the separation of the *Cx. pipiens* biotypes.

The application of molecular methods has greatly added to our understanding of insect populations and patterns of distribution; however, these methods are typically lethal for the individual (Gerken *et al.*, 1998). In recent years, non-lethal sampling methods for DNA extraction have gained attention from conservational and population genetic studies (Nguyen *et al.*, 2017), whilst also enhancing interest in isolation of environmental DNA (eDNA). One challenge encountered during the

development of tissue sampling methods for small creatures such as live insects is that, in order not to cause mortality, the tissue sample will have a small quantity of starting DNA present. Nonetheless, a single tibia has been shown to be a sufficient non-lethal source of DNA in several insect species including bumble bees (Holehouse *et al.*, 2003) and mosquitoes (Ruiz *et al.*, 2005). These challenges are exacerbated when attempting to study insect behaviour, where a fundamental assumption is that behaviour is not altered by the identification method. Hemolymph has been suggested as a source of DNA as demonstrated in the scorpion fly (*Panorpa communis*; Linnaeus, 1758) larvae and adults, although the downstream effects of sampling on adult behaviour and mortality rates were not defined (Gerken *et al.*, 1998; Kurtz and Sauer, 1999). Moreover, wing edges and tips from butterflies were shown to provide a sufficient source of DNA (Rose *et al.*, 1994; Lushai *et al.*, 2000). However, invasive sampling methods such as these have potential to impact subsequent behaviour and longevity of the sampled individual and can also have wider consequences on population size and structure (Starks and Peters, 2002). In mosquitoes a specific issue is that leg tapping was revealed to be an important precursor to mating within the *Cx. pipiens* group, with females observed to perform rejection kicks to prevent mating with incompatible males (Benelli, 2018; Kim *et al.*, 2018b).

Collection and identification of insect pupal exuviae has been an important tool in many ecological studies aiming to estimate population densities (Rodenhouse *et al.*, 1997), species distribution (Lee *et al.*, 2010; Ferrington and Coffman, 2014) and emergence periods (Sato and Sato, 2015). Successful extraction of DNA from exuviae has been demonstrated for a variety of species including butterflies (Feinstein, 2004), honey bees (Gregory and Rinderer, 2004), mosquitoes (Dhananjeyan *et al.*, 2010), dragonflies (Watts *et al.*, 2005), scarabs (Lefort *et al.*, 2012) and tarantulas (Petersen *et al.*, 2007). The exoskeleton of the pupal exuviae is comprised of extracellular chitin and does not itself contain any nucleic acids (Nation, 2008). Rather, successful DNA extraction from pupal exuviae relies on trace amounts of epithelial cells, hairs and muscle tissues remaining attached to the inner surface of the cuticle by the eclosing adult.

Trace DNA from Chironomid pupal exuviae has been shown to be sufficient for PCR amplification and sequencing of target genes (Krosch and Cranston, 2012). Initial attempts to extract DNA from Chironomid pupal exuviae were unsuccessful when a salting out method was used (Miller *et al.*, 1988), yet recent studies have found varying success depending on the extraction method used (Kranzfelder *et al.*, 2016). More recently, successful DNA sequencing of the COI target was performed in 46% of samples tested (Krosch and Cranston, 2012) whilst genomic DNA was isolated from an average of 61.2% of Chironomid pupal exuviae tested across five different extraction methods (Kranzfelder *et al.*, 2016). Furthermore, insect frass and exuviae has also been used to detect endogenous genes from

Mythimna separata (Walker, 1865) while food waste was used to determine gender and identify transgenic individuals early in the larval stage of *Drosophila melanogaster* (Ali *et al.*, 2019).

Pupal exuviae from *Aedes* and *Culex* mosquitoes were used successfully to obtain DNA for molecular speciation targeting the ITS2 and ITS1 regions, respectively, although success was limited to within the first 24 hours post-eclosion and no success was seen when tested in field collected samples (Dhananjeyan *et al.*, 2010). The authors postulated that microorganisms in the water may accelerate digestion of nucleic acids, however, this poses questions about the viability of this method for monitoring field populations of mosquitoes. Information regarding the sample sizes, success rates as well as the quantity and quality of DNA obtained during this study was also lacking. A subsequent study attempted to replicate this by comparing success of DNA extraction from pupal exuviae compared with individual legs of *Aedes aegypti* (Linnaeus 1762), however, the authors were unable to obtain enough DNA from pupal exuviae for downstream molecular testing (Wong *et al.*, 2012).

A wide variety of methods for DNA extraction are now available and the ideal technique should allow for the isolation of high quality and quantity DNA while facilitating high sample throughput by being efficient in terms of handling time and cost (Kranzfelder *et al.*, 2016). Traditional extraction methods, such as organic extractions using phenol/chloroform, are capable of efficiently extracting DNA from animal tissues and provide the added benefit of isolating DNA without the need for sample destruction allowing for specimens to be kept as a morphological reference. However, these methods often require the use of hazardous chemicals and can be time consuming (Hajibabaei *et al.*, 2005). More recently, a variety of dedicated commercially available kits have been developed which rely on silica-membrane-based technology and are becoming increasingly popular due to their ease-of-use and application to a wide range of biological samples including plant tissue and insects. However, the high cost of these kits can sometimes be prohibitive.

A low-cost alternative is direct PCR, which facilitates amplification of target DNA without the need for a dedicated extraction step (Thongjued *et al.*, 2019). However, this technique has had limited use due to low success rates, especially in samples with low quality DNA and inhibitors. A recent study comparing five extraction methods with direct PCR examined cost, handling time, DNA quantity, PCR and sequencing success for the extraction of DNA from Chironomid exuviae and found significant variation between the methods tested. In this study, commercially available kits generally performed better than the traditional extraction methods and direct PCR (Kranzfelder *et al.*, 2016).

The present study aims to assess the utility of *Cx. pipiens* pupal exuviae as a non-lethal source of DNA for distinguishing the two *Cx. pipiens* forms from a mixed population under both laboratory and field

conditions. Firstly, the effect of processing method on DNA yield and PCR amplification success was investigated with the hypothesis that commercially available kits would outperform other methods, whilst including an assessment of handling time and cost for each method. Secondly, the effect of processing time post-eclosion on DNA yield and PCR amplification success was investigated to determine time constraints of the assay and inform future work utilising the method. Subsequently, to test the hypothesis that this method would have limited utility in field collected samples when exuviae were processed in pools, DNA yield and PCR amplification success of samples processed individually and in pools of five exuviae were compared. Furthermore, this study aims to assess whether persistent single biotype colonies can be established from a mixed population, by extracting DNA from pupal exuviae to determine the biotype of emerging adults, and to estimate for how long these colonies remain pure in culture. If successful, creation of single biotype lines would facilitate work conducted in subsequent chapters.

2.2 Methods

2.2.1 Mosquito rearing

In order to establish mating success between the *Cx. pipiens* biotypes from a mixed population, single-pair mating was conducted. Experiments utilised an established mixed colony of *Cx. pipiens* biotypes originating from an allotment area in Brookwood, Surrey, UK established in 2011 and identified as the 'Brookwood' line (Manley *et al.*, 2015). Adults from this colony line were offered blood overnight using a Hemotek™ membrane feeding system (Hemotek™ Ltd., UK) with defibrinated horse blood (TCS Biosciences, UK). Egg rafts were collected and left to hatch in approximately 500 millilitres (ml) fresh tap water. Larvae were reared at a density of 200 larvae/litre (L) and maintained on a diet of 1 milligram (mg) of guinea pig pellets/larva (Pets at Home, UK) on alternate days. Larvae were reared in an environmentally controlled incubator maintained at a temperature of 25^{o}C ± 1^{o}C and relative humidity of 50% ± 1% and were exposed to a lighting regime of 16:8 hours light:dark with an hour of dusk and dawn on either side. Conditions in the incubator were monitored using a HOBO™ U12-012 temperature/relative humidity/light data logger (Measurement Systems Limited, UK). During the daylight period, light intensity was approximately 3500 lux whilst average light intensity during the night-time period was 3.5 lux. Dusk and dawn were simulated by an increase and decrease in light intensity over an hour period either dropping to approximately 2000 and then 1000 lux during dusk and increasing by the same increments for dawn.

2.2.2 Single-pair mating

Following the onset of pupation, pupae were collected daily and separated into males and females on the basis of external structure of the terminal segment (Christophers, 1960) by observation under a dissecting light microscope (Leica Microsystems, Germany). The terminal segment of male pupae is shield-shaped and bifurcate on the distal end whereas females are generally more broadly ovate in shape (Figure 2.1, panels A and B). Mating pairs consisted of males and females pupating within the same 24-hour period to minimise age difference of mosquitoes confounding results. Each pair was placed together in a small cup containing approximately 15 ml of water and cups were then placed into 12-ounce (oz) cardboard soup pots (Cater4you, UK) (Figure 2.1, panels C – E). Mosquito pairs were left for 10 days to allow for eclosion and mating before mating success was determined. Adult mosquitoes were maintained on 10% sucrose solution provided *ad libitum.*

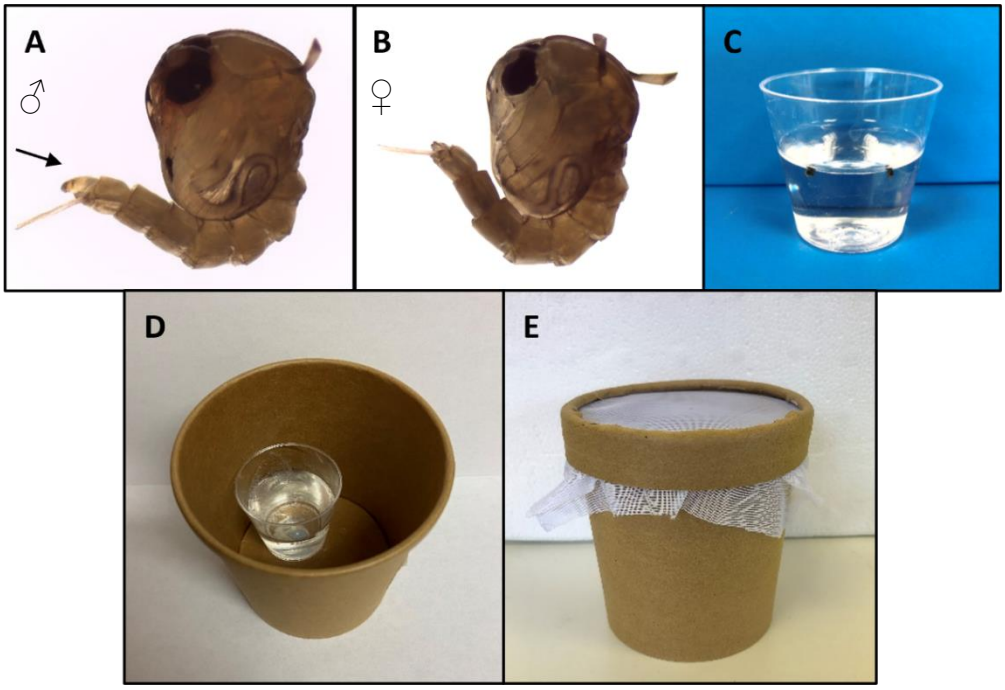

Figure 2.1. Construction of experimental arenas for single-pair mating experiments to assess mating success in *Culex pipiens*. Male and female *Culex pipiens* pupae were separated according to sex, identified by observation of the terminalia on the terminal segment of the body (arrow) of male (A) and female (B) mosquito pupae. Once separated by sex pupae were allocated into pairs in small plastic cups (25 ml capacity) in approximately 15 ml of water (C). These cups were placed into carboard soup pots (D) and sealed with netting (E).

Female mosquitoes were euthanised at -20°C before spermathecae were dissected from female mosquitoes to assess mating success (Figure 2.2). Briefly, female mosquitoes were placed ventral side up on a glass microscope slide under a dissecting microscope. The terminalia of the female was removed with forceps and separated from the rest of the body. The spermathecae were isolated from the terminalia, transferred to a new microscope slide, and observed for the presence of sperm under a compound microscope (Leica Microsystems, Germany) at 400x magnification. A successful mating event was characterised by the presence of sperm in the spermathecae (Rosay, 1969).

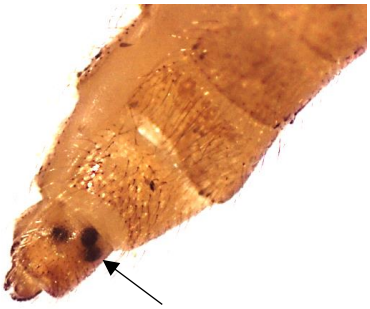

Figure 2.2. Spermathecae (arrow) of *Culex pipiens* female *in situ*, visualised under 40x magnification.

As the Brookwood colony line contained a mix of all three forms, blind-pairing of pupae meant biotyping of individuals within each pairing was carried out retrospectively. Therefore, following dissection, mosquitoes were decapitated, and heads collected for DNA extraction using the Wizard® SV Genomic DNA Purification System (Promega, UK) and molecular characterisation as described below in Sections 2.2.5 and 2.2.6. A total of 320 mating pairs were set up, of which only pairings with both adults surviving until the end of the study were processed to assess mating success and adult biotype (n= 240).

2.2.3 Pupal exuviae sample collection and preparation

Due to blind-pairing of pupae, low sample size numbers for some pairings were obtained, highlighting logistical challenges of using a mixed population, thus a non-invasive method of biotyping mosquitoes prior to experiments was sought. To achieve this, larvae from the Brookwood line were collected and reared as described above in Section 2.2.1**.** Following the onset of pupation, pupae were collected daily and placed into separate 2 ml tubes in approximately 1 ml of water. To compare the efficacy of DNA extraction kits, pupal exuviae and emerging adult mosquitoes were placed in 70% ethanol immediately following eclosion and stored individually at room temperature prior to DNA extraction. To assess the effect of time of processing following eclosion, adult mosquitoes and the pupal exuviae were placed individually into 70% ethanol at defined time points post-eclosion (0, 1, 6, 12, 18 and 24 hours post-eclosion). Pupal exuviae and adult mosquitoes were removed from ethanol and left to air dry for approximately 10 minutes prior to DNA extraction.

2.2.4 Use of pupal exuviae as a non-invasive method of sampling field populations

In order to collect *Cx. pipiens* pupal exuviae from field populations, an oviposition site was created using a 20 L black bucket with dimension of 28.3 cm x 47.8 cm x 33.0 cm (HxWxD), filled with 10 L of tap water and seeded with 5 g crushed guinea pig pellets (Pets at Home, UK) to attract gravid females. Pupal exuviae floating on the surface were collected each morning and placed into 70% ethanol for a total of 2 weeks and transferred to the laboratory for processing. Samples were either processed individually or in pools containing five exuviae using the ethanol precipitation method described below in Section 2.2.5. The bucket was placed in a residential garden in Guildford, Surrey (51.240, -0.578). Water temperature and light intensity in the bucket were measured using the HOBO™ temperature/light weatherproof pendant data logger (Measurement Systems Limited, UK). Two collections of pupal exuviae were undertaken. The first collection was performed in August 2020, collections undertaken during this time were used for comparison of individual and pooled processing.

The second collection period was between June to July 2021 and collections undertaken during this time were used for the comparison of processing methods in field collected samples.

2.2.5 DNA extraction method comparison

Three extraction methods and a direct PCR processing method were chosen to compare DNA yield and subsequent PCR amplification success. For each of the extraction methods, total genomic DNA was isolated from 20 individual pupal exuviae alongside a negative control, with equal numbers of males and females processed for each method. Extraction of the corresponding adult heads were included as positive controls. The three extraction methods included were an ethanol precipitation method and two commercially available kits, DNeasy® Blood & Tissue Kit (Qiagen, UK) and Wizard® SV Genomic DNA Purification System (Promega, UK), hereafter referred to as DNeasy® and Wizard® respectively. For the ethanol precipitation, DNA was extracted as follows: samples were placed into 200 µl of digestion solution comprised of 100 mM UltraPure™ 1 M Tris-HCL (pH 8.0) (Invitrogen™ by Thermo Fisher Scientific, UK), 200 mM sodium chloride (Invitrogen™, UK), 0.2% (w/v) sodium dodecyl sulphate (SDS) (Merck Life Science UK Limited, UK), 5 mM UltraPure™ 0.5 M Ethylenediaminetetraacetic acid (EDTA) (pH8.0) (Invitrogen™, UK), 200 µg/ml proteinase K (Qiagen, UK) made up to a total volume of 200 µl per sample using UltraPure™ water (Invitrogen™, UK). Samples were incubated overnight at 37^{o}C before proceeding with DNA extraction. Immediately following incubation, 500 µl of ice-cold 100% ethanol, 20 µl 3 M sodium acetate pH 5.5 (Invitrogen™, UK) and 2 µl of GlycoBlue™ coprecipitant (15 mg/ml) (Invitrogen™, UK) were added to each sample and incubated at -20^{o}C for 1 hour. DNA was pelleted by centrifugation at 14,000 revolutions per minute (rpm) at 4^{o}C for 30 minutes and supernatant was removed. Pellets were then washed in 400 µl 70% ethanol, re-pelleted by centrifugation under the same conditions for 15 minutes and supernatant was removed. Pellets were air-dried for 20 minutes to allow for the evaporation of excess ethanol, following which pellets were resuspended in either 15 µl or 200 µl nuclease free water for pupal exuviae and adult samples respectively.

For the commercially available kits, DNA was extracted according to the manufacturer's instructions with the following modifications to enhance DNA yield (Promega, 2012; Qiagen, 2020). Mosquito exuviae were subjected to a pre-homogenisation stage within the digestion solution detailed in each of the kit's instructions using 3 mm stainless steel homogenisation beads (Qiagen, UK) for 1 minute at 30 hertz using the Qiagen tissue lyser (Qiagen, UK). Samples were incubated overnight at 56^{o}C prior to DNA extraction. To maximise yield of DNA obtained from exuviae samples, elution was carried out

in 30 µl of elution buffer which was incubated on the membrane at room temperature for 5 minutes prior to elution. For DNA extraction from mosquito heads, elution was performed twice in 30 µl.

For the direct PCR method, samples were prepared according to Thongjued *et al.* (2019) with minor adjustments to sample preparation. Briefly, pupal exuviae were removed from ethanol and left to air-dry for approximately 10 minutes before being placed in 20 µl phosphate buffered saline (PBS) (pH 7.4) (Gibco™ by Thermo Fisher Scientific, UK). Samples were subsequently mixed before incubating at 98^{o}C for 4 minutes, following which 2 µl of supernatant was added directly to the PCR mix.

Nucleic acid concentrations were subsequently measured using the Qubit® dsDNA HS assay kit (Invitrogen™, UK) and read by a Qubit® 3.0 fluorometer (Invitrogen™, UK). For all samples, 2 µl of DNA template were used in 198 µl of the dsDNA HS assay.

2.2.6 Differentiation of *Culex pipiens* biotypes

Mosquitoes were simultaneously assigned to species and biotype level using a real-time PCR assay originally designed by Rudolf *et al.* (2013) with minor adaptations to primer concentrations. Primers *CxPip*F and *CxPip*R were used to amplify a segment of the CQ11 microsatellite locus of all *Cx. pipiens* species. Amplification of probe *CxPip*P was subsequently used to confirm species identity as *Cx. pipiens* whilst probes *CxPipPip*P and *CxPipMol*P were used to differentiate between the forms. *Culex torrentium* DNA was detected using the primers *CxTorr*F and *CxTorr*R and the probe *CxTorr*P which binds to the nucleotide positions 112 to 131 of the ACE-2 gene. Successful amplification of this probe confirmed the presence of *Cx. torrentium* DNA, whilst absence of amplification was indicative of *Cx. pipiens* species identification. Further details for primer/probe binding sites as well as assay validation is shown in Appendix 1.

Reactions were performed in 10 µl reaction volume consisting of 5 µl TaqMan™ multiplex master mix (2x) (Applied Biosystems™ by Thermo Fisher Scientific, UK), 0.3 µM *CxPip*F, 0.4 µM *CxPip*R, 0.2 µM *CxPip*P, 0.2 µM *CxPipPip*P, 0.2 µM *CxPipMol*P, 0.15 µM *CxTorr*F, 0.15 µM *CxTorr*R R, 0.1 µM *CxTorr*P, 0.16 µl bovine serum albumin (BSA) (20 mg/ml) (Merck Life Science UK Limited, UK), 1.14 µl UltraPure™ water (Invitrogen™, UK) and 2 µl DNA extract. Sequences for the primers and probes are shown in Table 2.1. The thermal profile started with an initial activation step of 95^{o}C for 20 seconds, followed by 40 cycles of 95^{o}C for 3 seconds and 60^{o}C for 1 minute using a QuantStudio™ 7 Flex Real-Time PCR machine (Applied Biosystems™, UK). All samples were run alongside appropriate positive controls consisting of pure *pipiens*, *molestus*, hybrid and *Cx. torrentium* DNA as well as negative controls including extraction and PCR negative controls. Quantification cycle (Cq) values, the number

of amplification cycles taken for the fluorescence signal of samples to cross the detection threshold, obtained from each of the probes for each sample were averaged for use in analysis.

Table 2.1. Primer and probe sequences for the simultaneous differentiation of *Culex pipiens* and *Culex torrentium* species through amplification of the ACE-2 gene as well as the *Culex pipiens* biotypes by the CQ11 microsatellite locus. Letters in the primer/probe names identify whether they are forward (F) primers, reverse (R) primers or probes (P).

Primer/probe name	Primer/probe sequence
*CxPip*F	5'- GCGGCCAAATATTGAGACTT-3'
*CxPip*R	5'-CGTCCTCAAACATCCAGACA-3'
*CxTorr*F	5'-GACACAGGACGACAGAAA-3'
*CxTorr*R	5'-GCCTACGCAACTACTAAA-3'
*CxPip*P	5'-VIC- GGAACATGTTGAGCTTCGG-QSY-3'
*CxPipPip*P	5'-ABY-GCTTCGGTGAAGGTTTGTGT-QSY-3'
*CxPipMol*P	5'-JUN-TGAACCCTCCAGTAAGGTATCAACTAC-QSY-3'
*CxTorr*P	5'-FAM-CGATGATGCCTGTGCTACCA-QSY-3'

2.2.7 DNA extraction cost and handling time

The cost per sample for each extraction method was estimated based on the price of the chemicals or kits used. The handling time per sample was calculated as the time required to complete all processing starting from dehydration of the pupal exuviae to obtaining a DNA extract ready for PCR. This was measured three times for the same number of samples and averaged.

2.2.8 Wing length measurements

Adult mosquitoes utilised in the kit comparison and time trial experiments were removed from ethanol and had their wing length measured as a proxy for body size (Siegel *et al.*, 1992). Wings were transferred to a piece of paper towel dampened with 70% ethanol, flattened, and left to air dry for approximately 5 minutes. Following evaporation of excess ethanol, wings were transferred to a strip of scotch magic tape with both wings from one adult placed together, ten pairs of wings were dissected per strip with placement of each pair noted to enable mosquito identification. Once the strip of tape was full this was stuck down to a glass microscope slide for processing. Wing images were taken using a Leica EZ4HD microscope (Leica Microsystems, Germany) alongside a size reference. Images were subsequently processed for size measurement using ImageJ (Schneider *et al.*, 2012). Wing length was measured by taking the distance from the axillary incision to the apical margin, excluding the fringe (Harbach and Knight, 1980). Measurements from both wings of the same adult were averaged for use in analysis and were considered to represent overall body size.

2.2.9 Creation of single biotype colony lines

Adults from the Brookwood colony line (F97) were offered defibrinated horse blood (TCS Biosciences, UK) overnight using a Hemotek™ membrane feeding system (Hemotek™ Ltd., UK). Approximately 5 days after blood-feeding, egg rafts were collected and separated into individual pots in approximately 15 ml of water for hatching. Egg rafts were reared individually under the same conditions described in Section 2.2.1. Following the onset of pupation, pupae were collected daily into individual 2.0 ml tubes and monitored for eclosion. Results from Section 2.3.3 were used to inform the limitation of the assay in terms of processing time post-eclosion therefore, pupal exuviae were collected into 70% ethanol at least every twelve hours and stored prior to processing. Exuviae were processed for DNA extraction by ethanol precipitation and biotype identified daily as described above in Sections 2.2.5 and 2.2.6. This method was chosen due to the superior DNA yield and lower processing cost compared with the other methods which facilitated the high through-put required to achieve sufficient sample sizes. Adult mosquitoes were subsequently allocated to separate colony cages (Bugdorm, Watkins and Doncaster, UK) according to biotype with approximately 80-100 individuals per biotype used to create single form colonies. Single biotype colonies were maintained according to Manley *et al.* (2015).

Following rearing for 10 generations, 40 adults from each line were tested to confirm the lines remained homologous or heterozygous according to their molecular classification as determined by the CQ11 microsatellite marker. Heads of adult mosquitoes were used to obtain DNA using the Wizard® SV Genomic DNA Purification System (Promega, UK) to prevent contamination with sperm stored within the spermathecae and biotype characterised, as described above in Sections 2.2.5 and 2.2.6.

2.2.10 Statistical analysis

Data were checked for normality using the Shapiro-Wilk test. As log transformation did not normalise data, to test the likelihood that DNA quantity varied between DNA extraction methods or time of processing post-eclosion, Kruskal-Wallis with Bonferroni correction for multiple comparisons was used. To assess the likelihood that PCR success varies between DNA extraction method or time post-eclosion a pairwise chi-squared test for independence with Bonferroni adjustment for multiple comparisons was performed. To assess the effect of processing method or year of collection on nucleic acid concentrations from field collected pupal exuviae, Mann-Whitney U test was used. To test whether wing length differed between males and females an unpaired t-test was used. To assess for correlation between concentration, wing measurement and Cq values, Spearman's correlation was

used. For all statistical tests, the statistical significance level was set at $P < 0.05$ and analyses were computed in R studio version 1.2 (RStudio Team, 2020).

2.3 Results

2.3.1 Single-pair mating

Mating success results for each of the cross combinations are shown in Table 2.2. A total of 240 mating pairs were assessed, of which 72 (30%) resulted in a successful mating event. *Culex pipiens* f. *pipiens* was the most abundant biotype in the mating pairs with 250 individuals (52.08%). In contrast *Cx. pipiens* f. *molestus* only contributed 29 individuals (6.04%) of the total population tested. Pairings containing a *molestus* biotype were the most underrepresented with no homologous *Cx. pipiens* f. *molestus* pairings created. Homologous *Cx. pipiens* f. *pipiens* crosses were the most represented pairing (30%) and were observed to have a low mating success rate under single-pair mating conditions with only 7 out of 72 (9.72%) crosses successfully mating. In contrast the hybrid male and *Cx. pipiens* f. *molestus* female cross had the greatest mating frequency with 4 of 8 (50%) female successfully mating.

Table 2.2. Analysis of mating success in homologous and reciprocal crosses of the *Culex pipiens* biotypes under single-pair mating conditions. A successful mating event was characterised by the presence of sperm stored in the female spermathecae.

Male (♂)	Female (♀)	Crosses (n)	Success (%)
f. *pipiens*	f. *pipiens*	72	9.72
f. *pipiens*	f. *molestus*	4	0
f. *pipiens*	Hybrid	46	36.96
f. *molestus*	f. *molestus*	0	-
f. *molestus*	f. *pipiens*	10	30.00
f. *molestus*	Hybrid	7	14.29
Hybrid	Hybrid	47	46.81
Hybrid	f. *pipiens*	46	39.13
Hybrid	f. *molestus*	8	50.00

2.3.2 Comparison of processing methods for successful DNA extraction and PCR amplification of *Culex pipiens* pupal exuviae from colony populations

DNA extraction cost and handling time

Cost was found to vary between the four processing methods. The direct PCR method was the cheapest option with no associated extraction costs, followed by the ethanol precipitation (Table 2.3). When comparing extraction methods, the ethanol precipitation was found to be much cheaper than the two commercial kit methods. The DNeasy® and Wizard® kits had a higher handling cost per sample

with the Wizard® kit having a ten-fold increase in price per sample compared with the ethanol precipitation. Moreover, the cost of the DNeasy® kit was substantially higher; double the Wizard® kit per sample (Table 2.3). Due to the high throughput nature of the experiment, the ethanol precipitation method was favoured over the commercial kits.

Table 2.3. Comparison of extraction cost per sample of three DNA extraction methods and direct PCR.

Method	Cost per sample
Qiagen DNeasy® Blood & Tissue kit	£3.21
Promega Wizard® SV Genomic DNA Purification System	£1.50
Ethanol precipitation	£0.15
Direct PCR	£0.00

Handling time also varied between the different processing methods (Table 2.4). The direct PCR was the quickest processing method, with no requirement for prior extraction of samples therefore eliminating the overnight incubation step. Of the three extraction methods tested, the ethanol precipitation had the longest processing time with the DNeasy® and Wizard® kits taking significantly less time.

Table 2.4. Comparison of processing time for three DNA extraction methods and a direct PCR to obtain DNA from individual *Culex pipiens* pupal exuviae. Processing time was defined as the total time taken to complete all processing steps starting from dehydration of the pupal exuviae to obtaining a DNA extract ready for PCR. This was recorded three times per method and an average taken.

Method	Processing time (Hours)	
	Mean	SD
Qiagen DNeasy® Blood & Tissue kit	18.527	0.025
Promega Wizard® SV Genomic DNA Purification System	18.707	0.040
Ethanol precipitation	20.573	0.087
Direct PCR	0.334	0.026

DNA yield and PCR amplification success

Genomic DNA was successfully extracted from a total of 80 samples (20 samples per method). The quantity of DNA extracted was shown to vary significantly between the four processing methods (KW $\chi^2 = 57.144$, df = 3, $P < 0.001$; Figure 2.3). The greatest total DNA yield was achieved using the ethanol precipitation method ($\bar{x}$ = 17.780 ng; sd= 6.874 ng) which was greater than both commercially available kits. The DNeasy® and the Wizard® kits had similar total concentrations ($\bar{x}$ = 2.193 ng; sd= 1.149 ng, $\bar{x}$ = 3.015 ng; sd= 0.999 ng, DNeasy® and Wizard® respectively) (P = 0.145) which both differed significantly from the ethanol precipitation method (P <0.001 for both the DNeasy® and

Wizard® kits). The total concentration of DNA from the direct PCR method was not significantly different to that from the DNeasy® ($P = 0.178$). However, total DNA yield obtained from the direct PCR was significantly lower than concentrations obtained from the Wizard® and ethanol precipitation methods ($P = 0.005$ and $P < 0.001$ for Wizard® and ethanol precipitation respectively).

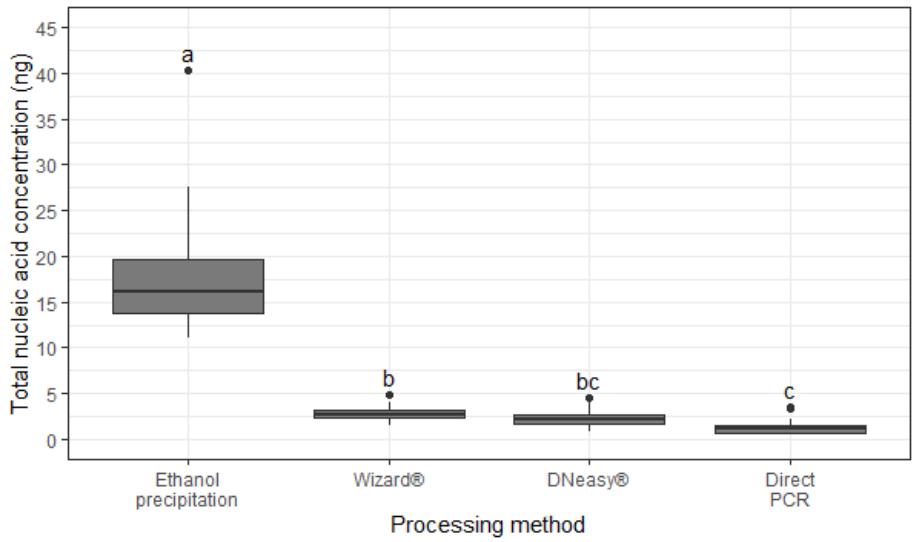

Figure 2.3. Comparison of total DNA quantity (ng) obtained from individual *Culex pipiens* pupal exuviae using three extraction methods plus direct PCR. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

Despite significantly different total concentrations obtained, PCR amplification success was not shown to vary significantly between the three extraction methods (Fisher's exact $P = 1.00$) with all showing 100% amplification success. However, the extraction methods tested were all shown to significantly differ from the direct PCR method ($P = 0.007$), with direct PCR amplification success decreasing to approximately 55% (Figure 2.4). When comparing nucleic acid concentrations with Cq values a general trend was seen of decreasing Cq with increasing nucleic acid concentrations (Figure 2.5). This correlation was significant for the ethanol precipitation (R= -0.47, $P = 0.037$), Wizard® (R=-0.51, $P = 0.021$) and DNeasy® (R= -0.75, $P = 0.018$) methods. The correlation between nucleic acid concentration and Cq value was not significant for the direct PCR method (R= -0.25, $P = 0.29$).

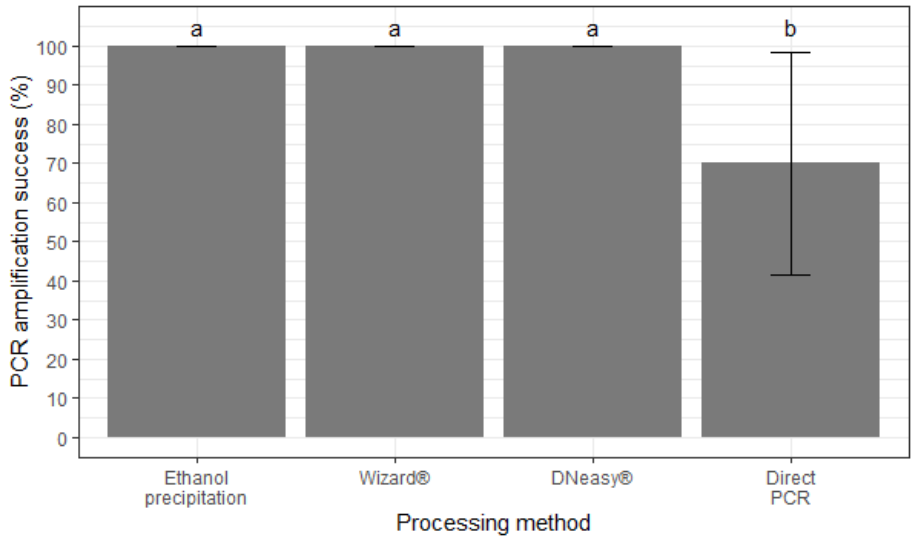

Figure 2.4. Comparison of PCR amplification success (%) for DNA extracts obtained from individual *Culex pipiens* pupal exuviae by three different extraction methods and direct PCR. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

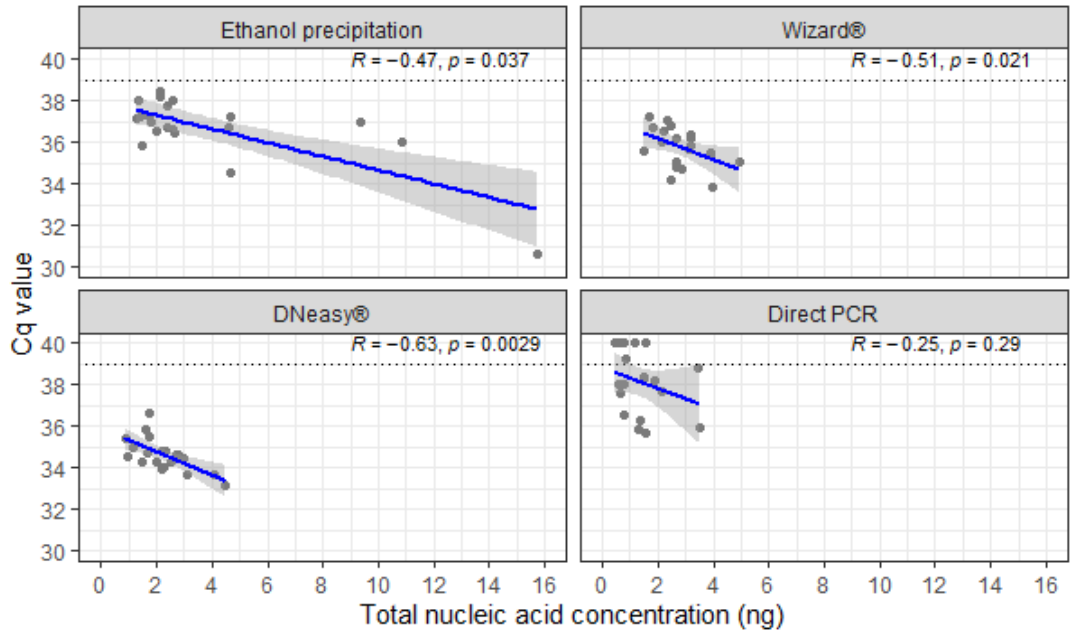

Figure 2.5. Comparison of total nucleic acid concentration (ng) compared with average Cq values following PCR amplification of DNA extracts obtained from individual *Culex pipiens* pupal exuviae by three different extraction methods and direct PCR. Grey shaded area indicates the 95% confidence interval.

2.3.3 Investigating the influence of processing time post-eclosion on DNA yield and PCR amplification success from *Culex pipiens* pupal exuviae from colony populations

DNA yield and PCR amplification success

Genomic DNA quantity was found to vary significantly between the different time points (KW χ^2 = 92.125, df= 5, $P < 0.01$; Figure 2.6). The highest DNA quantities resulted from the samples processed immediately after eclosion ($\bar{x}$ = 1.190 ng/µl; sd= 0.458 ng/µl) with mean nucleic acid concentrations decreasing as time post-eclosion increased. For samples processed immediately after eclosion, there was no significant difference between the yield of DNA obtained compared with those collected at 1-hour post-eclosion ($P = 1.00$), but significant differences were detected between all other time points ($P <0.05$). Similarly, for samples processed at 1-hour post-eclosion DNA yield did not differ significantly to those obtained from samples preserved at 6 hours post-eclosion ($P = 1.00$) but was significantly higher than those obtained from all time points 12 hours post eclosion and later ($P <0.05$).

Concentrations of DNA obtained from samples processed at 6 hours post-eclosion were not statistically different to those obtained at 1 and 12 hours post-eclosion ($P = 1.00$ and $P = 0.732$ for 1 hour and 12-hour time points respectively) however they differed significantly from all other time points ($P <0.05$). Concentrations obtained from samples processed at 12 hours post-eclosion were not significantly different to those obtained at 18 and 24 hours post-eclosion ($P = 0.105$ and $P = 1.00$ for 18 and 24-hour time points respectively), while no significant difference was detected between DNA concentrations obtained at 18 and 24 hours post-eclosion ($P = 1.00$) however, these all differed significantly from the other time points ($P <0.05$).

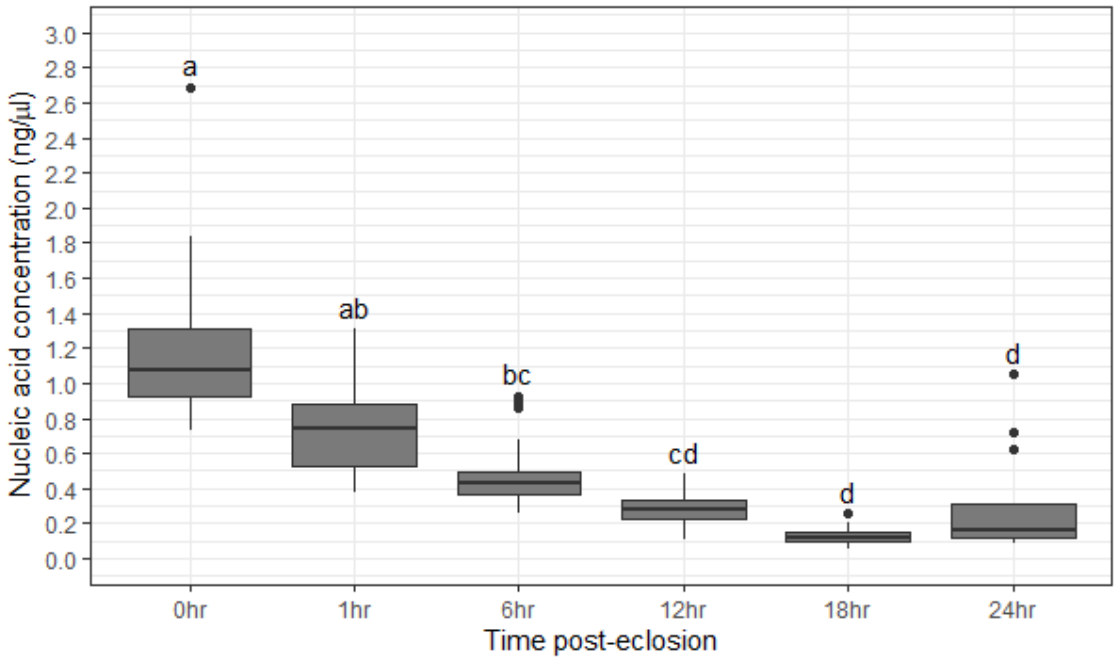

Figure 2.6. Comparison of DNA quantity (ng/µl) from all DNA extractions from individual *Culex pipiens* pupal exuviae at different time points post-eclosion. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

Time elapsed between collection and processing was shown to significantly affect PCR amplification success (Fisher's exact = P <0.001). Samples preserved at 18 or 24 hours post-eclosion demonstrated significantly lower PCR amplification success (55% and 45%) compared with samples preserved within 12 hours of eclosion ($P = 0.0184$ and $P < 0.001$ 18 and 24 hours respectively). However, these did not statistically vary from each other ($P = 1.00$; Figure 2.7). In contrast, no significant difference was observed between amplification success in samples preserved up to and including 12 hours post-eclosion ($P = 1.00$) with all samples showing amplification. Similarly, a trend was seen between concentration and Cq with a general decrease in Cq value as nucleic acid concentration increased (Figure 2.8) although the correlation was only significant for the first two time points (R = -0.57, $P = 0.0086$ and R= -0.64, $P = 0.0029$ for 0 hour and 1 hour respectively).

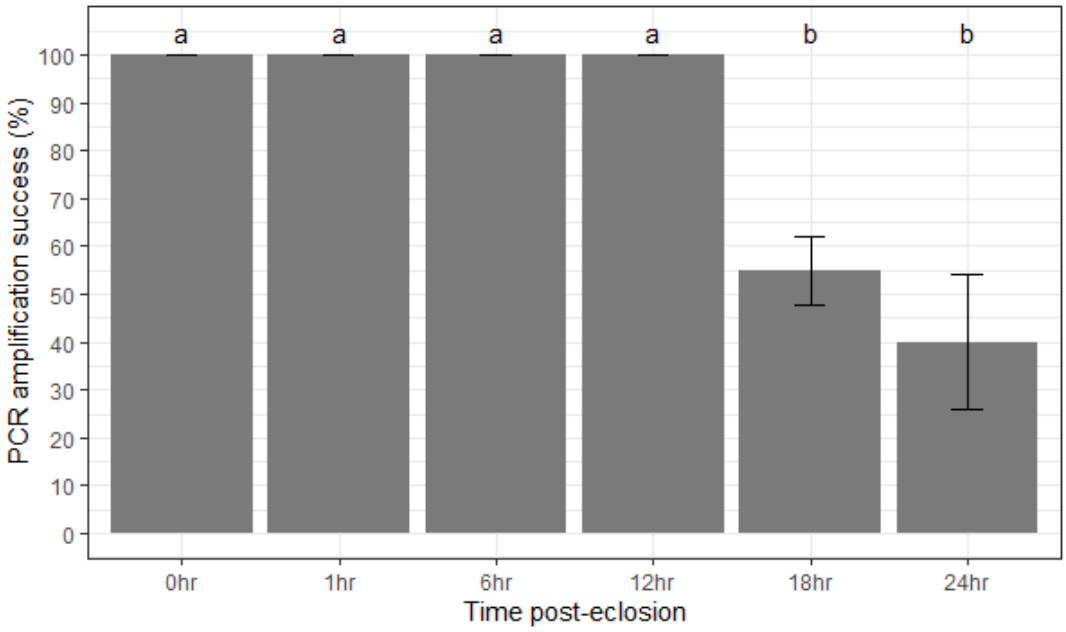

Figure 2.7. Comparison of PCR amplification success (%) of DNA extracts obtained from individual *Culex pipiens* pupal exuviae at different time points post-eclosion. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

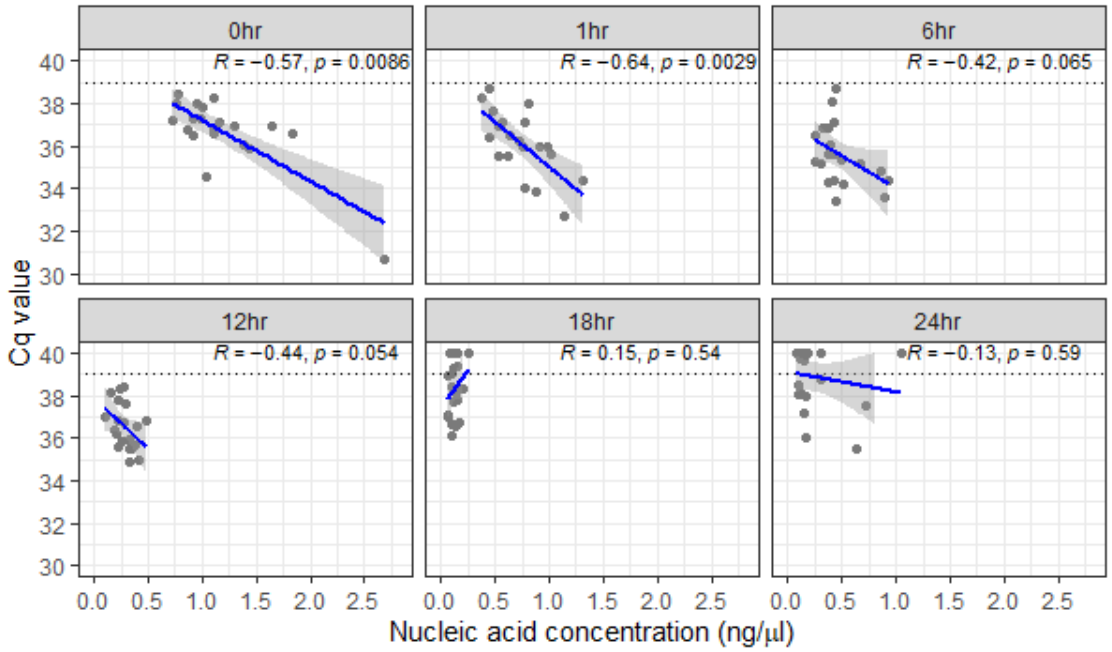

Figure 2.8. Comparison of nucleic acid concentrations (ng/µl) with mean Cq values for DNA extracts obtained from individual *Culex pipiens* pupal exuviae at six different time points post-eclosion. Grey shaded area indicates the 95% confidence interval.

Effect of body size on nucleic acid concentration

Wing measurements were used as a proxy for body size. No significant difference was detected between the left and right wing length of each individual mosquito (t= 0.0559, df= 224, *P* = 0.956). Wing measurements of males were found to be significantly smaller than those of female samples (t= 13.737, *P* < 0.001; Figure 2.9). Moreover, no statistical difference in wing length was detected between mosquitoes included in each time treatment group (F= 0.172, df= 5, *P* = 0.972). Wing length was overall shown to be positively correlated with nucleic acid concentration (t= 2.62, df= 111, *P* = 0.01), this interaction was shown to be significant in each time group except for the 18 and 24-hour time points (Figure 2.10). Finally, there was no statistically significant difference between the wing length measurements of different mosquito biotypes (F= 1.458, df= 2, *P* = 0.237; Figure 2.11).

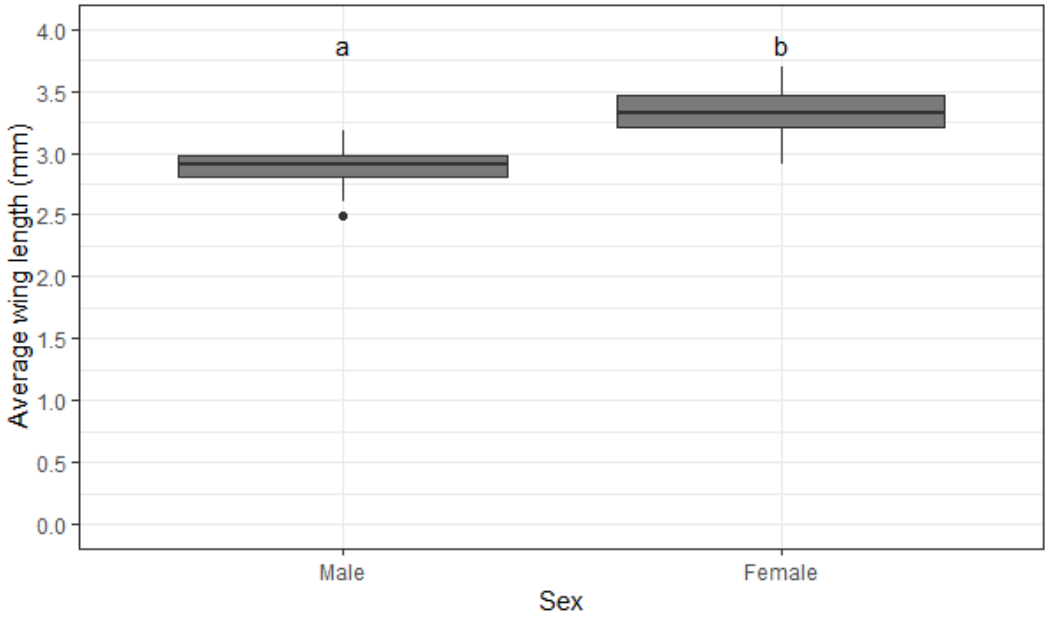

Figure 2.9. Comparison of male and female wing length measurements (mm) of eclosing adults used in experiments testing the efficiency of DNA extraction from individual *Culex pipiens* pupal exuviae. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. The letters above the boxplots indicate which groups differ significantly (*P* < 0.05) from one another. Specifically, groups which share a common letter are not significantly (*P* < 0.05) different from one another.

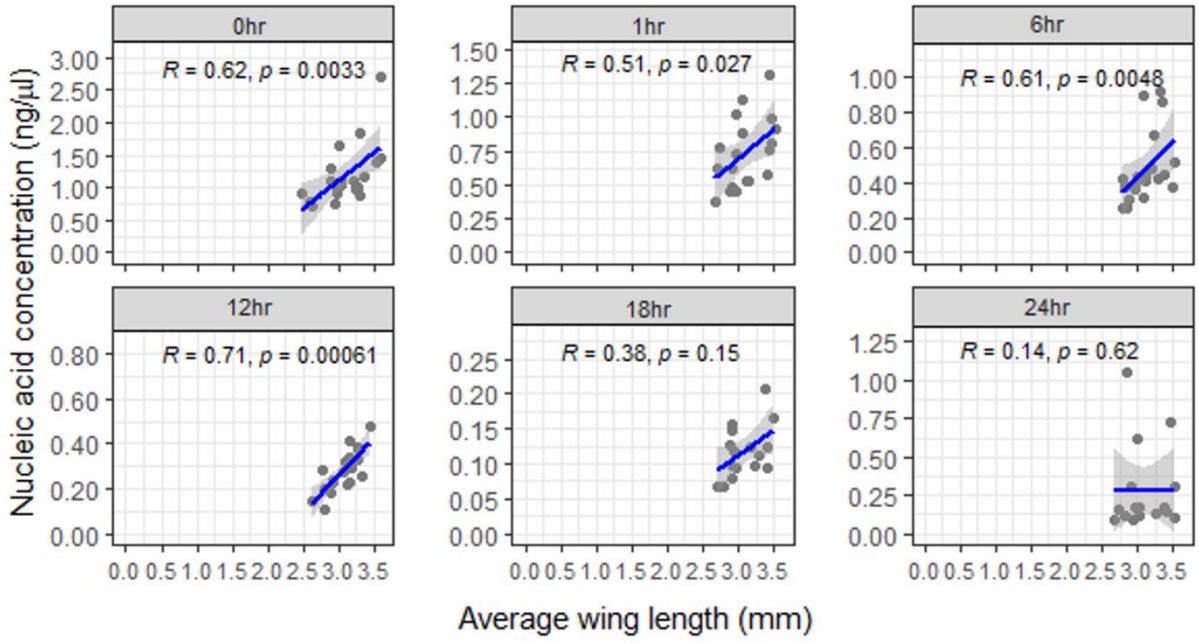

Figure 2.10. Comparison of nucleic acid concentrations (ng/µl) with mean wing length (mm) for DNA extracts obtained from individual *Culex pipiens* pupal exuviae at six different time points post-eclosion. Grey shaded area indicates the 95% confidence interval.

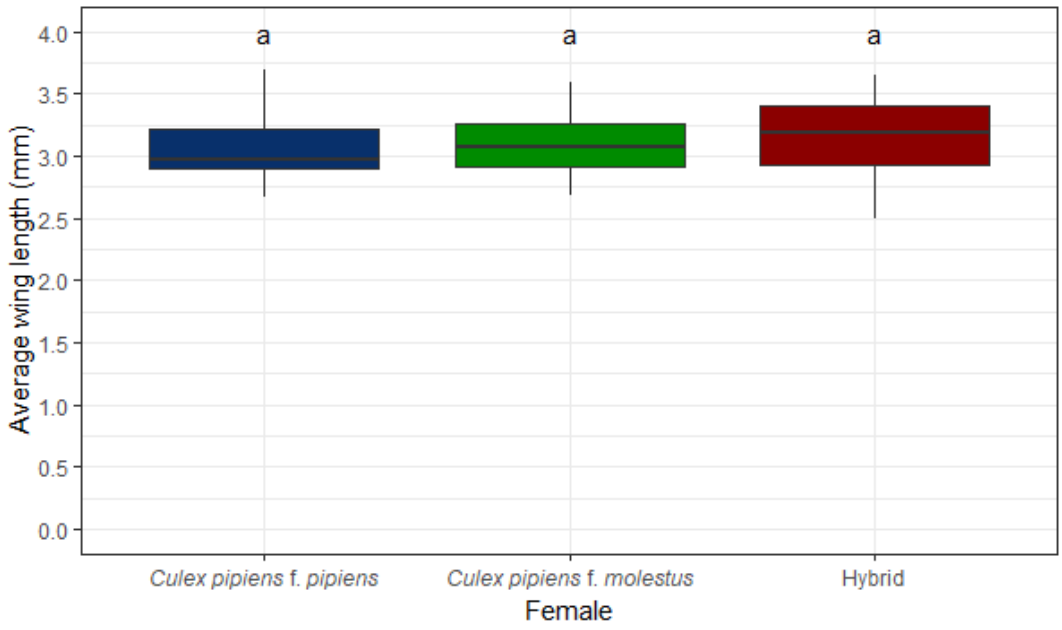

Figure 2.11. Comparison of wing length measurements (mm) for each of the *Culex pipiens* biotypes, according to the CQ11 assay. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

2.3.4 Assessment of processing methods from field collected pupal exuviae

Comparison of individually processed and pooled samples

Genomic PCR quantity was found to vary significantly between individually processed and pooled field exuviae samples (W= 16.6, $P < 0.001$; Figure 2.12). The highest PCR quantities resulted from the pooled samples ($\bar{x}$ = 1.000 ng/µl; sd= 0.181 ng/µl) which were approximately five times higher than the individually processed samples ($\bar{x}$ = 0.243 ng/µl; sd= 0.141 ng/µl). Amplification success was also affected significantly by the processing method with the pooled samples significantly more likely to successfully amplify compared with the individually processed samples (Fisher's exact P = 0.0292; Figure 2.13). Nucleic acid concentration was shown to be correlated with Cq value for both individually processed and pooled samples with Cq values shown to decrease as nucleic acid concentrations increased (Figure 2.14).

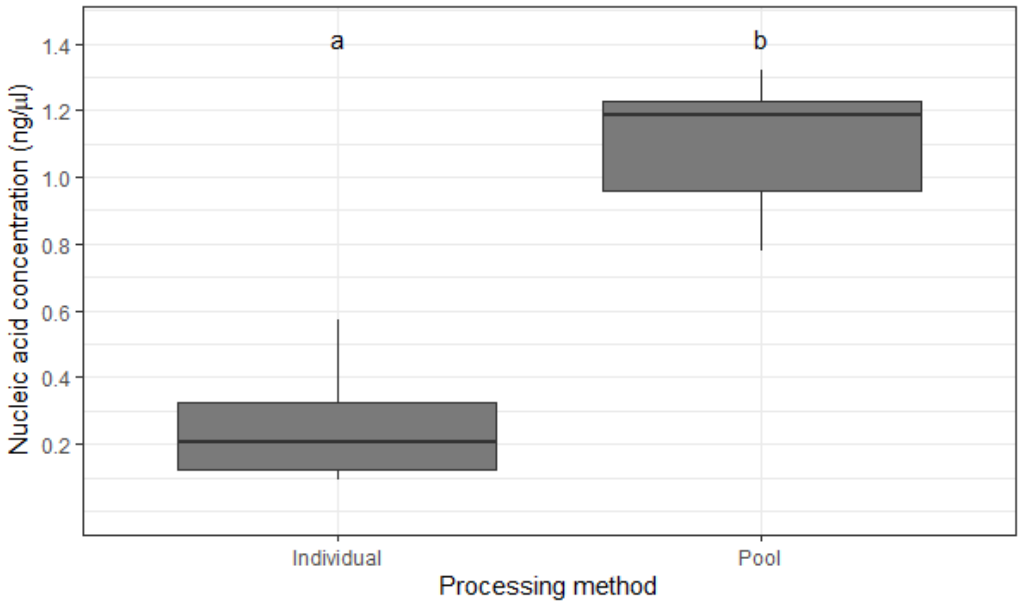

Figure 2.12. Comparison of PCR quantity (ng/µl) from PCR extracts obtained from individual and pooled field collected *Culex pipiens* pupal exuviae. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

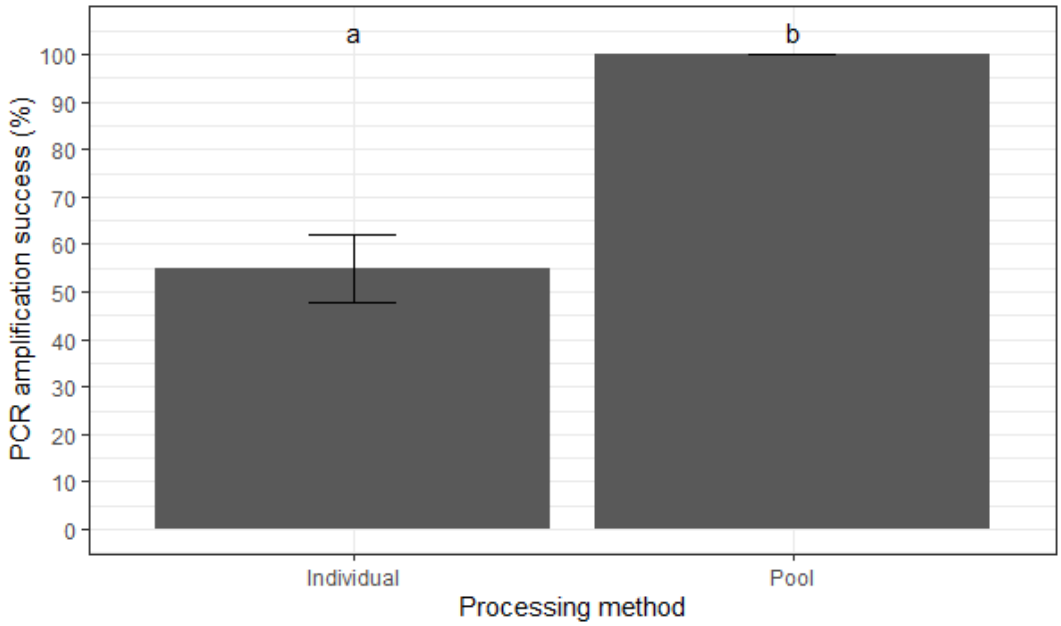

Figure 2.13. Comparison of PCR amplification success (%) for PCR extract obtained from individual and pooled field collected *Culex pipiens* pupal exuviae. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

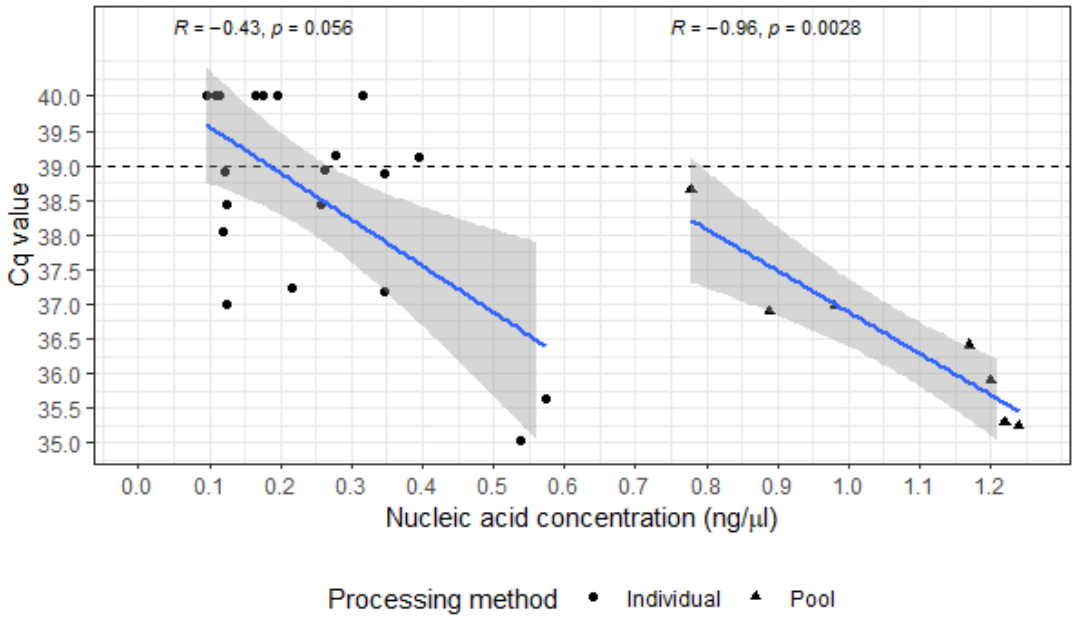

Figure 2.14. Comparison of nucleic acid concentrations (ng/µl) with mean Cq values for PCR extracts obtained from individual and pooled field collected *Culex pipiens* pupal exuviae. Grey shaded area indicates the 95% confidence interval.

Comparison of three PCR extraction methods and a direct PCR assay for field collected pupal exuviae

Quantity of PCR obtained from field samples was shown to vary significantly between the four processing methods (KW χ^2 = 45.82, df= 3, $P < 0.001$; Figure 2.15, Table 2.5). The greatest total PCR quantity was achieved using the ethanol precipitation method ($\bar{x}$ = 5.17 ng; sd= 1.75 ng) which was statistically higher than both the Dneasy® kit and the direct PCR ($P < 0.001$). The results obtained from the Wizard® kit did not statistically differ from the results from the ethanol precipitation method (P = 1.00), however, total concentration did vary significantly from both the Dneasy® and direct PCR (P = 0.00869 and P = 0.0.0276 for the Dneasy® and direct method respectively). The direct PCR was found to have no significant difference in total PCR concentration obtained when compared with the Dneasy® kit (P = 1.00).

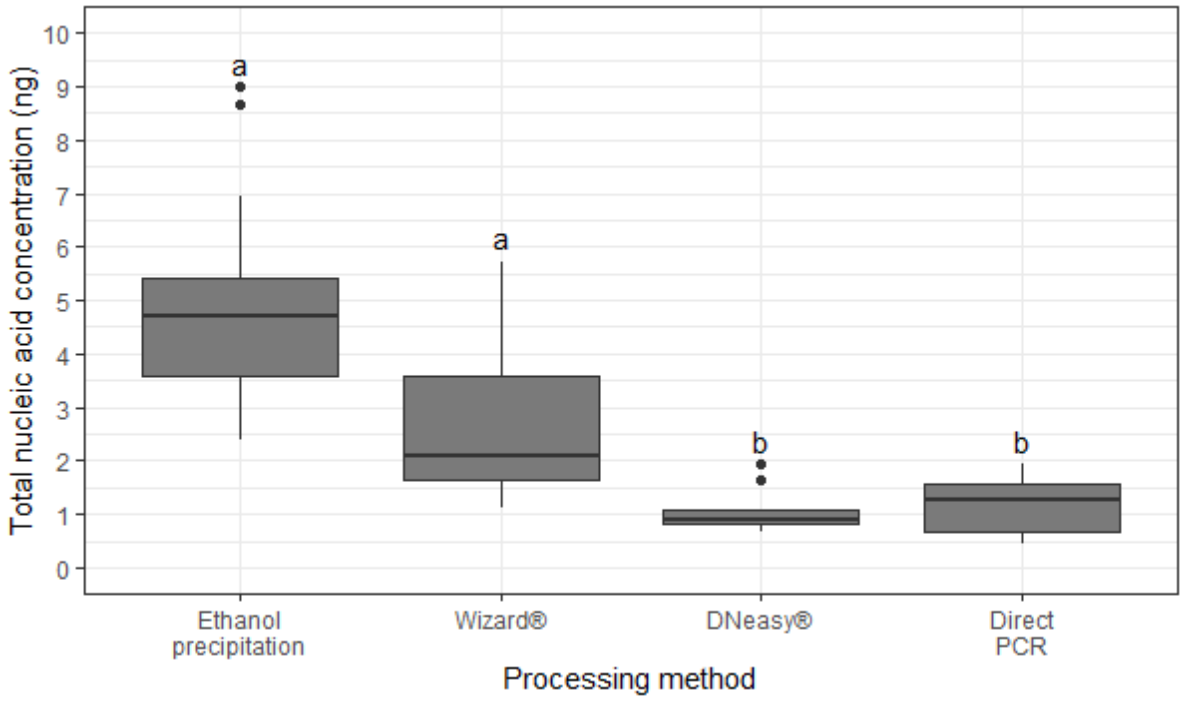

Figure 2.15. Comparison of total PCR quantity (ng) from PCR extractions between three extraction methods and direct PCR from field collected *Culex pipiens* pupal exuviae. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

There was no statistically significant differences in amplification success between the four processing methods (Fisher's exact $P = 0.367$) although the Wizard® kit yielded the highest amplification success (80%) (Figure 2.16, Table 2.5). There was a general trend in decreasing Cq with increasing nucleic acid concentrations (Figure 2.17), although this correlation was not statistically significant for any of the processing methods.

Table 2.5. Comparison of total PCR yield (ng) and PCR amplification success (%) for three extraction methods and direct PCR from individual field collected *Culex pipiens* pupal exuviae.

Method	Total PCR quantity (ng)		PCR success (%)	
	Mean	SD	Mean	SD
Dneasy®	1.07	0.437	60	0.00
Promega	2.71	1.37	80	0.00
Ethanol precipitation	4.76	1.87	70	0.00
Direct PCR	1.15	0.508	55	7.07

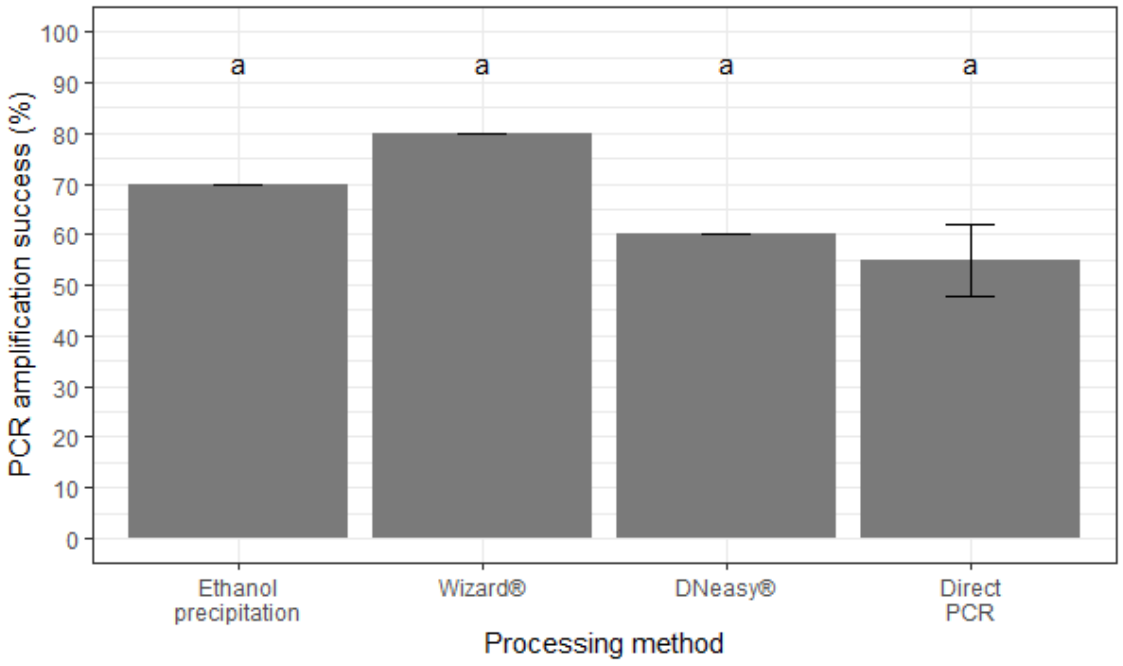

Figure 2.16. Comparison of PCR amplification success (%) for three different extraction methods and direct PCR for individual field collected *Culex pipiens* pupal exuviae. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

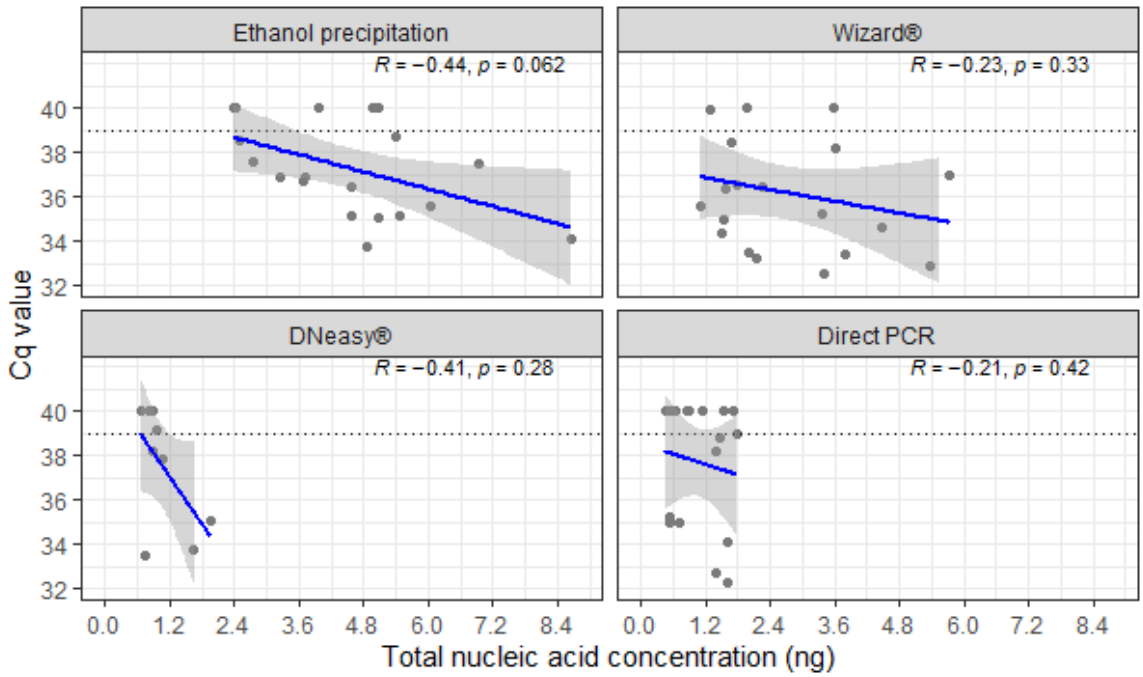

Figure 2.17. Comparison of total nucleic acid concentrations (ng) with mean Cq values for different processing methods of individual field collected *Culex pipiens* pupal exuviae. Grey shaded area indicates the 95% confidence interval.

When comparing nucleic acid concentrations obtained using the ethanol precipitation method between sampling years, a significant difference was detected (W=52, *P* =0.0366), with samples collected in 2021 yielding higher average concentrations compared with those collected in 2020 ($\bar{x}$ = 0.243 ng/µl; sd= 0.141 ng/µl and $\bar{x}$ = 0.345 ng/µl; sd= 0.117 ng/µl for 2020 and 2021 sampling period respectively; Figure 2.18). Although amplification success was higher for samples collected in 2021, this difference was not statistically significant (*P* = 0.5402; Figure 2.19).

Environmental conditions measured during the sampling periods were shown to vary between the two years. During 2020, average water temperatures of 21.3 ± 2.71°C were recorded which were significantly higher than those recorded during 2021 (17.7 ± 1.20°C; W = 354, *P* <0.001). Similarly, 2020 recorded significantly higher lux levels (34,444 Lux; W = 813, *P* <0.001) compared with those recorded during collections conducted in 2021 (10,066 Lux).

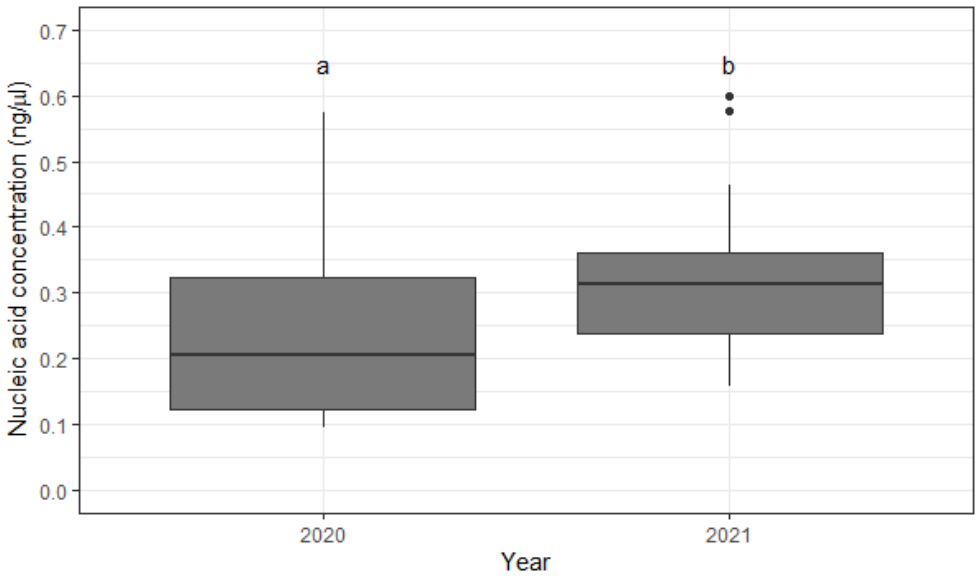

Figure 2.18. Comparison of DNA yield (ng/µl) from DNA extractions between different sampling periods from individual field collected *Culex pipiens* pupal exuviae using the ethanol precipitation extraction method. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

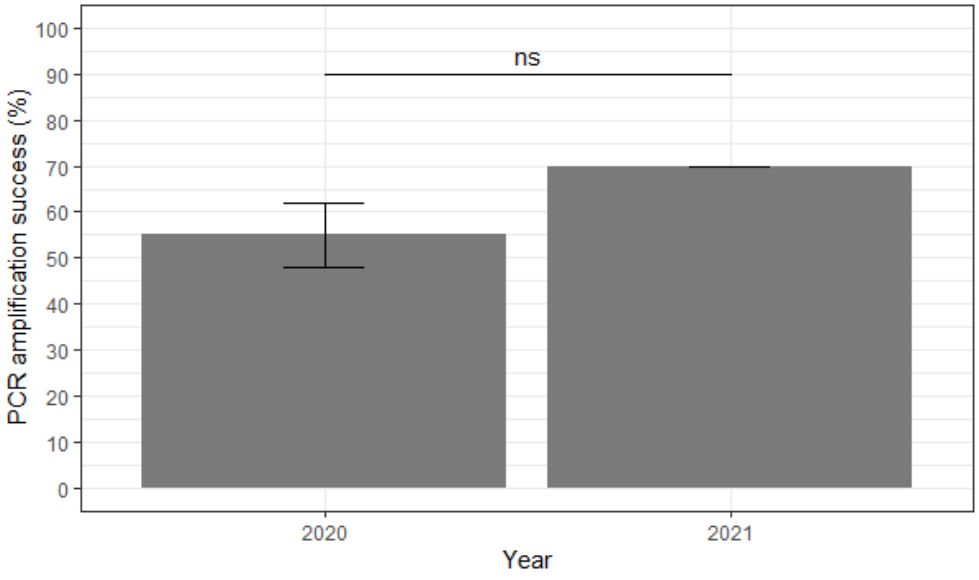

Figure 2.19. Comparison of PCR amplification success (%) for two different sampling periods for DNA extracts obtained from individual field collected *Culex pipiens* pupal exuviae using the ethanol precipitation method. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

Creation of pure colony lines using DNA extraction from pupal exuviae to select live individuals

Extraction of DNA from pupal exuviae was successfully used to select live individuals to create single biotype colonies from a mixed population, which were successfully reared for more than 10 generations. Mosquitoes tested from the 10th generation demonstrated that the colonies remained pure until this point (Table 2.6).

Table 2.6. Biotype of adult mosquitoes from the 10th generation of the single biotype colony lines created in Section 2.2.9, according to the CQ11 microsatellite marker.

Colony line	**Biotype**			**Total**
	Culex pipiens* f. *pipiens	***Culex pipiens* f. *molestus***	**Hybrid**	
Culex pipiens f. *pipiens*	40	0	0	40
Culex pipiens f. *molestus*	0	40	0	40
Hybrid	9 (22.50%)	8 (20.00%)	23 (57.50%)	40

2.4 Discussion

In the present study, successful DNA extraction and PCR amplification from individual pupal exuviae was demonstrated from colony and field reared *Culex pipiens* populations using three different extraction methods. Extracted DNA was successfully used to accurately identify biotypes within the *Cx. pipiens* species and was utilised to create single-biotype colony lines from a mixed population which are extant and remain pure. Time trials also revealed 100% amplification success in *Cx. pipiens* pupal exuviae collected up to 12 hours post-eclosion under laboratory conditions. Development of this method for biotype differentiation allowed for selection of live individuals of known biotype and creation of single-biotype colony lines which enabled work undertaken in subsequent studies (Chapters 3 and 4 of the thesis) that logistically would not have been possible using a mixed colony or field population.

Successful DNA extraction and PCR amplification from individual pupal exuviae was demonstrated for up to twenty-four hours post-eclosion although amplification success was shown to decrease as time post-eclosion increased. Time points up to and including twelve hours post-eclosion showed 100% amplification success, although a relatively small number of samples were tested, therefore with increased sample sizes this would be expected to slightly decrease. Indeed, amplification success was observed to drop slightly below 100% when the ethanol precipitation method was applied in high quantities for subsequent experiment set-ups. This slight decrease in amplification success could be due to the high throughput nature of the assay, as although every effort was made to ensure exuviae were collected every twelve hours, this cannot be guaranteed as some samples may have been missed. Alternatively, other factors may have accelerated degradation of nucleic acids in these samples such as the presence of microorganisms. Amplification success was seen to significantly decrease for the eighteen- and twenty-four-hour time points to 55% and 40% respectively, highlighting the limit of this assay, likely due to the degradation of nucleic acids.

Successful DNA extraction and PCR amplification from pupal exuviae of laboratory reared *Culex* and *Aedes* species within the first twenty-four hours post-eclosion has previously been reported (Dhananjeyan *et al.*, 2010). However, no successful amplification seen after twenty-four hours (and up to nine days) post-eclosion when testing pupal exuviae from colony reared individuals. Contrastingly, a subsequent study attempting to replicate this with *Ae. aegypti* pupal exuviae collected one-hour post-eclosion failed to amplify PCR targets (Wong *et al.*, 2012). Neither study quantified the resulting DNA extraction concentrations, therefore it is unknown whether the quantity of DNA used in the PCR was sufficient to meet the lower threshold of the reaction to allow detection. In contrast, the current study utilised a real-time PCR method with a high dynamic range, capable of amplifying

small quantities of DNA as low as $2x10^{-4}$ ng (Appendix 1). The real-time PCR method used in the current study is likely to have a lower detection threshold compared with the traditional PCR methods used in previous studies which could in part explain the increased amplification success seen. Further work should determine if DNA yield obtained from methods developed during this study are sufficient for successful amplification in endpoint PCR assays. This would facilitate the adaptation of this method to PCR assays targeting different markers and species.

The method used for processing samples was shown to significantly affect DNA quantity and PCR success for colony reared *Cx. pipiens* pupal exuviae. When considering all criteria, processing methods involving prior extraction of samples provided superior concentration of isolated trace DNA from single *Cx. pipiens* pupal exuviae. The isolation of DNA by ethanol precipitation provided the best results when considering all factors among methods tested. Although this method had the longest processing time, the DNA quantity was significantly higher than the other processing methods whilst also having the lowest processing cost compared with the other extraction methods. In contrast, previous studies comparing DNA extraction success from chironomid pupal exuviae using five different extraction methods found that overall, the commercial kits tested yielded the best results (Kranzfelder *et al.*, 2016). However, the chironomid study used a DTAB/CTAB lysis protocol to compare against commercially available kits, whereas the present study employed an ethanol precipitation method with a SDS lysis step. For the current project, the lower processing cost of the ethanol precipitation method facilitated processing of greater sample sizes. Therefore, the method used would depend on the resources available to different projects as well as the skill required for each method. For example, despite the higher cost per sample, processing times of commercial kits were reduced, this coupled with their ease of use and wide application to a variety of sample types, may overall favour the use of commercial kits for certain projects.

Both the DNeasy® and the Wizard® kits obtained high amplification success, similar to the ethanol precipitation method, despite obtaining lower total nucleic acid concentrations. Previous attempts to isolate DNA from individual mosquito pupal exuviae have excluded assessment of nucleic acid concentrations obtained therefore precluding direct comparison. However, higher concentrations were obtained from the present study compared with those achieved in similar studies with chironomids, despite chironomids have a larger body size (Kranzfelder *et al.*, 2016). Chironomids used in this previous study were obtained from field collections within 48 hours of eclosion, therefore it is difficult to draw direct comparisons as all specimens used in the current study were collected immediately following eclosion and thus are more likely to yield higher quantities of DNA. Despite

differences in sample collection parameters, target sequence amplification success was shown to be similar between the two studies, with both obtaining high rates of PCR amplification success.

Although the direct PCR method had a reduced amplification success rate (55% compared with 100%), this method was significantly cheaper and quicker to perform than the other processing methods tested, which would allow for rapid screening of populations. However due to reduced amplification success, this method would not be suitable for use in studies that only plan to use a small number of highly valuable specimens, for example those collected in hard to access areas. This method would also be of particular benefit for preliminary non-invasive screening of populations at a reduced cost or when utilising starting material with higher PCR success rates such as whole larvae for a quicker and cheaper screening method (Thongjued *et al.*, 2019).

When PCR success was assessed using a direct PCR method in chironomid specimens, very low success rates were obtained ($\bar{x}$= 2.11% ± 14.43). In contrast, the present study obtained significantly higher amplification success (55%), likely due to the use of an optimised direct PCR method. The direct PCR method in the present study incubated the exuviae in PBS which benefits from dilution of PCR inhibitors whilst also maintaining the pH of the reaction (Kitpipit *et al.*, 2014). Moreover, the heating step aids cell lysis, releasing DNA and denaturing proteins that could degrade DNA or inhibit the PCR (Grevelding *et al.*, 1996; Loto *et al.*, 2013). This method has been shown to be widely applicable to insect species identification with amplification success rates of 90.5% from Diptera specimens when using an approximately 1x1 mm^2 piece of tissue as template (Thongjued *et al.*, 2019). This previous study utilised a high-fidelity DNA polymerase specifically designed for use in direct PCR application with increased tolerance to PCR inhibitors. Contrastingly, amplification success in the present study was reduced, likely due to the type of tissue utilised as a template as well as the difference in polymerase used within the PCR master mix as the Phire™ DNA polymerase provides a two-fold increased fidelity compared with *Taq* DNA polymerases used here.

The present study also demonstrated successful DNA extraction and PCR amplification of field collected pupal exuviae when processed both individually and in pools of five exuviae, with individual processing applicable when comparing multiple processing methods. Processing of exuviae in pools yielded higher nucleic acid concentrations as well as consistent PCR success compared with individually processed exuviae (100% compared with 55%). Successful extraction and amplification from field collected insect exuviae has been demonstrated previously (Krosch and Cranston, 2012; Kranzfelder *et al.*, 2016; Kranzfelder *et al.*, 2017). However, attempts to extract PCR amenable DNA from field collected mosquito exuviae was not previously successful (Dhananjeyan *et al.*, 2010),

although the effects of larval habitat parameters such as exposure to sunlight and temperature were not considered.

Natural larval populations are exposed to fluctuating environmental conditions compared with colony reared larvae which can influence larval development and other life history traits. Most studies do not provide a comprehensive description of the larval habitat and environmental conditions at the time of sampling and, therefore it is difficult to understand the factors that may contribute to an accelerated degradation of nucleic acids in field exuviae compared with colony samples. Previous studies assessing the effects of different environmental factors on the degradation of environmental DNA (eDNA) in aquatic environments have found varying results with some studies demonstrating that factors including ultraviolet B radiation (UVB), pH, salinity and presence of microorganisms can influence degradation rate of eDNA (Strickler *et al.*, 2015; Tsuji *et al.*, 2017).

Nucleic acid concentration of field collected pupal exuviae were significantly affected by the year of sampling with higher results seen from the second sampling period when using the ethanol precipitation method. Although PCR success was also higher for the second sampling period, this difference was not significant. Average temperature and light intensity varied between the two sampling years which could have influenced the differences in nucleic acid concentrations obtained, as well as other unknown and unquantified factors such as microbial content of the water.

To conclude, the present study demonstrated that *Culex pipiens* pupal exuviae can be utilised as a source of non-invasive trace DNA for accurate biotype differentiation for up to twenty-four hours post-eclosion. Successful PCR amplification was demonstrated from both colony and field collected exuviae through various methods of processing and is therefore accessible to a wide range of projects with different levels of available resources. Successful application to field populations could add further benefit by facilitating initial screening of populations without the requirement of adult or larval collections. Finally, preliminary single-pair mating results highlighted logistical challenges with the use of mixed colony lines lacking morphological identifiers between forms, meaning biotype delineation of the mating pairs were carried out retrospectively. This posed challenges for achieving the required sample size numbers for all pairings as the *molestus* form was found to constitute a small proportion of the overall colony population. Thus, development of this method greatly enhanced the ability to investigate specific behaviours and life history traits of each of the *Cx. pipiens* forms from a mixed population through the creation of pure biotype colony lines, which remained pure after 10 generations. Further work should expand on testing the limitations of this assay, for example to determine if DNA yield is sufficient for use in endpoint PCR methods. This method could be of particular benefit to further studies examining behavioural differences between the forms from

sympatric populations and could be utilised to establish pure colonies from field derived individuals. Thus, this may provide a useful tool for work with other cryptic mosquito species.

Chapter 3 – Investigating barriers to gene flow between the *Culex pipiens* biotypes

3.1 Introduction

Culex pipiens complex mosquitoes are important vectors of several diseases worldwide, including West Nile virus (Kilpatrick *et al.*, 2005) and Usutu virus (Calzolari *et al.*, 2012). The taxonomy of the *Cx. pipiens* complex remains uncertain due to their morphological similarity, despite apparent disparity between their ecological, physiological, and behavioural traits (Fonseca *et al.*, 2004). These traits have the potential to influence the vectorial capacity of populations worldwide and play a major part in determining their role in the transmission of pathogens (Vogels *et al.*, 2017a). Despite their contribution in disease transmission, data regarding mating behaviour in *Cx. pipiens* are scant compared with that regarding *Aedes* and *Anopheles* mosquitoes (Benelli, 2018). The *Cx. pipiens* species further consists of two morphologically indistinguishable biotypes, *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*, which also have distinct ecological and behavioural traits that make studies of their potential role as disease vectors challenging.

Natural hybridisation between the two biotypes has been reported from several countries (for review see Brugman *et al.*, 2018). Gene flow between the two forms is thought to facilitate transfer of biotype specific traits, with hybrid individuals possessing intermediate behaviours between the two parental extremes. For example, *Cx. pipiens* f. *pipiens* has a strong bias for bird feeding, whilst the *Cx. pipiens* f. *molestus* biotype readily bites humans and other mammals (Fritz *et al.*, 2015). Thus, hybrid species have the potential to act as bridge vectors between avian reservoirs and a susceptible mammalian population for arboviruses such as West Nile virus and Usutu virus (Vinogradova, 2000). Understanding the abilities of the *Cx. pipiens* forms to produce viable progeny, and the maintenance of hybrid forms in natural populations, is of medical importance and has implications when considering viral transmission dynamics.

Speciation can occur through the evolution of isolating barriers which prevent gene flow between sympatric populations (Coyne and Orr, 1998). These barriers involve a variety of processes that can act during every stage of reproduction, from courtship displays through to sperm-egg interactions, and are usually divided into barriers acting either before (pre-zygotic) or after (post-zygotic) fertilisation. Pre-zygotic barriers include temporal, ecological, behavioural, mechanical and gametic isolation. While post-zygotic barriers include zygote non-viability and mortality, hybrid sterility and cytoplasmic incompatibility caused by the presence of microorganisms. Several barriers to gene flow between *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* have so far been described. Of these, it has

been hypothesised that ecological and behavioural isolation play pivotal roles in limiting hybridisation between the two biotypes (Fonseca *et al.*, 2004).

Ecological divergence and reproductive isolation can arise from an accumulation of genetic differences necessary for survival and reproduction in different niches (Boake, 2000). Mating barriers have been demonstrated between insect strains from distinct geographical areas, for example in the South American fruit fly (Vera *et al.*, 2006). Reasons for these mating barriers include disparity in life history traits due to exposure to different selection pressures (Stearns, 1992; Roff, 2002; Lounibos *et al.*, 2003; Armbruster and Conn, 2006), variation in wing morphology (Imasheva *et al.*, 1995; Azevedo *et al.*, 1998; Gilchrist *et al.*, 2000; Hoffmann and Shirriffs, 2002; Sendaydiego *et al.*, 2013; Hidalgo *et al.*, 2015), and adult fitness (Leisnham *et al.*, 2008; O'Donnell and Armbruster, 2009).

Traditionally, the two *Cx. pipiens* forms were thought to occupy distinct ecological niches with the *pipiens* biotype associated with aboveground habitats whilst the *molestus* biotype was predominantly found in underground environments such as tunnels, basements and sewers (Byrne and Nichols, 1999). Thus, ecological isolation was thought to be the major barrier limiting gene flow between the two forms, however, more recent studies have led to the identification of sympatric populations in several countries (Brugman *et al.*, 2018). Despite this, natural hybridisation rates have been shown to vary significantly between studies, ranging from as little as 1.7% in Germany (Rudolf *et al.*, 2013) up to 36.8% in Algeria (Korba *et al.*, 2016), suggesting that barriers to gene flow are multifaceted.

Behavioural differences between the forms have also been suggested as a limiting factor to their hybridisation. In general, *Cx. pipiens* f. *pipiens* preferentially mates in swarms (eurygamous) whilst biotype *molestus* are capable of mating under stenogamous conditions (Byrne and Nichols, 1999). A recent study assessing mating behaviours of the two forms under stenogamous conditions reported a significantly higher mating success rate of homologous *Cx. pipiens* f. *molestus* crosses compared with *Cx. pipiens* f. *pipiens* and reciprocal crosses (Kim *et al.*, 2018b). Mating success was however seen to increase to comparable levels for some combinations of F1 hybrid crosses. Additionally, t has been hypothesised that due to the ability of *Cx. pipiens* complex species to obtain high mating success in confined laboratory cages, swarming was not essential to mating (Benelli, 2018). A mating success rate of 56.84% of *Cx. pipiens* under laboratory conditions suggested that male swarming is not essential to mating success, however, the proportion of the biotypes within the population was not determined (Benelli, 2018).

Furthermore, high mating success and insemination in *Cx. quinquefasciatus* within relatively small laboratory cages, supporting the hypothesis that male swarming was not essential to mating under

laboratory conditions (Sebastian and de Meillon, 1967). Likewise, Kim *et al.* (2018b) observed that although *Cx. pipiens* mating pairs take flight together prior to mating, the duration of the co-flying was brief compared with swarming, suggesting that auditory mechanisms play a minor role in mate recognition and mating under confined conditions. Rather, repetition of certain behaviours within the mating sequence such as tapping suggest olfactory and gustatory mechanisms may be heavily relied upon for successful mating in *Cx. pipiens* mosquitoes under stenogamous conditions.

A further two post-zygotic barriers have also been suggested between *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* which may result in reduced fitness of the hybrids. One of these is the inheritance of diapause as a recessive trait (Vinogradova, 2003). Previous studies have demonstrated that F1 hybrids and a significant proportion of F2 hybrids were unable to undergo diapause meaning they could not survive winter conditions as adults (Fonseca *et al.*, 2004; Gomes *et al.*, 2015). In addition, reciprocal crosses of two pure colony lines of *Cx. pipiens* demonstrated a reduction in fecundity and fertility when compared with homologous crosses of *Cx. pipiens* f. *molestus* under stenogamous conditions (Kim *et al.*, 2018b). These reciprocal crosses were also shown to have a significantly lower survival rate, decreasing over six-fold, however, survival of progeny from homologous crosses of F1 hybrids increased again to comparable levels of homologous parental crosses. Survivability in this study was defined as the survival of first instar larvae to adult and did not consider hatching success.

Finally, members of the *Cx. pipiens* complex are exposed to cytoplasmic incompatibility of hybrids infected with different strains of *Wolbachia* (Dumas *et al.*, 2013). For example, hybridisation was significantly reduced between *Cx. pipiens* and *Cx. quinquefasciatus* in South Africa by natural infection of *Cx. quinquefasciatus* with *Wolbachia* (Cornel *et al.,* 2003). Furthermore, differential *Wolbachia* infection of the two biotypes from Eurasian populations has been reported, with *Cx. pipiens* f. *molestus* infected by one strain of *Wolbachia* while *Cx. pipiens* f. *pipiens* was infected with two strains (Khrabrova *et al.*, 2009). However, the extent to which *Wolbachia* induced cytoplasmic incompatibility contributes to reproductive isolation between the biotypes is currently unknown (Brugman *et al.*, 2018).

A major challenge for studies working with mixed or undefined populations is the differentiation of the *Cx. pipiens* biotypes. Numerous authors have suggested morphological features of eggs, larvae, pupae, and adults for the differentiation of the forms, but this is not well established. Ecological and physiological differentiation of the two forms is well established and widely implemented as a method of delineation. However, more recent research suggests that these biotype definitions are not as rigid as previously thought with genetically identified *Cx. pipiens* f. *pipiens* mosquitoes showing signs of autogenous behaviour in rare circumstances (Gomes *et al.*, 2009; Beji *et al.*, 2017) as well as the lack

of autogeny in *Cx. pipiens* f. *molestus* individuals from certain habitat types (Osorio *et al.*, 2014; Beji *et al.*, 2017).

The lack of clarity in the differentiation of the biotypes has meant that previous behavioural mating studies have utilised two individual, genetically pure colonies from distinct geographic locations (Kim *et al.*, 2018b). Mixed colonies of both biotypes have rarely been used to assess mating success with differentiation of the forms omitted from the study due to the complexity (Benelli, 2018). Geographic mating polymorphisms have been noted between three colonies of *An. stephensi* (Rutledge *et al.*, 1970; Mahmood and Reisen, 1982), however the same was not observed when a population of *An. arabiensis* from La Réunion Island mated freely with continental African colonies (Girod *et al.*, 2001). Any differences observed in mating behaviours between genetically distinct *Cx. pipiens* forms may be confounded by the effects of laboratory maintenance or local adaptation. Therefore, non-invasive methods for DNA extraction can allow for biotyping of mosquitoes from sympatric populations prior to these experiments which would permit formation of targeted mating pairs arising from a mixed population.

The present study aims to investigate mating barriers between the *Cx. pipiens* biotypes from colony lines originating from a mixed population. To assess the hypothesis that hybrid progeny will suffer fitness costs, a series of experiments were conducted to investigate the effects of different cross combinations on a variety of life history traits. This study includes assessment of adult traits including blood-feeding success, fecundity, fertility, and mating success. Larval traits including larval development times, larval survival, pupation success and sex ratio will also be assessed. Mating success under single-pair and grouped mating conditions will be investigated to test the hypothesis that the single-pair mating conditions will provide a barrier to mating within the *pipiens* form. Assessment of assortative mating of each female form will be undertaken to test the hypothesis that female *Cx. pipiens* will preferentially mate with conspecific males. Understanding factors that may influence levels of hybridisation between the biotypes of *Cx. pipiens* within natural populations is imperative to accurately assess risk of disease transmission.

3.2 Methods

3.2.1 Mosquito rearing

Single-pair mating experiments utilised a mixed colony of *Cx. pipiens* biotypes originating from an allotment area in Surrey, United Kingdom established in 2011 (Manley *et al.*, 2015). Grouped mating experiments utilised pure biotype colony lines derived from this mixed population as per Chapter 2. Adult mosquitoes from the 'Brookwood' lines were offered blood overnight using Hemotek feeding system (Hemotek™ Ltd., UK) with defibrinated horse blood (TCS Bioscience, UK). For all experiments, egg rafts were collected and left to hatch in approximately 500 millilitres (ml) of water. Hatching larvae were reared at a density of 200 larvae/litre (L) and were maintained on a diet of 1 milligram (mg) of guinea pig pellets/larva (Pets at Home, UK) on alternate days. Larvae were reared in an environmentally controlled incubator for the duration of the experiment. Environmental conditions were maintained at 25^{o}C ± 1^{o}C and relative humidity of 50% ± 1%. Lighting regimes consisted of a 16:8 light: dark cycle with an hour of dusk and dawn either side. Adult mosquitoes were provided with 10% sucrose *ad libitum*.

3.2.2 Single-pair mating

Pupae were collected daily and separated individually into 2.0 ml tubes in approximately 1 ml of water and monitored for eclosion. Following eclosion, pupal exuviae were collected at least every 12 hours and transferred individually to 70% ethanol for storage prior to DNA extraction and biotyping (described in Sections 3.2.6 and 3.2.7). Following biotype identification, one male and one female mosquito pupating within the same 24-hour period were released using an aspirator into a 12-ounce (oz) cardboard pot (Cater4you, UK) through a hole in the bottom which was sealed with cotton wool. Pots were left for 1 week to allow for mating under the same conditions described in Section 3.2.1. Adult mosquitoes were maintained on 10% sucrose provided *ad libitum*. For each combination of biotypes 15 mating pairs were created.

Mating success was assessed by dissecting the spermathecae of female mosquitoes and looking for the presence of sperm within the spermathecae. Briefly, female mosquitoes were placed ventral side up on a glass microscope slide under a dissecting microscope. The terminalia of the female was removed with forceps and separated from the rest of the body. The spermathecae were isolated from the terminalia, transferred to a new microscope slide and observed for the presence of sperm under

a compound microscope at 400x magnification (Leica Microsystems, Germany). A successful mating event was characterised by the presence of sperm in the spermathecae (Rosay, 1969).

3.2.3 Assessment of reproductive characteristics of different crosses

Experiments to assess the effects of hybridisation on life history traits utilised single biotype colonies created as per Chapter 2. Larvae from these lines were reared as described above in Section 3.2.1. Following the onset of pupation, pupae were collected daily and separated into males and females on the basis of external structure of the terminal segment (Christophers, 1960) under a dissecting light microscope (Leica Microsystems, Germany) for the *pipiens* and *molestus* colonies. The terminal segment of male pupae is shield-shaped and bifurcate on the distal end whereas females are generally more broadly ovate in shape. Pupae from the hybrid line were collected into 2.0 ml tubes and processed to obtain individuals testing as heterozygous for both alleles as described above in Section 3.2.2. Experimental cages to assess the fecundity and fertility of different crosses were created by mixing 1-3 day-old adults, for each pairing cages consisted of 20 males and 20 females.

Mosquitoes were left for seven days to allow for mating, following which female mosquitoes were offered defibrinated horse blood (TCS Bioscience, UK) using a Hemotek feeding system (Hemotek™ Ltd., UK) overnight for three consecutive nights with fresh blood provided each evening. Blood-fed mosquitoes were removed from the cage and transferred individually to 12 oz cardboard pots with a small oviposition cup containing approximately 15 ml water. Oviposition cups were monitored for the presence of egg rafts over seven days (Richards *et al.*, 2012), following which female mosquitoes were collected in 70% ethanol for dissection to determine mating frequency (described above in Section 3.2.2) and to check for egg retention. Laid egg rafts were collected and monitored for hatching, following which surviving hatched larvae were counted and fecundity and fertility of the crosses were calculated. Egg rafts were observed under a light microscope (Leica Microsystems, Germany) to determine the size and hatching success of egg rafts. Female fecundity corresponded to the number of eggs laid per female whilst fertility was estimated by the number of larvae hatched from each egg raft.

3.2.4 Larval development characteristics of different crosses

A subset of six hatched egg rafts per cross were used to assess the survival and development traits of larvae from each of the crosses. Larvae from each egg raft were reared individually under the same conditions as described above in Section 3.2.1. Numbers of surviving larvae were counted on alternate days when food was provided. Following the onset of pupation, pupae were collected daily, with

proportion of males and females counted by method described above in Section 3.2.3 (Christophers, 1960). Pupae were subsequently separated into individual pots by cross and sex to monitor eclosion success. Larval development times were defined as the time taken for 50% of larvae to reach pupation according to (Kassim *et al.*, 2012b).

3.2.5 Assessment of mating choice

Single biotype colonies created from the Brookwood mixed colony line were reared as described above (Section 3.2.1.). Following the onset of pupation, pupae were collected daily and separated into males and females of the *pipiens* and *molestus* biotypes. Hybrid individuals were obtained by collected individual pupal exuviae testing as heterozygous for both alleles as describe above (Section 3.2.2). Experimental arenas to assess mating choice were created by mixing 1-3 day-old adults. Cages consisted of single biotype females mixed with all three male biotypes at a ratio of 3:1 in a cage (17.5 x 17.5 x 17.5 cm BugDorm, Watkins and Doncaster, UK) with a total number of 30 mosquitoes per cage. A separate set of experimental cages of the same dimensions were also prepared consisting solely of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* to assess polyandry frequency. Here, single biotype females were mixed with *pipiens* and *molestus* male biotypes in a ratio of 2:1 per cage consisting of a total number of 20 mosquitoes. For all experiments four replicates were completed. Experimental cages were left for one week to allow for mating, following which females were collected and placed into 70% ethanol. Storage of females in 70% ethanol causes the proteinaceous fluid in the sperm to coagulate meaning a sperm bundle free of contaminating female DNA can be collected (Tripet *et al.*, 2001).

Female mosquitoes were transferred from ethanol to distilled water for rehydration two to three days prior to dissection. Sperm bundles were dissected under a stereo microscope (Leica Microsystems, Germany) at 60x magnification and collected for DNA extraction and biotype identification (Figure 3.1). Dissecting instruments were cleaned between each dissection with a three-stage wash step consisting of Microsol4 (Anachem, UK), 70% ethanol and distilled water to prevent cross contamination between samples.

3.2.6 DNA extraction

Pupal exuviae were removed from ethanol and left to air dry for approximately 10 minutes prior to DNA extraction to allow evaporation of excess ethanol. Subsequently exuviae were transferred into 200 µl digestion solution consisted of 100 mM UltraPure™ 1 M Tris-HCL (pH 8.0) (Invitrogen™ by Thermo Fisher Scientific, UK), 200 mM sodium chloride (Invitrogen™, UK), 0.2% (w/v) SDS (Merck Life

Science UK Limited, UK), 5 mM UltraPure™ 0.5 M EDTA (pH8.0) (Invitrogen™, UK), 200 µg/ml proteinase K (Qiagen, UK), made up to a total volume of 200 µl per sample using UltraPure™ water (Invitrogen™, UK).

Extraction of DNA from sperm bundles was performed using a modified version of a forensic protocol (Walsh *et al.*, 1991). Sperm bundles from individual females were placed into 200 µl of digestion solution consisted of 100 mM UltraPure™ 1 M Tris-HCL (pH 8.0) (Invitrogen™, UK), 200 mM sodium chloride (Invitrogen™, UK), 0.2% (w/v) SDS (Merck Life Science UK Limited, UK), 5 mM UltraPure™ 0.5 M EDTA (pH8.0) (Invitrogen™, UK), 200 µg/ml proteinase K (Qiagen, UK), 50 mM Dithiothreitol (DTT), made up to a total volume of 200 µl per sample using UltraPure™ water (Invitrogen™, UK).

All samples were incubated overnight at 37°C before proceeding with DNA extraction. Immediately following incubation, 500 µl of ice-cold 100% ethanol, 20 µl 3 M sodium acetate pH 5.5 (Invitrogen™, UK) and 2 µl of GlycoBlue™ coprecipitant (15 mg/ml) (Invitrogen™, UK) were added to each sample and incubated at -20°C for 1 hour. DNA was pelleted by centrifugation at 14,000 rpm at 4°C for 30 minutes and the supernatant removed. Pellets were then washed in 400 µl 70% ethanol and re-pelleted by centrifugation under the same conditions for 15 minutes and the supernatant removed. Pellets were air-dried for 20 minutes to allow for the evaporation of excess ethanol, following which pellets were resuspended in 15 µl nuclease free water.

3.2.7 Biotype Identification

Biotype identity was confirmed using a real-time PCR assay adapted from Rudolf *et al.* (2013) with minor adaptations to primer concentrations. Primers *CxTorr*F and *CxTorr*R were used to amplify a section of the ACE-2 gene of *Culex torrentium* DNA which was identified using probe *CxTorr*P. Amplification of this probe confirmed mosquito identity as *Cx. torrentium,* whilst failure to amplify indicated species identity as *Cx. pipiens*. Primers *CxPip*F and *CxPip*R were used to amplify a segment of the CQ11 microsatellite locus of *Cx. pipiens* species, amplification of probe *CxPip*P confirmed identity as *Cx. pipiens* whilst probes *CxPipPip*P and *CxPipMol*P were used to determine biotype.

Reactions were performed in 10 µl consisting of 5 µl TaqMan™ multiplex master mix (2x) (Applied Biosystems™ by Thermo Fisher Scientific, UK), 0.3 µM *CxPip*F, 0.4 µM *CxPip*R, 0.2 µM *CxPip*P, 0.2 µM *CxPipPip*P, 0.2 µM *CxPipMol*P, 0.15 µM *CxTorr*F*,* 0.15 µM *CxTorr*R R, 0.1 µM *CxTorr*P, 0.16 µl bovine serum albumin (BSA) (20 mg/ml) (Merck Life Science UK Limited, UK), 1.62 µl UltraPure™ water (Invitrogen™, UK), 2 µl DNA. Sequences for the primers and probes are shown in Table 3.1. The thermal profile started with an initial activation step of 95°C for 20 seconds, followed by 40 cycles of 95°C for

3 second and 60°C for 1 minute using a QuantStudio™ 7 Flex Real-Time PCR machine (Applied Biosystems™, UK). All samples were run alongside appropriate positive controls consisting of pure *pipiens*, *molestus* and hybrid DNA and negative controls including extraction and PCR negatives.

Table 3.1. Primer and probe sequences for the simultaneous differentiation of *Culex pipiens* and *Culex torrentium* species through amplification of the ACE-2 gene as well as the differentiation of *Culex pipiens* biotypes by the CQ11 microsatellite locus. Letters in the primer/probe names identify whether they are forward (F) primers, reverse (R) primers or probes (P).

Primer/probe name	Primer/probe sequence
*CxPip*F	5'- GCGGCCAAATATTGAGACTT-3'
*CxPip*R	5'-CGTCCTCAAACATCCAGACA-3'
*CxTorr*F	5'-GACACAGGACGACAGAAA-3'
*CxTorr*R	5'-GCCTACGCAACTACTAAA-3'
*CxPip*P	5'-VIC- GGAACATGTTGAGCTTCGG-QSY-3'
*CxPipPip*P	5'-ABY-GCTTCGGTGAAGGTTTGTGT-QSY-3'
*CxPipMol*P	5'-JUN-TGAACCCTCCAGTAAGGTATCAACTAC-QSY-3'
*CxTorr*P	5'-FAM-CGATGATGCCTGTGCTACCA-QSY-3'

3.2.8 Wing measurements

Adult mosquitoes were removed from ethanol and wing length measured as a proxy for body size (Siegel *et al.*, 1992). Wings were transferred to a piece of paper towel dampened with 70% ethanol, flattened, and left to air dry for approximately 5 minutes. Following evaporation of excess ethanol, wings were transferred to a strip of scotch magic tape with both wings from one adult placed together, 10 pairs of wings were dissected per strip with placement of each pair noted to enable mosquito identification. Once the strip of tape was full this was stuck down to a glass microscope slide for processing. Wing images were taken using Leica EZ4HD microscope (Leica Microsystems, Germany) alongside a size reference. Images were subsequently processed for size measurement using ImageJ (Schneider *et al.*, 2012). Wing length was measured by taking the distance from the axillary incision to the apical margin excluding the fringe (Harbach and Knight, 1980). Measurements from both wings of the same adult were averaged for use in analysis (Mpho *et al.*, 2000).

3.2.9 Statistical analysis

To assess the likelihood that cross combination affected the fecundity of female mosquitoes a One-way ANOVA with Tukey's correction for multiple comparisons was used. For data that could not be normalised by log transformations (larval development times and fertility), the influence of cross combination on these factors were analysed using Kruskal-Wallis with Bonferroni correction for

multiple comparisons. To assess whether survival to pupation, sex ratio, mating success or female mating choice were influenced by the biotype cross, chi-squared analysis was used. To determine whether wing length was influenced by any categorical variables, unpaired t-tests or ANOVA with Tukey's correction were performed. To assess the relationship between wing length and fecundity or fertility, Pearson's and Kendall's correlations were used respectively. All tests were performed in R studio version 1.2 (RStudio Team, 2020) and the statistical significance level was set at $P < 0.05$.

3.3 Results

3.3.1 Single-paired mating

Cross combinations for each of the three forms were observed for successful mating under single-paired mating conditions. Mating success was validated by the presence of sperm in the female spermathecae (Figure 3.1). Cross combination was found to have a significant affect on mating success between the three forms of *Cx. pipiens* (χ^2 = 25.113, df= 8, *P* = 0.001). Homologous *Cx. pipiens* f. *molestus* crosses were found to have the highest insemination rates across all crosses (93.34%), which differed significantly from the homologous *Cx. pipiens* f. *pipiens* crosses (*P* <0.001) which only had a mating success rate of 6.67% (Figure 3.2).

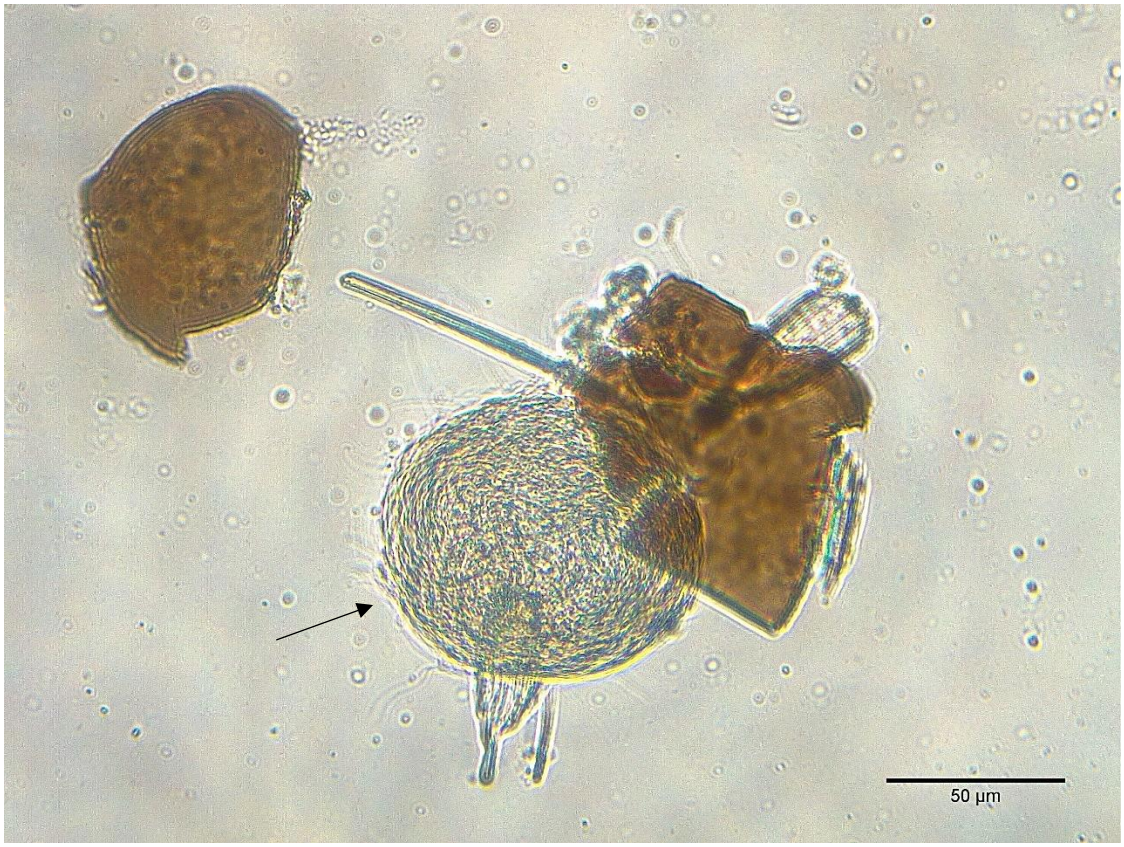

Figure 3.1. Sperm bundle (arrow) dissected from one *Culex pipiens* spermatheca after being stored in 70% ethanol, visualised at 400x magnification.

Homologous hybrid crosses had a mating success rate intermediate of the homologous *pipiens* and *molestus* forms (53.34%). This was significantly higher than the homologous *pipiens* cross ($P = 0.0426$), however, was not significantly different from the homologous *molestus* cross ($P = 0.106$). Mating success of reciprocal crosses ranged from 37.50% (P♂xH♀) to 61.12% (H♂xM♀) which did not vary significantly from each other ($P > 0.05$) or from the homologous *molestus* or hybrid crosses ($P > 0.05$). The homologous *pipiens* cross had a significantly lower mating success rate compared with the reciprocal crosses containing the female *molestus* biotype ($P = 0.0248$ for both P♂xM♀ and H♂xM♀ pairings) however, was not shown to vary significantly from any of the other reciprocal crosses ($P > 0.05$).

Female biotype, irrespective of male pairing, was shown to have a significant affect on mating success ($\chi^2 = 12.758$, df= 2, $P = 0.0017$) with the *molestus* biotype having the highest mating success rate under single-pair mating conditions (Table 3.2). This was shown to be significantly higher than mating success of the *pipiens* biotype ($P = 0.00178$) however did not differ significantly from hybrid mating success ($P = 0.0732$). No significant difference between the *pipiens* and hybrid females was detected ($P = 0.897$).

Table 3.2. Mating success, as determined by the presence of sperm stored in the spermatheca, of the female biotypes of *Culex pipiens*, independent of male pairing, under single-paired and grouped mating conditions.

Female biotype	**Mating success (%)**	
	Single	**Grouped**
Culex pipiens f. *pipiens*	34.69	72.74
Culex pipiens f. *molestus*	70.00	89.58
Hybrid	46.81	80.85

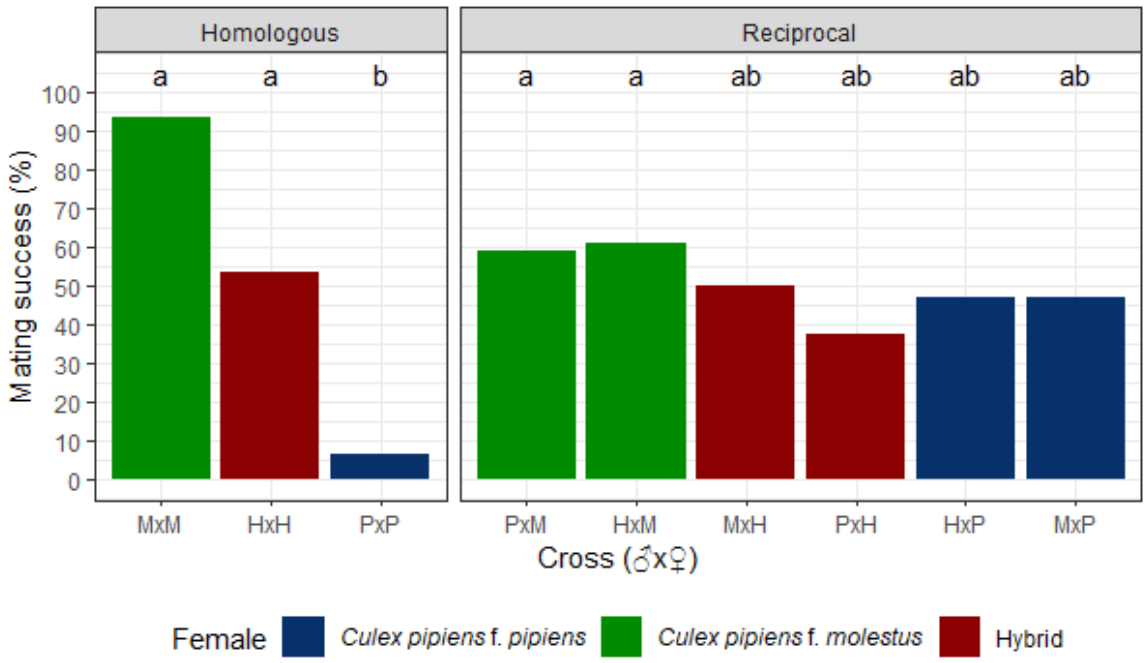

Figure 3.2. Comparison of mating success (%) of homologous and reciprocal crosses of the different forms of *Culex pipiens* under single-paired mating conditions. Successful mating events were characterised by visualising the presence of sperm stored in the female spermathecae under 400x magnification. The letters above the bars indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

3.3.2 Grouped mating

A group of 20 males and 20 females of each of the cross combinations were assessed for mating success under grouped mating conditions, with a successful mating event characterised by the presence of sperm in the female spermathecae. In contrast to single-pair mating conditions, no significant difference was seen between the different cross combinations ($\chi^2 = 5.83$, df= 8, $P = 0.6907$; Figure 3.3). As above, *Cx. pipiens* f. *molestus* females were more likely to accept a mating attempt compared with *Cx. pipiens* f. *pipiens* (89.58% and 72.74% mated respectively) however, this was not shown to be statistically significant ($P = 0.109$) (Table 3.2).

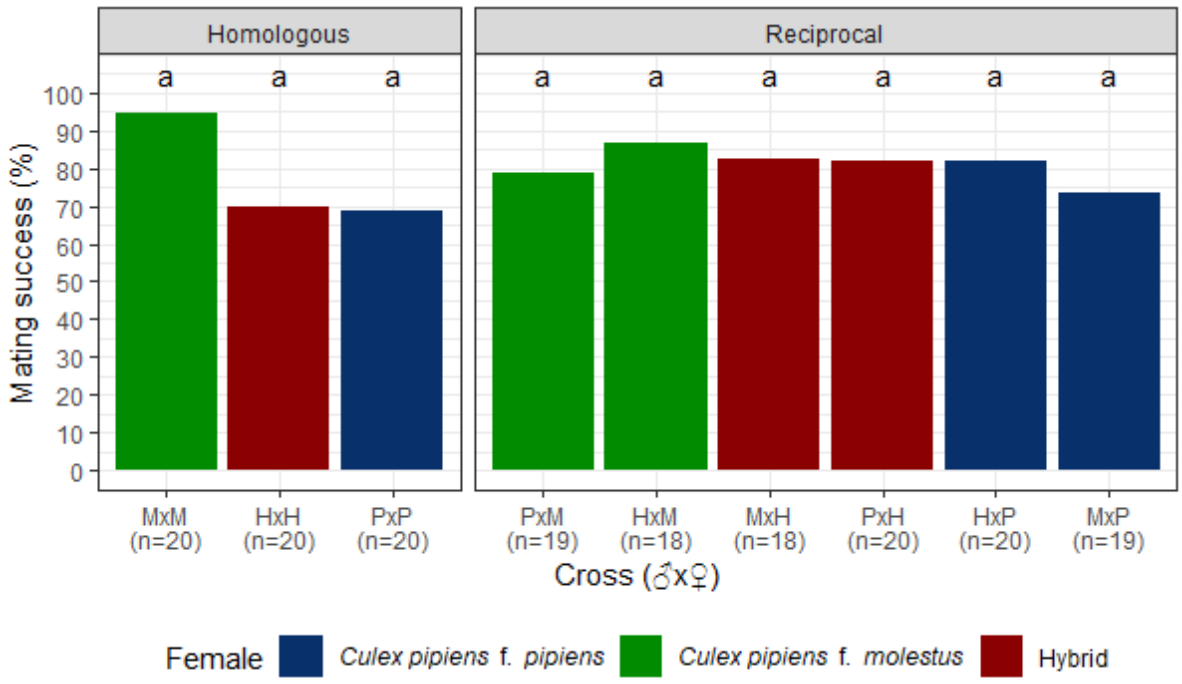

Figure 3.3. Comparison of mating success (%), as characterised by the presence of sperm stored in the female spermathecae, of homologous and reciprocal crosses of the different forms of *Culex pipiens* under grouped mating conditions. The letters above the bars indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

3.3.3 Comparison of single and grouped mating

The method of mating was shown to have a significant affect on mating success ($\chi^2 = 27.567$, df= 1, $P < 0.001$; Table 3.2, Figure 3.4). When considering the homologous *Cx. pipiens* f. *pipiens* cross a significant increase in mating success was seen under grouped compared with single-pair mating with a 10-fold increase in success ($\chi^2 = 10.097$, df= 1, $P = 0.00149$). Moreover, a significant difference was seen between single and grouped mating success in the P♂xH♀ pairing, increasing from 37.50% to 80.00% ($\chi^2 = 4.130$, df=1, $P = 0.0421$). Although an increase in mating success under grouped mating conditions was seen for all other cross combinations compared with single pairs, this difference was not shown to be significant ($P > 0.05$).

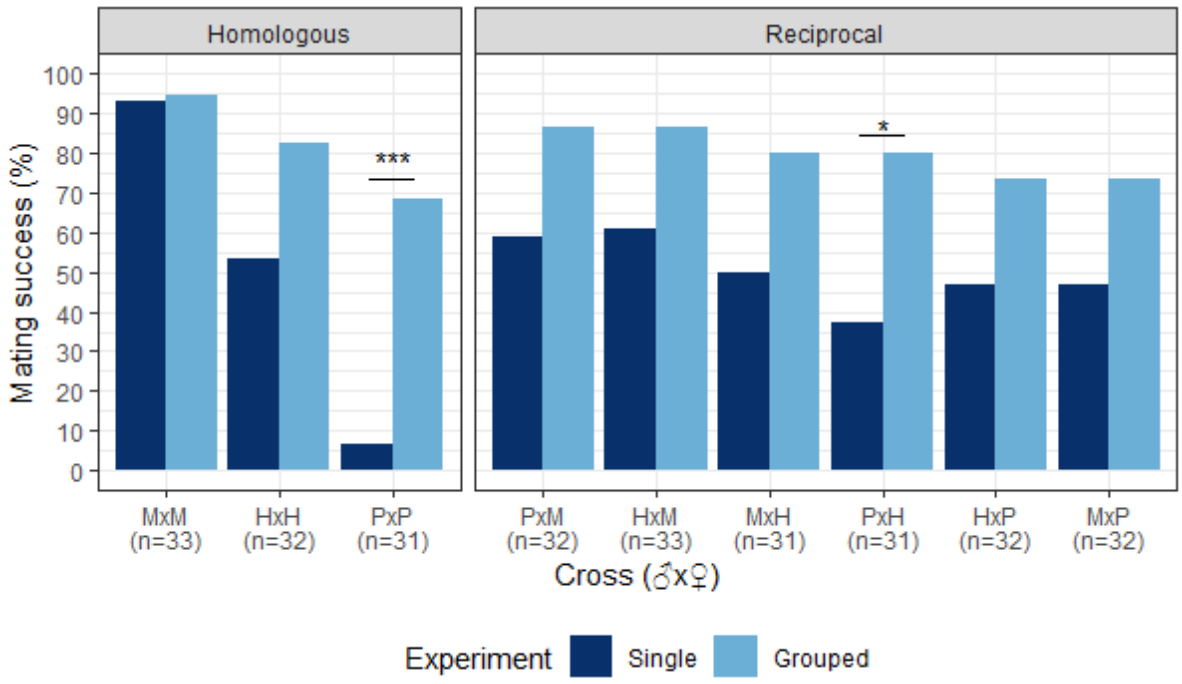

Figure 3.4. Comparison of mating success (%) of homologous and reciprocal crosses of the different forms of *Culex pipiens* under single-paired compared with grouped mating conditions. A successful mating event was characterised by the presence of sperm stored in the female spermathecae under 400x magnification. Asterix (*) indicate significance groups (*P* <0.05 "*"; *P* <0.01 "**"' *P* <0.001 "***").

When comparing mating success of each of the female forms using single-pair mating compared with grouped mating, all combinations of forms showed an increase in mating success percentages under grouped mating conditions (χ^2= 46.31, df= 5, *P* <0.001; Table 3.2, Figure 3.5). The largest increase was detected in the female *Cx. pipiens* f. *pipiens* which had a mating success of 34.69% when tested under single-pair mating, rising to 72.74% under grouped mating conditions. The mating success of *Cx. pipiens* f. *molestus* females under single and grouped mating, irrespective of male pairing, was not shown to differ significantly (*P* = 0.348). Conversely, *Cx. pipiens* f. *pipiens* and hybrid females were shown to have a significantly higher mating success under grouped mating compared with single-pair mating (*P* = 0.00652 and *P* = 0.0168 respectively).

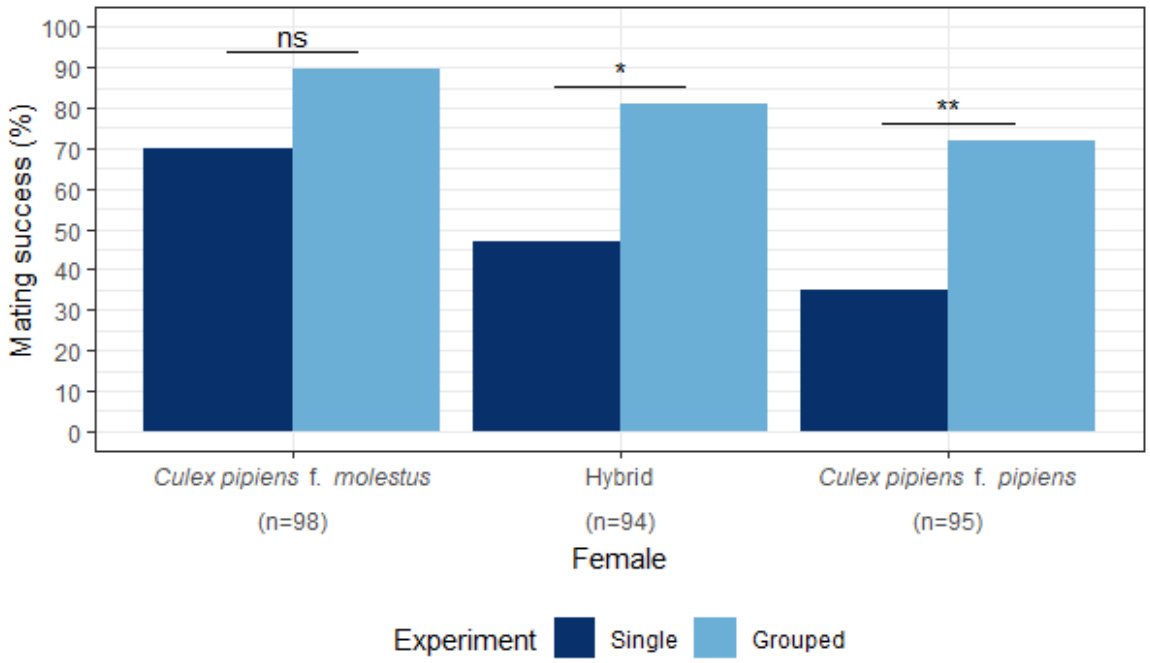

Figure 3.5. Assessment of mating success (%) of each of the female biotypes, independent of male biotype, under single-paired compared with grouped mating conditions. Successful mating was scored by visualisation of sperm stored within the female spermathecae at 400x magnification. Asterix (*) indicate significance groups (*P* <0.05 "*"; *P* <0.01 "**"; *P* <0.001 "***").

3.3.4 Larval development

A total of 2,507 larvae from six replicates of nine cross combinations were assessed to estimate larval development time. Cross combination was shown to significantly affect larval development time (KW χ^2= 28.368, df= 8, *P* <0.001; Table 3.3, Figure 3.6). Homologous *Cx. pipiens* f. *molestus* crosses were found to have the longest development time, taking approximately 14.3 ± 2.28 days for 50% of the population to reach pupation. This was significantly longer compared with the homologous *Cx. pipiens* f. *pipiens* cross (*P* = 0.0158) which had a mean development time of 10.5 ± 0.81 days. Larvae from the homologous hybrid cross took approximately 11.3 ± 0.45 days to reach pupation which was not significantly different to either of the homologous crosses (*P* = 0.869 and *P* = 1.00 for *Cx. pipiens* f. *molestus* and *Cx. pipiens* f. *pipiens* respectively). Development time of reciprocal crosses was not shown to vary significantly from any of the homologous crosses (*P* >0.05) except for the P♂xH♀ pairing which developed significantly faster compared with the homologous *molestus* cross (*P* <0.001), however, this was not shown to differ significantly from the homologous *pipiens* or hybrid cross (*P* = 1.00).

Table 3.3. Comparison of mean development times (days) for 50% of the larval population to reach pupation for homologous and reciprocal crosses of the *Culex pipiens* biotypes.

Cross (♂x♀)	Development time (days)	
	Mean	**SD**
MxM	14.3	2.28
HxH	11.3	0.447
PxP	10.5	0.808
MxH	11.9	0.498
MxP	11.6	0.530
HxM	12.0	1.06
HxP	10.4	0.550
PxH	10.0	0.728
PxM	11.0	1.82

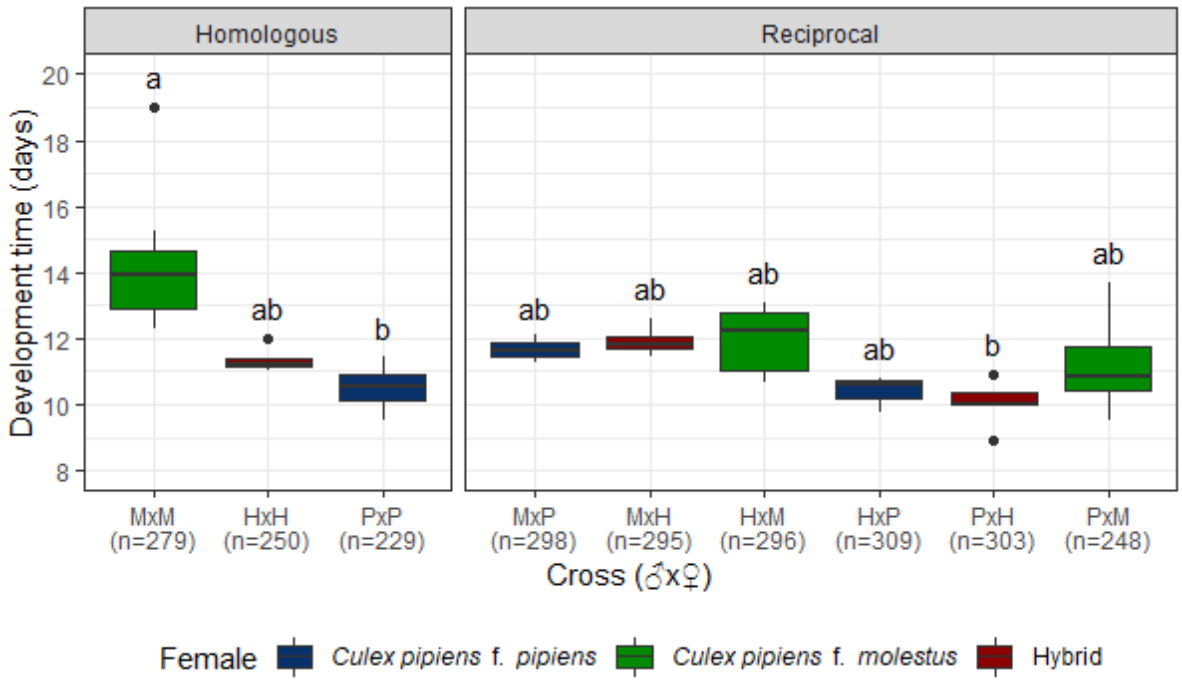

Figure 3.6. Comparison of development time (days) for 50% of the larval population to reach pupation for homologous and reciprocal crosses of the *Culex pipiens* biotypes. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another. N= 6 replicates per cross.

3.3.5 Larval survival and pupation success

Larval survival to pupation was shown to be significantly affected by cross combination with the homologous crosses having a significantly higher chance of survival than the reciprocal crosses (χ^2= 92.216, df= 8, *P* <0.001; Figure 3.7). The homologous hybrid cross was shown to have the highest survival rate (91.3% ± 3.46) closely followed by the homologous *molestus* cross (86.4% ± 2.11) with no significant difference detected between the two groups (*P* = 1.00). Likewise, the homologous *pipiens* cross had an 83.1% ± 4.49 chance of survival which did not differ significantly from the homologous *molestus* (*P* = 1.00) or hybrid (*P* = 0.352) crosses. Survival of all reciprocal crosses was found to differ significantly from the homologous *molestus* and hybrid crosses (*P* <0.05 for *Cx. pipiens* f. *molestus* and *P* <0.001 for hybrids), however, the homologous *pipiens* cross was only shown to have a significant difference from both parental reciprocal crosses of *pipiens* and *molestus* (*P* = 0.0464 and *P* = 0.0029 for P♂xM♀ and M♂xP♀ respectively).

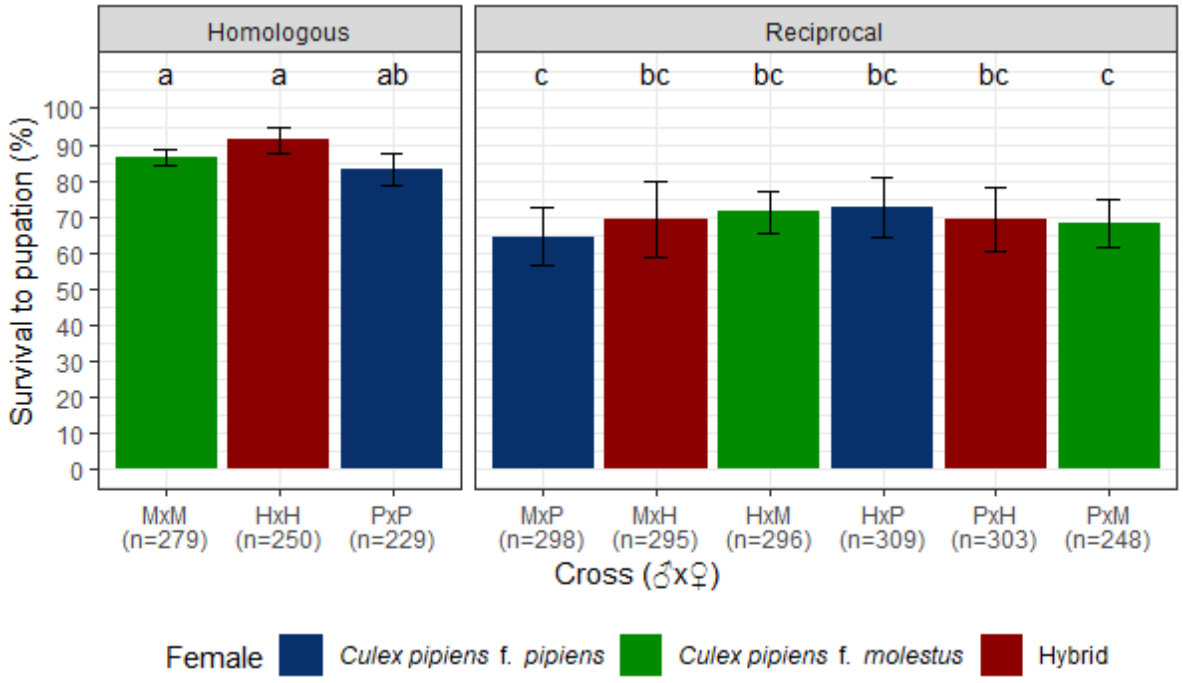

Figure 3.7. Comparison of mean survival (%) of hatching larvae to pupation of homologous and reciprocal crosses of the *Culex pipiens* biotypes. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly (*P* < 0.05) from one another. Specifically, groups which share a common letter are not significantly (*P* < 0.05) different from one another.

All cross combinations were shown to have high levels of pupation success ranging from 84.5 ± 13.30% to 91.9 ± 3.99% (Figure 3.8). Collectively, the reciprocal crosses were shown to have a marginally higher survival rate of 88.5 ± 7.41% compared with the homologous crosses (87.2 ± 6.37%) however this difference was not statistically significant (χ^2= 14.418, df= 1, *P* = 0.0715). Cross combination did not significantly affect pupation success (χ^2= 14.418, df= 8, *P* = 0.0715).

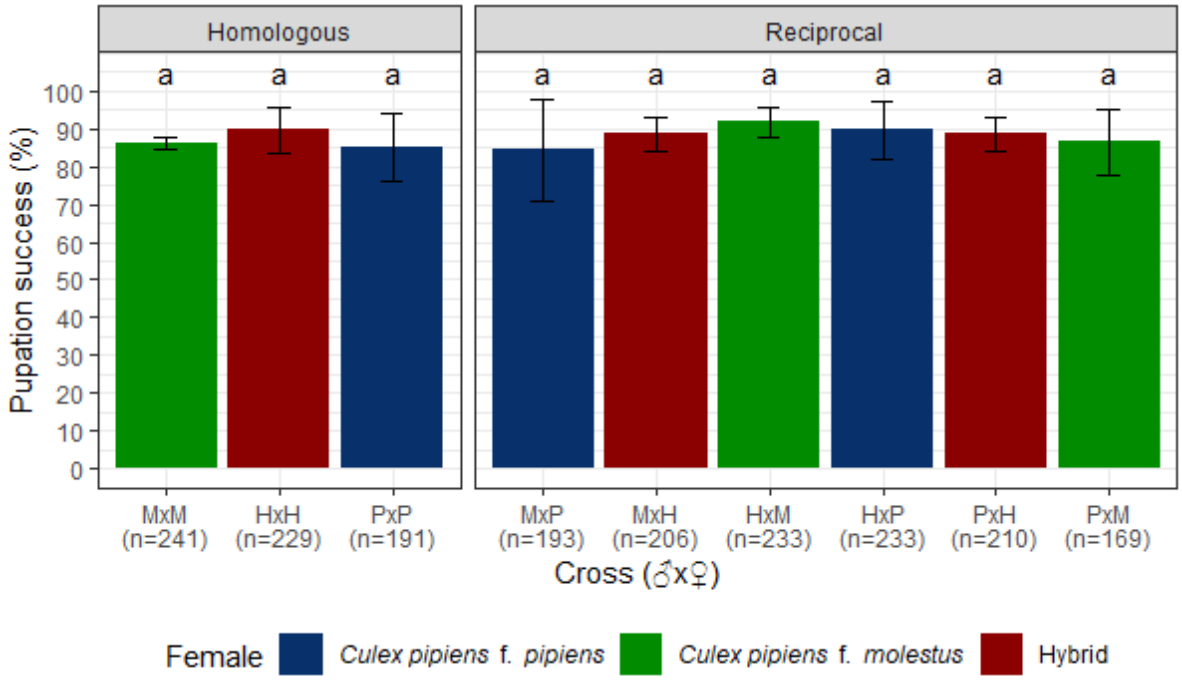

Figure 3.8. Comparison of survival (%) from pupae to eclosed adults for homologous and reciprocal crosses of the *Culex pipiens* biotypes. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly (*P* < 0.05) from one another. Specifically, groups which share a common letter are not significantly (*P* < 0.05) different from one another.

3.3.6 Blood-feeding

Cross combination was shown to significantly affect blood-feeding success (Fisher's exact: *P* <0.001). Homologous crosses were shown to have the highest blood-feeding success with 100% of individuals feeding to repletion (Figure 3.9), which was significantly different to the reciprocal crosses (χ^2= 12.723, df= 1, *P* <0.001). The M♂xH♀ cross had the lowest feeding rate of 52.63%, which was significantly lower than all homologous crosses (*P* = 0.0407). Reciprocal crosses containing the *molestus* female were also shown to have high blood-feeding success (94.45%) however these did not differ significantly from the other reciprocal crosses (*P* >0.05). Female biotype was also shown to significantly influence blood-feeding success (χ^2= 7.569, df= 2, *P* = 0.0227). In general, *molestus* females had an overall higher blood-feeding success compared with the other female forms (*P* = 0.0429 and *P* = 0.0456 for the *pipiens* and hybrid forms respectively; Figure 3.10). Overall blood-

feeding rate of *Cx. pipiens* f. *pipiens* was not shown to differ significantly from that of hybrid females (*P* = 1.00). No correlation between blood-feeding success and wing length was found (F= 1.703, *P* = 0.19).

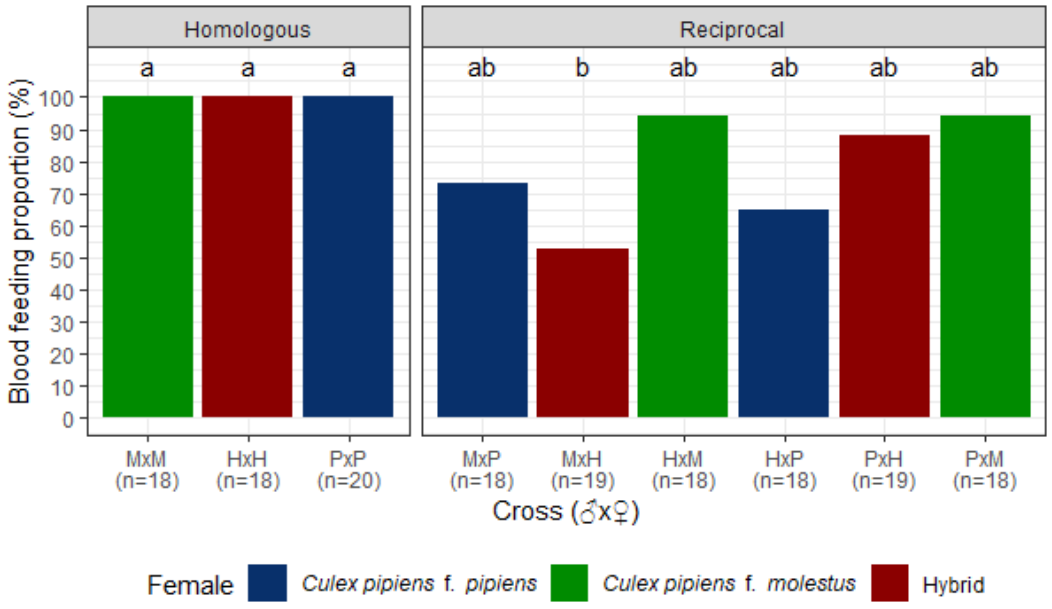

Figure 3.9. Comparison of the proportion (%) of female *Culex pipiens* blood-feeding following mating with homologous or reciprocal males. The letters above the bars indicate which groups differ significantly (*P* < 0.05) from one another. Specifically, groups which share a common letter are not significantly (*P* < 0.05) different from one another.

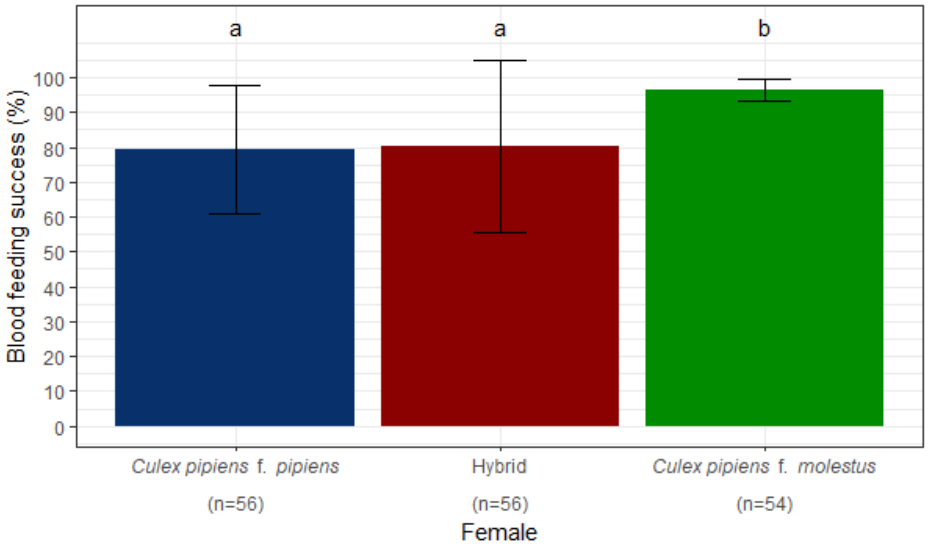

Figure 3.10. Comparison of blood-feeding success (%) by different female biotypes, independent of male pairing. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly (*P* < 0.05) from one another. Specifically, groups which share a common letter are not significantly (*P* < 0.05) different from one another.

3.3.7 Fecundity and fertility

A total of 200 egg rafts were assessed with an average raft size of 75.63 ± 23.11. Neither cross combination or cross type were shown to significantly affect total raft size (F= 1.868, *P* = 0.0764; t= 0.845, *P* = 0.4002 for cross combination and cross type respectively; Figure 3.11). There was also no statistically significant affect of female biotype on raft size (F= 0.869, *P* = 0.23).

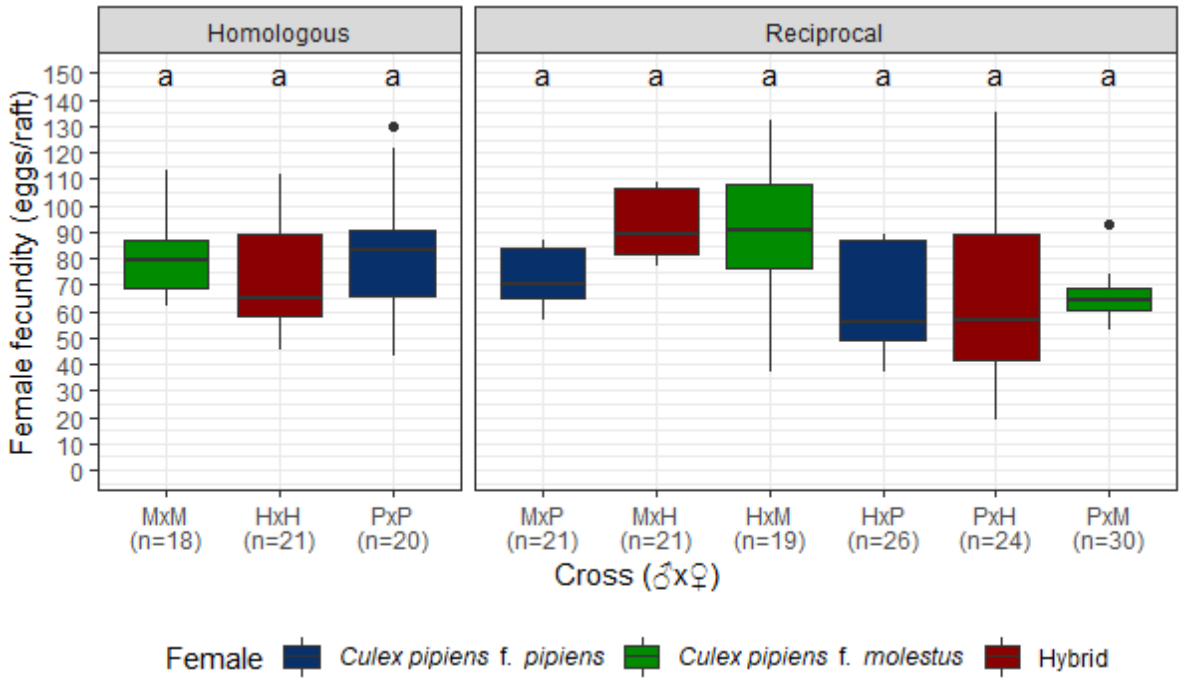

Figure 3.11. Comparison of female fecundity (number of eggs/raft) of homologous and reciprocal crosses of the *Culex pipiens* biotypes. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

Mating success was shown to significantly influence the size of egg rafts that were laid by *Cx. pipiens* females (t= -4.018, *P* = 0.00266), with mated females producing an average of 75.9 ± 22.1 eggs per raft compared with 48.8 ± 17.6 for unmated females (Figure 3.12). In contrast, mating success was not shown to significantly influence size of retained egg rafts or total number of eggs per raft (t=0.691, *P* = 0.538 and t= -1.303, *P* = 0.218 for retained and total eggs per raft respectively).

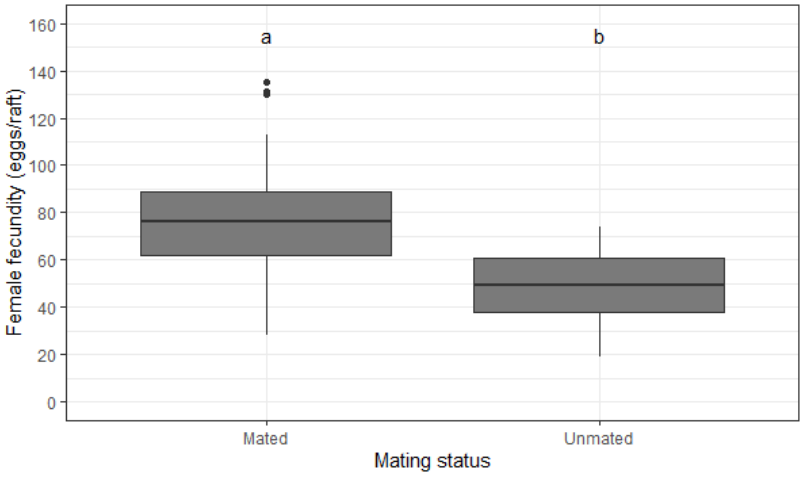

Figure 3.12. Assessment of the affect of mating success on the number of eggs per raft laid by *Culex pipiens* females. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

Female fecundity was also shown to be significantly affected by wing length. Total raft size (t= 6.627, p<0.001) as well as size of laid and retained egg rafts (t= 6.268 and p<0.001 and t= 3.783 and p= 0.00431 for laid and retained, respectively) was positively correlated with wing length. Positive correlations were also seen between fecundity and wing length for each female biotype (t= 3.355, P = 0.0026; t= 3.462, P = 0.0018; t= 5.891, P <0.001 for *pipiens*, *molestus*, and hybrid females respectively; Figure 3.13).

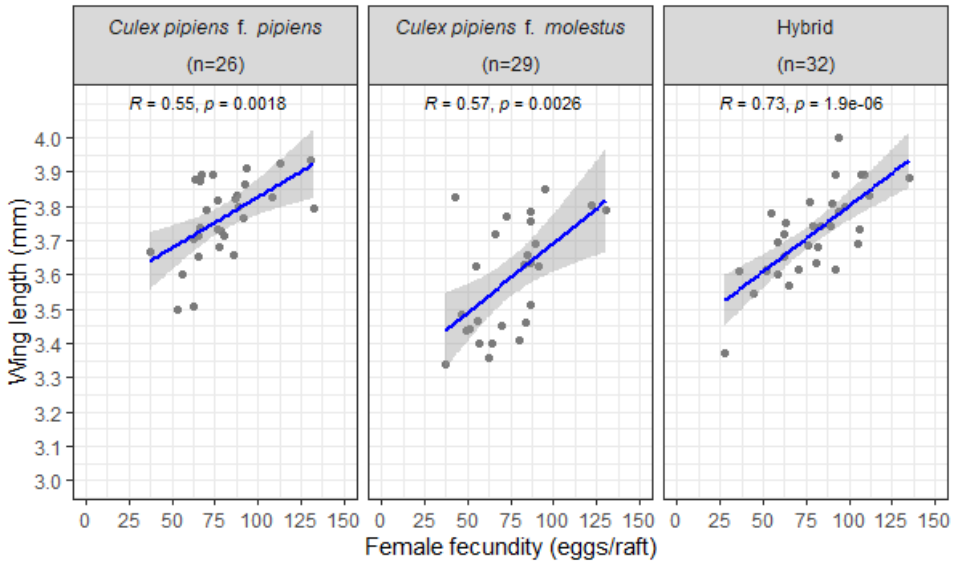

Figure 3.13. Assessment of the correlation between average wing length (mm) and total number of eggs per raft produced by *Culex pipiens* females. Grey shaded area indicates the 95% confidence interval.

Biotype was also shown to influence female wing length significantly (F= 29.02, *P* <0.001) with the *molestus* forms shown to have larger average wing lengths (3.79 ± 0.129 mm) compared with *pipiens* (3.56 ± 0.169 mm) and hybrid (3.69 ± 0.129 mm) individuals (Figure 3.14). *Culex pipiens* f. *molestus* females were shown to have significantly larger wing lengths compared with *pipiens* (*P* <0.001) and hybrids (*P* = 0.00496). Wing lengths were also shown to vary significantly between the *pipiens* biotypes and hybrids (*P* <0.001).

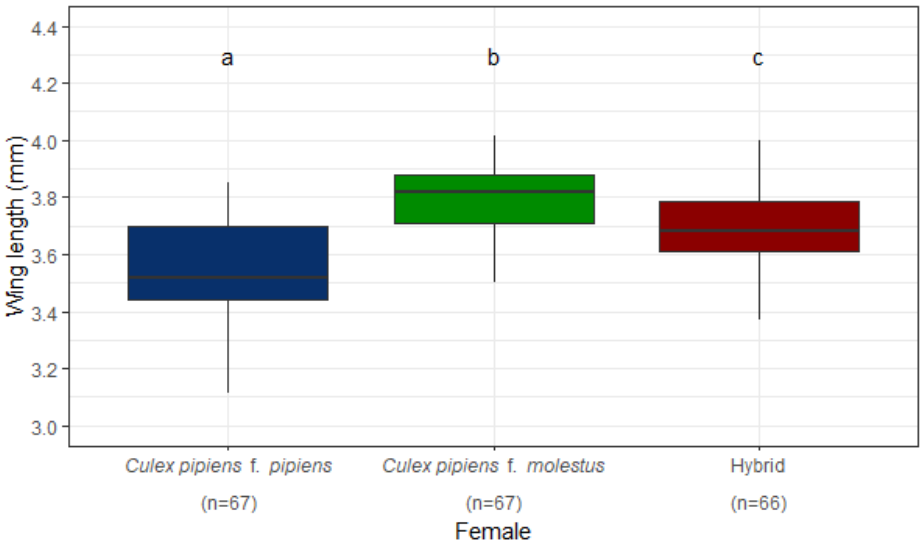

Figure 3.14. Comparison of wing length measurements (mm) for each of the *Culex pipiens* biotypes. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles. The letters above the boxplots indicate which groups differ significantly (*P* < 0.05) from one another. Specifically, groups which share a common letter are not significantly (*P* < 0.05) different from one another.

Mean hatching success of egg rafts produced by the different crosses were all shown to be above 50% success rate with the homologous hybrid cross having the highest mean hatching success of 92.5% ± 5.55 (Figure 3.15). Neither cross combination nor cross type were shown to significantly affect hatching success (KW χ^2 = 10.116, df= 8, *P* = 0.257 and W= 635, *P* = 0.363 for cross combination and cross type respectively). No correlation between fertility and wing length was identified (z= 0.987, *P* = 0.323).

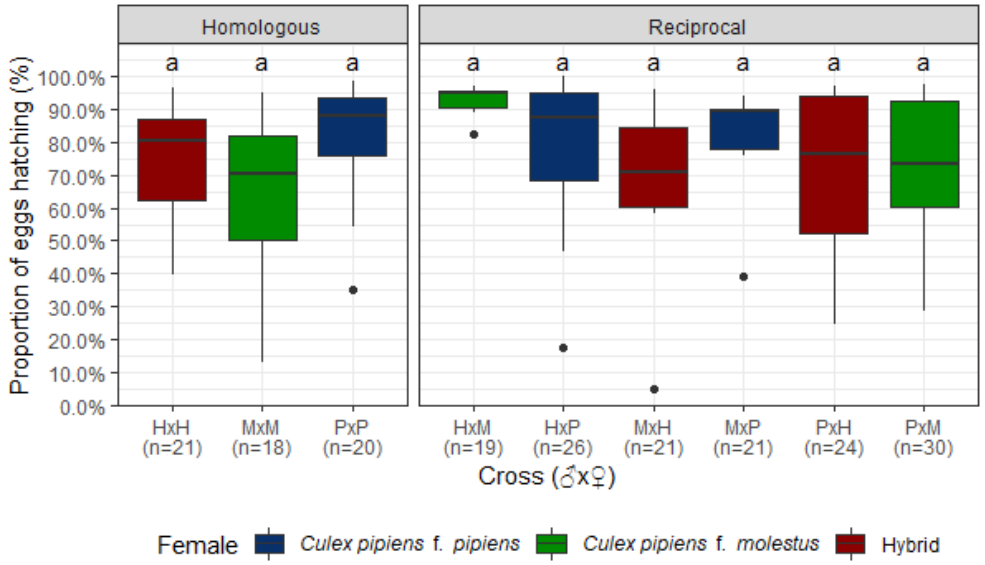

Figure 3.15. Comparison of hatching success (%) of laid egg rafts of homologous and reciprocal crosses of the *Culex pipiens* biotypes. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

3.3.8 Mating choice

When comparing mating choice of the three female biotypes, all were significantly more likely to mate with homologous males than either of the reciprocal male biotypes present (χ^2= 51.838, df=1, P <0.001; Figure 3.16), however, no significant difference was detected between the three forms (χ^2=0.572, df=2, P = 0.751). A significant difference was seen when considering which male biotype females preferentially mated with (χ^2= 23.242, df=4, P <0.001; Figure 3.17).

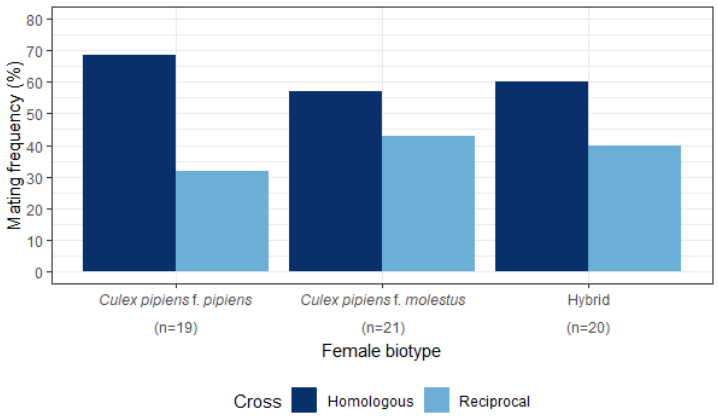

Figure 3.16. Assessment of mating choice of female *Culex pipiens* biotypes for homologous and reciprocal males. A successful mating event was defined by the presence of sperm stored in the female spermathecae.

When *Cx. pipiens* f. *pipiens* were provided with a choice of male, females were significantly more likely to mate with conspecific males compared with the other biotypes (P = 0.0244 and P = 0.00189 for *molestus* and hybrid males respectively). No significant difference was seen between mating frequencies with *molestus* or hybrid males (P = 1.00). For *Cx. pipiens* f. *molestus*, again females were significantly more likely to mate with conspecifics compared with *pipiens* biotype males (P = 0.0265), however, although *molestus* males outcompeted hybrid males, this difference was not found to be significant (P = 0.354). Hybrid males were shown to outcompete both *pipiens* and *molestus* males with significantly more females mating with hybrid males (P = 0.0338 for both *pipiens* and *molestus*), however, no significant difference was seen between mating successes for *pipiens* and *molestus* males (P = 1.00).

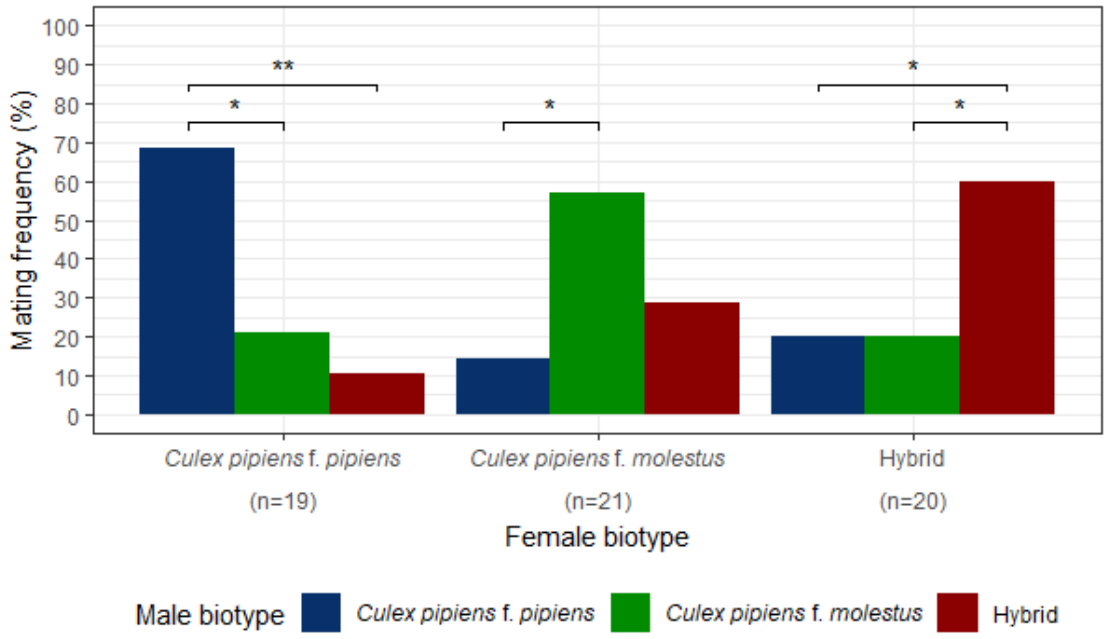

Figure 3.17. Assessment of mating choice of female *Culex pipiens* biotypes when provided with a choice of three different male biotypes. Mating choice was determined by DNA extraction and PCR amplification of the CQ11 locus of sperm stored in the female spermathecae. Asterix (*) indicate significance groups (P <0.05 "*"; P <0.01 "**"; P <0.001 "***").

When experiments were repeated excluding hybrid individuals, again, *pipiens* and *molestus* females were shown to be significantly more likely to mate with conspecific males (P <0.001 for both *pipiens* and *molestus*). Only one occurrence of polyandrous mating was detected during this study which was detected in a *Cx. pipiens* f. *pipiens* female (Figure 3.18).

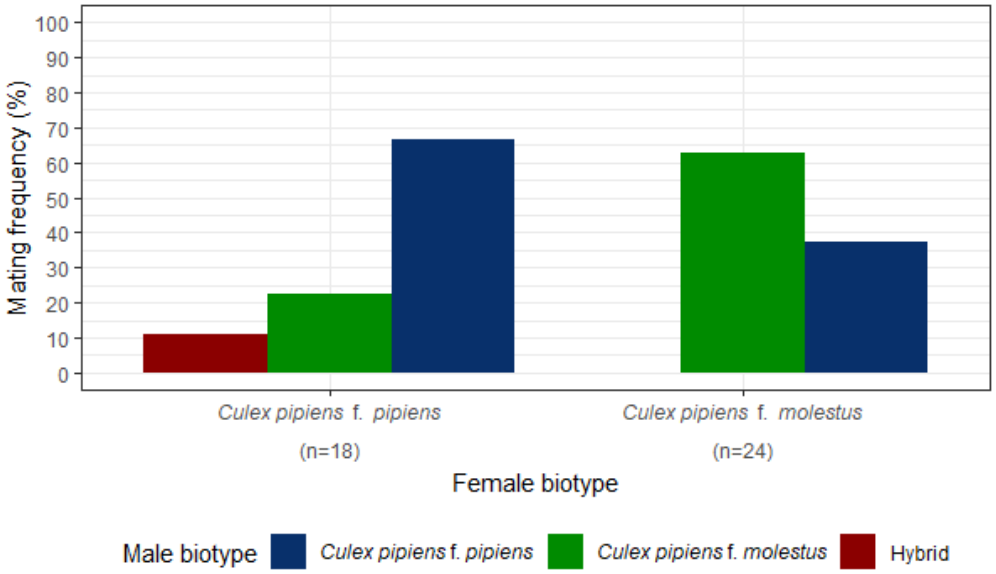

Figure 3.18. Mating choice of female *Culex pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* females when hybrid individuals were excluded from the experiment. Mating choice was determined by DNA extraction and PCR amplification of the CQ11 locus of sperm stored in the female spermathecae.

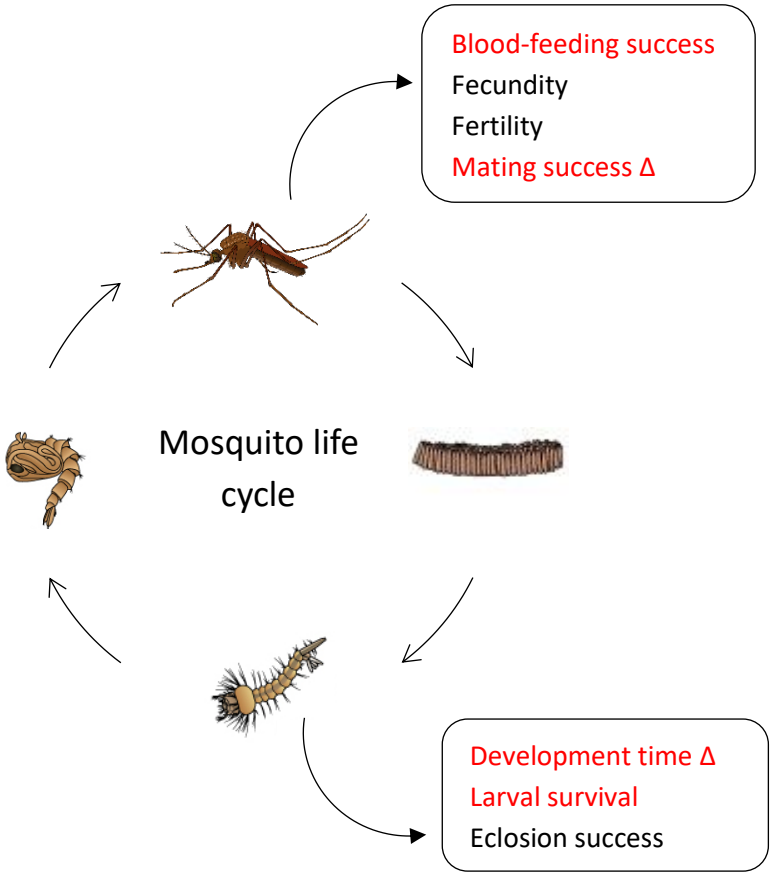

Figure 3.19. Schematic diagram showing the life history traits assessed in response to hybridisation between the *Culex pipiens* forms. Traits highlight in red demonstrate life history traits that were significantly affected by hybridisation, while disparity in life history traits between the two parental forms are also indicated (Δ).

3.4 Discussion

This study demonstrated plasticity in life history traits between the different forms and crosses of *Cx. pipiens* under colony conditions and determined a variety of mating barriers that potentially contribute to the variable rates of hybridisation seen in natural populations. Mating success, larval development time and larval survival were all shown to differ between the crosses under colony conditions (Figure 3.19). Understanding how these results translate into field populations is of the utmost importance to determine which factors limit hybridisation under natural conditions.

Homologous crosses of *Cx. pipiens* f. *pipiens* did not mate effectively under single-pair mating conditions with approximately 7% of females successfully mating compared with 93% of the homologous *molestus* cross females. *Culex pipiens* f. *pipiens* are described as being eurygamous thus low mating success under single-pair mating conditions is not surprising, representing a pre-zygotic behavioural barrier to gene flow between the forms under these conditions. In contrast, previous single-pair mating studies in *Cx. quinquefasciatus* have demonstrated successful mating under these conditions suggesting that mating in flight is not always necessary, facilitating mating under stenogamous conditions (Grover, 1981). Previous research has suggested that female mosquitoes predominantly determine whether a mating attempt is successful by performing rejection kicks (Benelli, 2018; Kim *et al.*, 2018b). However, in the present study, when paired with other male biotypes, 47% of encounters for the *pipiens* females resulted in a successful mating event, suggesting that mating success is not solely controlled by the female mosquito.

Previous studies assessing sperm transfer in homologous and reciprocal crosses of *pipiens* and *molestus* biotypes under stenogamous conditions also demonstrated low mating success rates of homologous *pipiens* crosses (Kim *et al.*, 2018b). However, in the current study, the homologous *pipiens* cross was shown to have significantly higher mating success under grouped compared with single-pair mating conditions, although this was still lower than in the other homologous crosses. Densities varied between these two studies, with mosquitoes maintained at a slightly higher density of 0.007 mosquitoes/cm^3 in the present study compared with 0.006 mosquitoes/cm^3 previously (Kim *et al.*, 2018b). It is therefore surprising that the *pipiens* form had a higher mating success in the present study despite being exposed to similar conditions, suggesting that barriers to mating between the forms cannot be attributed to swarming behaviour alone.

All pairings were shown to have higher mating success under grouped compared with single-pair mating experiments, suggesting that mate selection behaviour and swarming influences mating success under colony conditions, however, this difference was not shown to be significant in most

pairings. *Culex pipiens* f. *molestus* is known to be capable of mating in confined spaces (Byrne and Nichols, 1999) with previous studies suggesting that auditory recognition and nuptial flight play a less crucial role in successful mating compared with the *pipiens* biotype (Kim *et al.*, 2018b). This behavioural adaptation of the *molestus* biotype facilitates its mating under colony conditions hence is not surprising that the *molestus* female biotype saw the smallest variation in mating success between single and grouped mating conditions.

Reciprocal crosses were observed to have lower mating success rates than homologous crosses under both single-pair and grouped mating conditions, although these were significantly higher than previously reported rates (Kim *et al.*, 2018b). For example, 11.65 ± 5.79% mating success of reciprocal crosses has been reported but this was shown to increase to 64.15 ± 26.16 for F1 crosses (Kim *et al.*, 2018b), which is comparable to reciprocal mating success reported in the current study. The use of colonies originating from a mixed population in the present study, rather than the mixing of genetically distinct colonies as used previously, could account for the differences observed in mating success. Limited data are available regarding mating behaviours and cross-mating in mixed populations of *Cx. pipiens*, however a recent study reported similar overall insemination rates from a mixed population under colony conditions, although omission of biotype identification limits direct comparison (Benelli, 2018).

When provided with a choice of male biotype, female mosquitoes were shown to preferentially mate with conspecific males, although interform mating was reported 38.33% of the time. In contrast when levels of assortative mating between *Cx. pipiens* f. *molestus* with *Cx. quinquefasciatus* and *Cx. globocoxitus* was previously examined, no interspecific mating was identified (Miles, 1977). Characterisation of cross mating was performed through assessment of the alcohol dehydrogenase locus of progeny produced by the crosses, therefore the potential for mixed mating cannot be fully eliminated as females producing no or non-viable egg rafts were excluded from examination. Mating choice experiments were repeated, containing solely *pipiens* and *molestus* forms to examine the potential for polyandry through testing biotype of sperm bundles extracted from mated females. Only one occurrence of multiple mating was identified through the amplification of both *pipiens* and *molestus* alleles, supporting previous research suggesting that *Cx. pipiens* predominantly mate only once (Kitzmiller and Laven, 1958; Craig, 1967).

Satyrization is a form of mating interference where males will mate with heterospecific females resulting in decreased fitness and non-viable offspring. Investigation of hybridisation and satyrization between *Ae. aegypti* and *Ae. albopictus* demonstrated that retention of sperm does not always occur following mating with heterospecific males, whilst such mating also made females refractory to

further mating attempts with conspecifics (Carrasquilla and Lounibos, 2015). Very little research in this area has been undertaken in *Cx. pipiens*, and the experimental design used herein did not allow for detection of sperm which had not been successfully stored, meaning some mating events may have gone undetected. Moreover, research in *Aedes* mosquitoes has revealed that age significantly affects interspecific mating rates, with older mosquitoes engaging in heterospecific mating more frequently than younger counterparts (Bargielowski *et al.*, 2019), suggesting a breakdown of mating barriers with age. All mosquitoes used in the present study were within the same age range therefore an assessment of how rates of hybridisation could be influenced in an aging population were not addressed. Further work in the area should focus on the frequency of satyrization between the different *Cx. pipiens* forms, as well as interspecific mating within an aging population, and whether this influences rates of hybridisation within a population.

Larval development times were shown to differ significantly between the two forms of *Cx. pipiens* with the *molestus* biotype taking an average of 3.8 days longer than the *pipiens* form to reach pupation. Similarly, a recent study assessing the effect of different larvicides on life history traits of *Cx. pipiens* forms noted a significant variation in development times between *pipiens* and *molestus* biotypes within the control groups (Rumbos and Athanassiou, 2020). The *molestus* form was shown to reach adulthood approximately 5.6 days later than the *pipiens* form (Rumbos and Athanassiou, 2020), although direct comparisons with the present study are limited by differences in rearing conditions and data collection parameters.

In *Drosophila,* differential development times of hybrids between two sister species have been suggested as a post-zygotic mating barrier, with hybrids shown to have significantly longer development times compared with the parental strains (Matute and Coyne, 2010). The increased development times were suggested to reduce fitness in natural populations by exposing larvae to elevated risk of larval food depletion, predation, and desiccation. In contrast, development time of hybrids in the current study was not shown to be significantly longer, rather, maturation time of reciprocal crosses was intermediate of the two parental strains. Research in *Anopheles* has demonstrated fluctuation in development times between different species, with shorter growth periods frequently observed in mosquitoes such as *An. gambiae* that breed in unstable and transient sites (Gimnig *et al.*, 2001), or when exposed to predation (Knight *et al.*, 2004). Differential development times due to environmental interactions could have implications for *Cx. pipiens* ability to hybridise under natural conditions by reducing their interactions, thus providing a barrier to gene flow between the forms.

Homologous crosses of the *Cx. pipiens* biotypes were demonstrated to have a significantly higher larval survival rate compared with reciprocal crosses (86.9 ± 4,45% compared with 70.7 ± 3.49%), suggesting a fitness cost to hybridisation within this population. Previous studies assessing hybridisation between the two forms have reported reduced larval survival of reciprocal crosses with only 12% of the population surviving to adult (Kim *et al.*, 2018b). However, an increase in survival rate to approximately 80% in subsequent F1 crosses was observed; higher than the initial homologous *molestus* cross (Kim *et al.*, 2018b). Likewise, in the present study the homologous hybrid cross was shown to have the highest larval survival rate, although this was not significantly different to the other homologous crosses.

Disparity between larval survival rates of the parental crosses has been reported previously (Rumbos and Athanassiou, 2020), with the *pipiens* form shown to have decreased survival compared with *molestus*. In the present study, although survival rates in the homologous *pipiens* cross were slightly lower than those for *molestus*, this was not statistically significant. Although differences in survival were seen between homologous and reciprocal crosses, these variations were not as great as previously reported (Kim *et al.*, 2018b; Rumbos and Athanassiou, 2020). This is likely due to the influence of colony origin, with the present study utilising colonies from a mixed population, with reduced differences in life history traits between the forms.

The proportion of females taking a bloodmeal was shown to significantly differ, with females of homologous crosses more likely to take a bloodmeal than those in reciprocal crosses. Results also demonstrate that the *molestus* form was more likely to blood-feed under colony conditions than the *pipiens* females, irrespective of male pairing. Previous research has demonstrated that mating can alter behaviours in mosquito females, with male accessory gland (MAG) proteins increasing blood-feeding propensity (Villarreal *et al.*, 2018) and the rate of bloodmeal digestion in *Aedes* mosquitoes (Downe, 1975). Research regarding reciprocal mating between *Ae. aegypti* and *Ae. albopictus* demonstrated that rate of feeding of female *Ae. aegypti* was significantly reduced when mated with *Ae. albopictus* compared with conspecifics (Soghigian *et al.*, 2014). Contrastingly, the same relationship was not observed for *Ae. albopictus* with the authors proposing that the insensitivity to male activity could be attributed to more aggressive rate of blood-feeding of *Ae. albopictus.* In the current study, *pipiens* females exhibited alterations in blood-feeding behaviour depending on male mate, unlike the *molestus* form, suggesting that the *pipiens* form is more impacted by heterospecific mating than the *molestus* biotype.

In the current study, fecundity and fertility rates did not vary between different cross combinations, instead number of eggs per raft were shown to be positively correlated with female wing length

corroborating reports from other studies (Kassim *et al.*, 2012b). Previous research has found significant differences in female fecundity between homologous *molestus* crosses compared with parental and F1 reciprocal crosses (Kim *et al.*, 2018b). However, only the number of surviving L1 larvae counted following hatching was considered, rather than the overall size of the egg raft, or the total number of hatching larvae (Kim *et al.*, 2018b). Moreover, both autogenously and anautogenously produced egg rafts were collected without comparison between the two (Kim *et al.*, 2018b). Previous research has demonstrated that expression of autogeny can significantly influence egg raft size (Korba *et al.*, 2016) which may have influenced results observed, complicating direct comparison between the two studies.

As well as autogeny, the effect of bloodmeal source on the fecundity of *Cx. pipiens* mosquitoes has been well documented (Richards *et al.*, 2012), whilst larval and adult nutrition has also been shown to influence adult fecundity (Kassim *et al.*, 2012b; Kassim *et al.*, 2012a). As all female mosquitoes were maintained under standardised conditions and provided with a bloodmeal from the same source, it is unsurprising that the size of egg raft did not vary significantly here. Despite this, horse blood is not a natural host for the *pipiens* form and therefore may not be indicative of field fecundity rates. However, fecundity and fertility rates of two forms of *An. gambiae* have also been shown to be unaffected under conditions of reciprocal crossing and backcrossing when provided with the same bloodmeal source (Diabaté *et al.*, 2007), suggesting this does not provide a significant barrier to hybridisation.

Mated females were shown to produce larger egg rafts compared with unmated females, implying that mating status impacts fecundity of individual mosquitoes. Similarly, research regarding the influence of various factors on the expression of autogeny in *Cx. pipiens* f. *molestus* showed that mated females developed more eggs than unmated mosquitoes (Kassim *et al.*, 2012b). Moreover, unmated and mating induced stimulator of oogenesis gene (MISO) knockout *An. gambiae* mosquitoes were shown to produce significantly fewer eggs than their mated counterparts (Baldini *et al.*, 2013), demonstrating the effect of MAG proteins on physiology of female mosquitoes. Furthermore, studies in *Cx. quinquefasciatus* have demonstrated a positive relationship between bloodmeal size and fecundity (Akoh *et al.*, 1992; Lima *et al.*, 2003). Although measurement of bloodmeal size was omitted from the current study, female fecundity was shown to be positively correlated with female size. Whilst the volume of bloodmeal acquired would likely be linked to body size, the influence of body size and bloodmeal size on fecundity cannot be separated from the current data.

Overall, results from the current study demonstrate disparity in life history traits between the different cross combinations of the *Cx. pipiens* biotypes in colony populations. The factors highlighted here have the capacity to influence the levels of hybridisation seen within a population, such as

differential development periods and reduced survival of larvae from reciprocal crosses. Despite this, mixed populations containing all three forms have been maintained under colony conditions for over 100 generations with hybridisation rates of approximately 60% (Manley et al 2015), suggesting that although hybridisation is limited it is not completely prevented.

Laboratory environments are inherently artificial, with colonised mosquitoes exposed to different selective pressures compared with natural populations (Leftwich *et al.*, 2016). Previous studies have noted disparity between response of colony and field populations to environmental stressors, demonstrating colony populations are not representative of natural populations. Thus, results obtained using colony individuals may not be directly relatable to field populations. Therefore, understanding how these factors may influence field population dynamics is imperative for assessing disease risk. Hybrid populations of *Cx. pipiens* are thought to pose the largest risk for disease transmission by acting as a bridging vector between the avian reservoir and the susceptible human population. Any changes to population dynamics that may alter the hybridisation rates will therefore have important implications when considering *Cx. pipiens* ability to vector disease and consequently risk of spill over events into human populations. Further work should focus on expanding current knowledge of barriers to gene flow between the forms. Future work would particularly benefit from conducting similar cross mating experiments using field populations displaying characteristic traits of the pure biotype forms rather than individuals that have been kept in colony over several generations and been exposed to significant levels of bottlenecking and inbreeding.

Chapter 4 – Investigating the plasticity of life history traits of *Culex pipiens* in response to environmental factors

4.1 Introduction

The *Culex pipiens* complex includes the most geographically widespread of mosquitoes that are implicated in the transmission of several human pathogens including West Nile virus (WNV) (Fonseca *et al.*, 2004) and human filariasis (Bogh *et al.*, 1998). Their success can be attributed to variation in several key physiological adaptations that promote their survival in diverse environments (Vinogradova, 2000). The taxonomy of the complex is debated, due to the morphological similarity of forms that exhibit different physiological traits that confer a selective advantage within different ecological niches (Harbach, 2012). This has been further complicated by evidence of genetic introgression between members of the complex in many, but not all areas where their ranges overlap (Cornel *et al.*, 2003). The *Culex pipiens* species is the only member of the complex endemic to the United Kingdom (UK) and has been reported widely across Europe (Medlock and Jameson, 2010; Vogels *et al.*, 2016).

Two forms of the *Cx. pipiens* species have been identified, *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*, with hybrids between the two forms also reported (Fonseca *et al.*, 2004). The forms were originally differentiated on the presence or absence of behavioural or physiological traits such as the expression of autogeny (Marshall and Staley, 1935a) and habitat associations (Byrne and Nichols, 1999). However, separation by these methods has not always been accurate with identification of anautogenous populations of *Cx. pipiens* f. *molestus* (Beji *et al.*, 2017) as well as sympatric occurrence of the two forms. Hybrids between the biotypes are hypothesised to pose a major risk for disease transmission as they display behaviours intermediate of the two forms and are therefore able to act as bridging vectors of disease between avian and mammalian hosts.

The origin of *Cx. pipiens* f. *molestus* and its taxonomic classification is still contested, with two alternative hypotheses proposed to account for difference in ecology and physiology between the forms. Several studies from Europe have suggested that *molestus* is a distinct species from *Cx. pipiens* which arose through a single speciation event, where *Cx. pipiens* f. *molestus* is considered a separate evolutionary entity to *Cx. pipiens* f. *pipiens* (Yurchenko et al., 2020). In contrast, other studies have suggested that *molestus* populations arise from repeated and independent colonisation events where local populations of *Cx. pipiens* f. *molestus* are closely related to local populations of *Cx. pipiens* f. *pipiens*, considering *molestus* an eco-physiological variant of *Cx. pipiens* (Kothera et al., 2010).

Culex pipiens is known to be widely present across the UK, however, the distribution of the different biotypes and their hybrids is less well known as differentiation of the forms has only recently been implemented (Medlock *et al.*, 2005; Danabalan *et al.*, 2012). A few studies in the UK have delineated the different forms, demonstrating that both biotypes plus their hybrids have been found in sympatry in aboveground habitats (Danabalan *et al.*, 2012; Manley *et al.*, 2015). A study sampling larval habitats from six counties across England and Wales demonstrated presence of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* from all sampling locations, while hybrids between the forms were present at three of these sites according to identification by the CQ11 microsatellite maker (Danabalan *et al.*, 2012). Molecular identification using the CQ11 microsatellite marker suggested that hybridisation rates varied between 7-14% at the locations identified, but their study relied on morphological separation of *Cx. pipiens* from its sister species *Cx. torrentium* so rates obtained may be overestimated due to cross amplification of *Cx. torrentium* DNA with *Cx. pipiens* primers. A mixed population from an allotment area in Brookwood, Surrey demonstrated an initial hybridisation rate of 20%, although only five individuals from the field population were sampled, and this rate increased to 62.5% after maintenance of the population in colony for 20 generations (Manley *et al.*, 2015).

In contrast, an extensive survey of drains across London did not find any evidence of *Cx. pipiens* f. *molestus* or any hybrid individuals, with all larval habitats sampled dominated by *Cx. pipiens* f. *pipiens* (Curtotti, 2009). Rather, larval *Cx. pipiens* f. *molestus* populations were concentrated in a few niche environments, most notably sewage treatment works, whilst adult *Cx. pipiens* f. *molestus* were found to be present in high numbers on an urban farm in London when tested using the CQ11 microsatellite locus (Curtotti, 2009). More recently, an assessment of mosquito composition on dairy farms in the south of England failed to identify presence of *molestus* or hybrid individuals when using the same molecular identification methods (Brugman *et al.*, 2017a; Brugman *et al.*, 2017b) although a limited sample size was tested.

The presence of sympatric populations of closely isomorphic species can complicate accurate species identification. In the UK, the presence of the morphologically similar *Cx. torrentium* and two indistinguishable biotypes within *Cx. pipiens* s.l. makes identification challenging. An endpoint PCR assay exploiting differences in the CQ11 microsatellite locus between the forms has aided differentiation of the two (Bahnck and Fonseca, 2006). This is usually preceded by an assay targeting the ACE-2 gene to separate *Cx. pipiens* from *Cx. torrentium*. The CQ11 assay has since been further adapted to a real-time PCR assay (Rudolf *et al.*, 2013) which also incorporates amplification of the ACE-2 gene to simultaneously identify *Cx. torrentium* as well as differentiate the biotypes. More recently three high resolution melt (HRM) markers have been designed to differentiate *Cx. pipiens* f.

pipiens, *Cx. pipiens* f. *molestus* and *Cx. quinquefasciatus* from North American populations (Kang and Sim, 2013; Kim *et al.*, 2018a). Yet, assessment of one of these markers in field populations of *Cx. pipiens* from Sweden failed to corroborate the assay's ability to differentiate the biotypes (Luande *et al.*, 2020). To date, the endpoint PCR assay exploiting variation in the CQ11 microsatellite locus is still the most widely implemented assay for biotype differentiation (Bahnck and Fonseca, 2006; Brugman *et al.*, 2018).

Environmental conditions experienced during immature stages of the lifecycle can impact development of mosquitoes, particularly the quantity and quality of larval nutrition which can influence expression of phenotypes, such as autogeny, which may be important determinants of vectorial capacity (O'Meara, 1979; Kassim *et al.*, 2012b). The response to larval diet can differentially affect individual species with larval nutrition reported to increase, as demonstrated in *Ae. albopictus* (Zhang et al., 1993); decrease, as shown in *Ae. aegypti* (Nasci and Mitchell, 1994), or have no effect on vector competence (Kay *et al.*, 1989). Differential response to fluctuation in environmental conditions between the *Cx. pipiens* biotypes may facilitate their local adaptation to distinct ecological niches and influence their survival within different environments. However, understanding how fitness of populations is influenced by environmental conditions as well as the differential response of the forms to environmental selective pressures is imperative for quantifying risk of disease transmission.

The effect of larval nutrition on adult longevity has been researched extensively, with increased mortality seen with larval nutrition limitation in *Cx. pipiens* (Muturi 2010). In contrast, no association between longevity and larval nutrition were reported for either *Ae. aegypti* or *Ae. albopictus* (Mori, 1979; Maciel de Freitas *et al.*, 2007). In *Anopheles* mosquitoes, increased nutritional availability during development was shown to elevate adult blood-feeding propensity with females shown to have higher levels of reproductive success (Takken *et al.*, 2013). Larval development times and survival rates were also reported to be closely linked to nutritional availability during development, with elongated development times reported in mosquitoes reared under larval nutrition deficit (Kassim *et al.*, 2012b).

Although larval nutrition was shown to influence life history traits of mosquitoes, under field conditions, this parameter is not constant, rather larvae are exposed to fluctuating conditions. Other additional factors including temperature, light, competition, and predation were shown to influence larval development and adult traits in field populations (Couret, 2013), which have also been shown to vary among different habitat types. The forms of *Cx. pipiens* were reported to be adapted to separate niches with variable biotype ratios between different habitat types reported, with high association of the *molestus* biotype to underground environments (Korba *et al.*, 2016; Vogels *et al.*,

2016; Beji *et al.*, 2017). Adaptation to different habitat types was shown to influence key life history traits in *Cx. pipiens*. For example, increased expression of autogeny was recorded in populations sampled from underground environments compared with aboveground habitats in Tunisia (Beji *et al.*, 2017) and Algeria (Korba *et al.*, 2016). More recently, adaptive changes to artificial lights at night, typically associated with urban environments, was shown to alter gene expression in *Cx. pipiens* f. *molestus* in genes related to gametogenesis and immune response (Honnen *et al.*, 2016). Moreover, no significant differences in wing length and fecundity between female mosquitoes from urban and rural habitats was found in a UK study (Townroe and Callaghan, 2015), whilst increased larval densities in urban populations compared with rural counterparts was demonstrated (Townroe and Callaghan, 2014). Body size and fecundity were closely linked to seasonal fluctuations, with reduced body sizes observed later in the season compared with the summer peak (Townroe and Callaghan, 2014).

The aims of the present studies were to determine the genetic composition of UK *Cx. pipiens* populations, with an assessment of methods used to differentiate biotype, and to investigate the plasticity of life history traits in field-derived and colonised *Cx. pipiens* mosquitoes according to development site and biotype. Firstly, sampling of field collected adult and larval *Cx. pipiens* from inner London sites was undertaken to determine biotype ratios within the populations according to the CQ11 microsatellite analysis. These samples were subsequently reanalysed using other markers, previously reported to be diagnostic for biotype with the hypothesis that results from these assays would correlate correctly with initial biotype identification according to CQ11. Secondly, the hypothesis that adult life history traits, including blood-feeding and fecundity, vary in response to the environmental conditions experienced during larval development within an urban to rural gradient was tested. Subsequently, variation in response to larval nutrition availability in field derived and colonised *Cx. pipiens* was investigated with the hypothesis that biotype would influence response to larval nutrition level and be indicative of their adaptation to different ecological niches with the *molestus* form requiring greater larval nutrition to facilitate autogenous egg production. Finally, genetic characterisation of aboveground *Cx. pipiens* specimens was conducted to infer population structure and compare genetic similarity and diversity of specimens from across the UK with colony derived individuals from Sweden and the UK.

4.2 Methods

4.2.1 Biotype ratios of *Culex pipiens* populations from inner London

Three sites located across Inner London were selected to represent three different urban green spaces where human-mosquito-host interactions were predicted to be high. The sites were a residential garden, an allotment area, and a zoo (Figure 4.1). Collections at each site consisted of host-seeking adult collections, larval collections, and collections from adult resting sites. Temperature and humidity were measured at each site using Tinytag data loggers (Gemini Data Loggers, UK) while wind speed and wind direction was measured using an anemometer and wind vane throughout the collection period at 30-minute intervals (WINDLogger, UK).

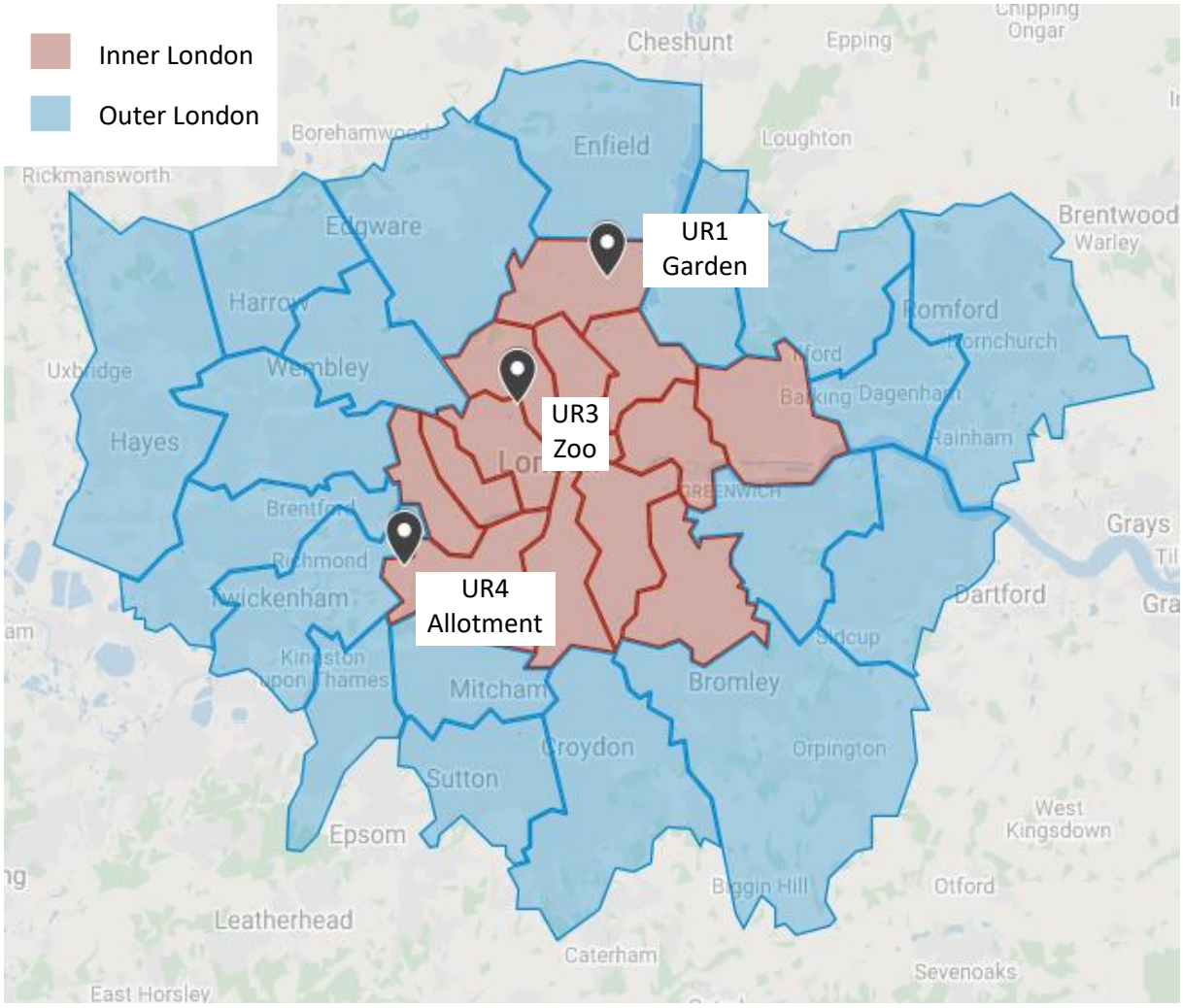

Figure 4.1. Map of sites selected within Inner London to represent different green spaces where human-mosquito-host interactions were predicted to be high. These sites included a garden, an urban zoo, and an allotment area.

Adult host-seeking mosquitoes were collected using BG sentinel traps (Biogents, Germany) baited with CO_2 placed at ground level at each site, with traps positioned within 2 meters of larval habitats (Figure 4.2). A mixture of 17.5 g dry active yeast (Allinson's, UK), 250 g white granulated sugar, and 2 litres (L) of tap water in a 5 L container were used for the production of CO_2 (Smallegange *et al.*, 2010). Silicon tubing, 7 mm internal diameter, was connected to the outlet of the container and attached to the base of the fan within the BG sentinel trap. Host-seeking adult mosquitoes are attracted towards the traps by the production of CO_2, following which a fan within the trap draws mosquitoes into a collection bag. Collections were conducted weekly throughout August and September 2019 with collections carried out at all three sites on the same day by the same person to account for differences in meteorological conditions.

Figure 4.2. BG sentinel traps (Biogents, Germany) used for the collection of host-seeking adult *Culex pipiens* mosquitoes. Plastic bottles (5L) were filled with 17.5 g dry active yeast, 250 g granulated sugar and 2 L of tap water to produce CO_2 as an attractant. Silicon tubing with an internal diameter of 7 mm was used to feed CO_2 produced into the container into the trap.

Permanent water containers at each site were identified and sampled for mosquito larvae weekly, where available (Appendix 2-4). Permanent water containers at the allotment site consisted of eight metal troughs (approximately 60 x 40 x 65 cm, WxDxH) located throughout the area which were used to provide water for plants, while the zoo site also consisted of a metal trough (approximately 1500x 1800 x 60 cm, WxDxH) filled with rainwater. The garden site did not contain a water container therefore a larval habitat was created using a 14 L black bucket. Larvae were sampled by up to three

consecutive dips using a 500 ml plastic pot (Brugman, 2016). Dipping was conducted towards the edge of the containers, where mosquito density was highest, by carefully submerging one side of the dipping pot into the water and allowing water to fill the pot. One minute was left between dips to allow for the water to settle and any disturbed larvae to return to the surface.

Resting boxes with final exterior dimensions of 30.5 x 30.5 x 30.5 cm were constructed from oak frames overlaid with exterior grade plywood (9 mm) (Figure 4.3). Boxes were coated with a waterproof primer and then painted black. One box was positioned at both the garden and the allotment site, while four boxes were placed at London Zoo (Appendix 2-4). To increase the success of the boxes, and to circumvent the potential loss of samples, resting boxes were lined with acetate sheets which were manually coated with rat glue (The Big Cheese Trading Company Ltd., UK) so that any mosquitoes resting would become stuck (Pombi *et al.*, 2014). Acetate sheets were fixed into the boxes with Velcro and were replaced with fresh sheets weekly and returned to the laboratory for mosquito ID. Mosquitoes to be used in molecular identification were removed by cutting out a small square of the acetate sheet containing the individual and soaking in acetone for approximately 5 minutes. Any additional resting mosquitoes observed outside of the boxes were also collected by aspiration.

Figure 4.3. Resting boxes used for the collection of *Culex pipiens* mosquitoes. Boxes had a final exterior dimension of 30.5 x 30.5 x 30.5 cm and were made from oak frames (panels A and B) overlaid with 9 mm exterior grade plywood (panel C). Boxes were constructed to leave the top half of the front open to allow mosquito entry. Boxes were coated with waterproof primer (Panel E), following which they were painted black and placed in areas where *Culex pipiens* were likely to rest (panel F).

Bloodmeal hosts of collected engorged females were identified using a triple primer cocktail (VF1_t1 + VF1d_t1 + VF1i_t1/VR1_t1 + VR1d_t1 + VD1i_t1) amplifying an approximately 685 base pair region of the COI gene, previously described by Ivanova *et al.* (2007). Reactions were run in a final volume of 25 µl consisting of 2.5 µl PCR buffer (10X) (Invitrogen™, UK), 0.75 µl $MgCl_2$ (50 mM) (Invitrogen™, UK), 0.5 µl deoxyribonucleotide triphosphate (dNTP) mix (10 mM) (Merck Life Science UK Limited, UK), 0.2 µl bovine serum albumin (BSA) (20 mg/ml) (Merck Life Science UK Limited, UK), 0.1 µl Platinum™ *Taq* DNA polymerase (Invitrogen™, UK), 0.1 µM each primer VF1_t1, VF1d_t1, VR1_t1, VR1d_t1, VR1i_t1, 0.2 µM primer VF1i_t1 and 5 µl DNA made up to 25 µl with UltraPure™ water (Invitrogen™, UK). The thermal cycling consisted of 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 40°C for 30 seconds, 72°C for 1 minute, followed by a final elongation step of 72°C for 10 minutes. All reactions were carried out in an Applied Biosystems™ 96 well Veriti thermal cycler (Applied Biosystems™ by Thermo Fisher Scientific, UK).

Products were separated on a 2% agarose gel stained with SYBR™ safe (Invitrogen™, UK) with positive samples represented by a band at approximately 685 base pairs. Samples were run alongside a colony blood-fed mosquito as a positive control, a PCR negative control and the TrackIt™ 100 bp DNA ladder (Invitrogen™, UK) as a size marker. Samples displaying a strong band at the expected size were selected for sequencing. Purification of PCR products was conducted using the MinElute® PCR purification system (Qiagen, UK) following the manufacturer's instructions. Samples were subsequently sent to Source Bioscience for bidirectional sequencing using the M13 primers. Sequences were aligned in MEGA version 7 (Kumar *et al.*, 2016) and compared with entries in GenBank via BLAST search.

A subset of 20% individuals from each site, as well as all blood-fed females were processed for DNA extraction and biotype identification as described below in Sections 4.2.4.2 and 4.2.4.3.1. A subset of these samples were reanalysed using the conventional CQ11 assay as described below in Section 4.2.4.3.2.

4.2.2 Assessment of environmental factors influencing reproductive traits of field collected *Culex pipiens*

This study was conducted to assess the effect of environmental parameters, experienced during larval development, on adult life history traits including fecundity and stenogamy. To achieve this, six sites were established across Inner London and Surrey to represent urban ("UR1" and "UR2"), peri-urban ("PUR1" and "PUR2"), and rural ("RU1" and "RU2") habitats within residential gardens (Figure 4.4). Definitions for each of the habitat types were as follows:

Urban – densely built-up areas inside towns and cities with multi-storey buildings and few green spaces (mainly parks).

Peri-urban – residential areas and small towns predominately consisting of residential homes with gardens or allotments.

Rural – areas located outside towns and cities with significant land cover consisting of farmland or unmanaged areas.

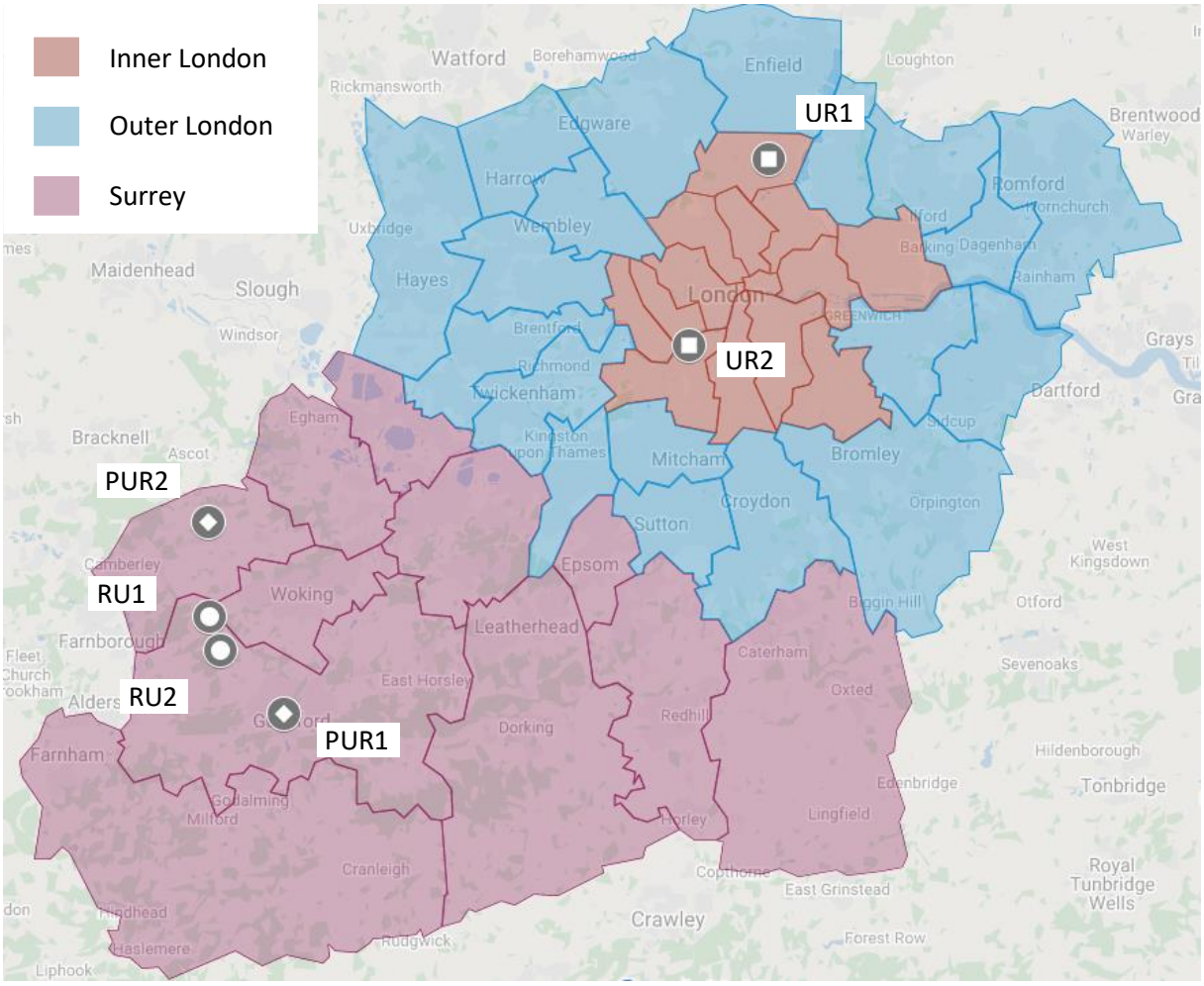

Figure 4.4. Map of sites selected to monitor the effect of environmental conditions on reproductive traits of *Culex pipiens*. Sites were selected to represent urban (square), peri-urban (diamond) and rural (circle) residential gardens.

Black 20 L buckets (dimensions: 28.3 cm x 47.8 cm x 33.0 cm (HxWxD) containing 10 L of water and 5 g of ground guinea pig pellets (Pets at Home, UK) were placed at each site to encourage colonisation by mosquitoes (Figure 4.5). Water temperature and light intensity in the buckets were measured using a HOBO™ U12-012 temperature/relative humidity/light data logger (Measurement Systems Limited, UK) with measurements taken at 15-minute intervals. Biological oxygen demand (BOD_5) was measured

using a Lutron dissolved oxygen meter (Lutron Electronic Enterprise Co. Ltd., Taiwan) following a five-day incubation. Briefly, water samples were collected and dissolved oxygen (DO) measured, following which samples were returned to The Pirbright Institute where they were placed into BOD bottles which consisted of a ground-glass stopper and flared mouth that formed a water seal to prevent air from entering the bottle (Brand™ by Thermo Fisher Scientific, UK). These were incubated in the dark at 20°C for five days, following which DO levels were remeasured. Dissolved oxygen readings taken at day five were subtracted from those taken during collection to calculate BOD_5 for each container.

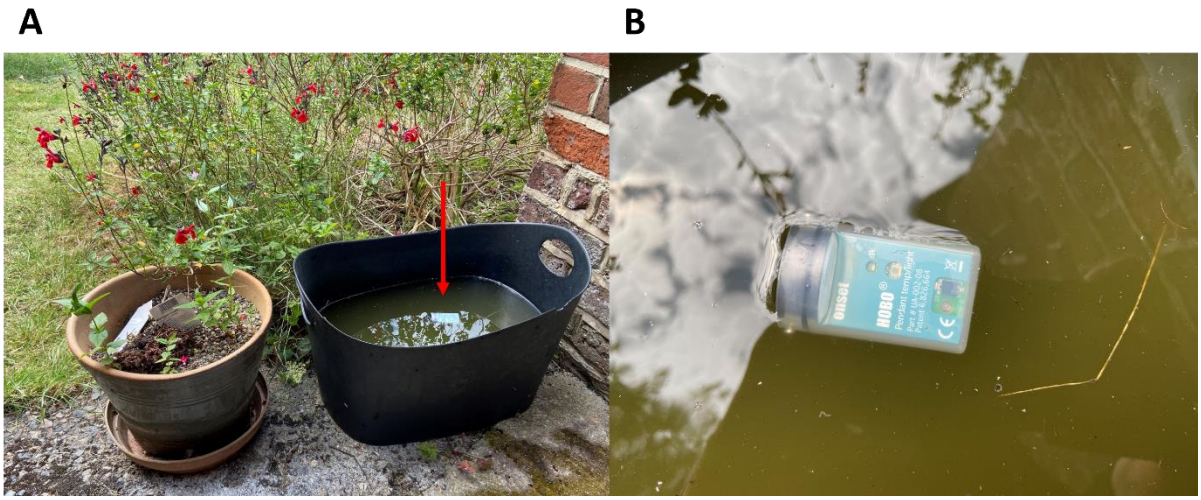

Figure 4.5. Collection and sampling of *Culex pipiens* larvae. **A:** black 20 L buckets (dimensions: 28.3 cm x 47.8 cm x 33.0 cm (HxWxD)) were filled with 10 L of tap water and seeded with 5 g of guinea pig pellets and placed at each site to act as a larval habitat for *Culex pipiens* larvae. **B:** Data loggers (arrow) were used to measure water temperature and light intensity in each bucket.

Two weeks after commencement, larval containers were sampled with three consecutive dips using a 1 L plastic pot as described above in Section 4.2.1. The number of larvae at each life stage were counted to estimate larval density and any late-stage larvae and pupae were removed from the bucket and returned to The Pirbright Institute for eclosion. Late-stage larvae were maintained in the insectary facilities at The Pirbright Institute in water from the larval habitat with volume of water adjusted to maintain larvae at the same density as had been observed in the field at time of collection.

Pupae were separated according to sex and site, with 50 males and 50 females allocated to each experimental cage for eclosion. For sex separation, following the onset of pupation, pupae were collected daily and separated into males and females on the basis of external structure of the terminal segment (Christophers, 1960) by observation under a dissecting light microscope (Leica Microsystems,

Germany). The terminal segment of male pupae is shield-shaped and bifurcate on the distal end whereas females are generally more broadly ovate in shape.

Adult mosquitoes were kept in cages at a density of 0.019 mosquitoes/cm^3 in experimental cages measuring 17.5 x 17.5 x 17.5 cm (Bugdorm, Watkins and Doncaster, UK) and were provided with 10% sucrose solution *ad libitum.* Three technical replicate cages per site were provided with a bloodmeal to monitor fecundity traits, whilst an additional three replicate cages were provided with an oviposition cup to monitor for the presence of autogeny.

For the replicates examining autogeny, following the eclosion of the last pupae, adult mosquitoes were provided with a black oviposition cup (dimensions 14 x 8 cm height x diameter) filled with approximately 200 ml of tap water and observed for 14 days. Oviposition cups were checked every other day to monitor for the presence of egg rafts. Females able to lay eggs without the need for a bloodmeal were classed as autogenous whilst those unable to lay prior to taking a bloodmeal were classified as anautogenous. After 14 days a total of 900 females were dissected under a light microscope (Leica Microsystems, Germany) to check for egg retention.

For the replicates measuring fecundity, following the eclosion of the last pupae, cages were left for 7 days to allow for mating, following which female mosquitoes were provided with defibrinated horse blood (TCS Bioscience, UK) via artificial membrane (Hemotek ™ system, UK) overnight. This was repeated for 5 subsequent nights with fresh blood provided each evening. Blood-fed mosquitoes were removed from the cage and were transferred individually to 12 oz cardboard pots (Cater4you, UK) with a small oviposition cup containing approximately 15 ml water. Oviposition cups were monitored for the presence of eggs rafts for 7 days (Richards *et al.*, 2012). Following hatching of the egg raft, the number of surviving larvae and eggs per raft were counted. Female fecundity corresponded to the number of eggs laid per female whilst fertility was estimated by the number of larvae hatched from each egg raft.

Mating success for all replicates was assessed by dissecting the spermathecae of female mosquitoes and observing for the presence of stored sperm. Briefly, female mosquitoes were placed ventral side up on a glass microscope slide under a dissecting microscope. The terminalia of the female was removed with forceps and separated from the rest of the body. The spermathecae were isolated from the terminalia, transferred to a new microscope slide and observed for the presence of sperm under a compound microscope at 400x magnification (Leica Microsystems, Germany). A successful mating event was characterised by the presence of sperm in the spermathecae (Rosay, 1969). Females able to mate under colony conditions within the 17.5 x 17.5 x 17.5 cm cages were considered stenogamous.

Fifty females per site which survived until the end of experiments were chosen at random and wing length was measured as a proxy for body size (Siegel *et al.*, 1992). Wings were transferred to a piece of paper towel dampened with 70% ethanol, flattened, and left to air dry for approximately 5 minutes. Following evaporation of excess ethanol, wings were transferred to a strip of scotch magic tape with both wings from one adult placed together, 10 pairs of wings were dissected per strip with placement of each pair noted to enable mosquito identification. Once the strip of tape was full this was stuck down to a glass microscope slide for processing. Wing images were taken using Leica EZ4HD microscope (Leica Microsystems, Germany) alongside a size reference. Images were subsequently processed for size measurement using ImageJ (Schneider *et al.*, 2012). Wing length was measured by taking the distance from the axillary incision to the apical margin excluding the fringe (Harbach and Knight, 1980). Measurements from both wings of the same adult were averaged for use in analysis (Mpho *et al.*, 2000).

4.2.3 Influence of larval diet quantity on life history traits of field derived and colonised *Culex pipiens* populations

These studies were conducted to assess the effect of variable levels of nutritional availability during larval development on the life history traits of field collected populations and three colony lines of *Cx. pipiens*. To achieve this, egg rafts were collected from each larval habitat created in Section 4.2.2 and returned to the Pirbright Institute for hatching in approximately 500 ml of water. Egg rafts were also collected from the *pipiens*, *molestus* and hybrid *Cx. pipiens* colony lines created as per Chapter 2. Upon hatching, larvae from each larval habitat and colony line were separated into three treatment groups, and fed high, mid-, and low protein diets (Kassim *et al.*, 2012b). Larvae were reared at a density of 200 larvae/L and were maintained on a diet consisting of equal amounts of Wardley's tropical fish food (Hartz, USA) and Brewer's yeast provided daily, quantities for which varied according to the treatment group (Table 4.1). Survival of larvae was recorded daily during maintenance. Development times of larvae were defined as the time taken for 50% of larvae to reach pupation according to (Kassim *et al.*, 2012b). Larvae were maintained in an environmentally controlled incubator at 25^{o}C ± 1^{o}C and relative humidity of 50% ± 1% with a 16:8 light:dark cycle with an hour of dusk and dawn either side. Adult mosquitoes were provided with 10% sucrose solution *ad libitum*.

Following the onset of pupation, pupae were separated into males and females on the basis of external structure of the terminal segment (Christophers, 1960) as described above in Section 4.2.2 and allocated to experimental cages according to site/colony and diet with 50 males and 50 females per cage. A total of three replicates per site/colony and diet treatment were constructed. Two

replicates were blood-fed and assessed for fecundity, fertility and stenogamy whilst the third replicate was provided with an oviposition cup to monitor for the expression of autogeny as described above in Section 4.2.2. A subset of 600 individuals across all diets and populations were selected to dissect to check for egg retention as described above in Section 4.2.2. Wing length was measured for a subset of 50 individuals per diet and population as described above in Section 4.2.2.

A total of 90 individuals (30 from each diet treatment) from the hybrid colony line as well as a selection of 40 individuals from each site (combination of individuals used in Sections 4.2.2 and 4.2.3 above) were processed for DNA extraction and biotype identification as describes below in Sections 4.2.4.2 and 4.2.4.3.1.

Table 4.1. Quantities of larval diet in milligrams (mg) provided to each of the life stages of field derived and colonised *Culex pipiens* within the three treatment groups. Larval diets consisted of equal ratios of Wardley's tropical fish food (Hartz, USA) and Brewer's yeast provided daily to treatment groups (Kassim et al., 2012b).

Diet	L_1-L_2	L_3-L_4
Low protein	0.4	0.8
Mid-protein	0.8	1.6
High protein	1.6	3.2

4.2.4 Mosquito identification

4.2.4.1 Morphological identification

All adults and larvae collected were returned to the Pirbright Institute and adult mosquitoes were stored at -20°C prior to identification. Larvae were reared in the insectary according either to methods described above for individuals to be used in experiments (Sections 4.2.2 and 4.2.3) or the methods of Manley *et al.* (2015). All adult mosquitoes were morphologically identified using the key of Snow (1990), and subsequently stored in 70% ethanol.

4.2.4.2 DNA extraction

DNA was extracted from individual mosquito heads using the Wizard® SV 96 Genomic DNA Purification System (Promega, UK) following the manufacturer's instructions for vacuum extraction with minor adaptations to increase DNA yield. Mosquito heads were subjected to a pre-homogenisation stage within the digestion solution detailed in the kit's instructions using 3 mm stainless steel homogenisation beads (Qiagen, UK) for 1 minutes at 30 hertz using the Qiagen tissue lyser (Qiagen,

UK). Elution of DNA was performed once in 60 µl of nuclease free water. All extractions were carried out alongside two negative controls per extraction plate.

For recently blood-fed females (females with a visible, undigested bloodmeal), abdomens were separated from the rest of the body and placed into individual 1.5 ml Eppendorf tubes containing 200 µl digestion buffer described by the DNeasy® Blood & Tissue Kit (180 µl buffer ATL and 20 µl proteinase K) (Qiagen, UK). Abdomens were homogenised in the digestion solution using pellet pestles (Sigma-Aldrich, UK). The remaining head and thorax of each mosquito were stored at -20^{o}C for morphological reference. Dissecting instruments were cleaned between each dissection with a three-stage wash step consisting of Microsol4 (Anachem, UK), 70% ethanol and distilled water to prevent cross contamination between samples. Samples were incubated at 56^{o}C overnight prior to extraction. Following complete digestion of samples, DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen, UK) following the manufacturer's instructions for spin columns with minor adjustment. Elution of DNA was performed in two stages of 40 µl each. Extracted DNA was used to identify bloodmeal host as well as speciation of mosquito.

4.2.4.3 Biotype identification

Mosquitoes were characterised using a range of published assays designed to differentiate the *Cx. pipiens* biotypes to assess the congruence between assay for determination of biotype.

4.2.4.3.1 Biotype differentiation by real time PCR

Mosquitoes morphologically identified as *Culex pipiens/torrentium* by the key of Snow (1990) were simultaneously assigned to species and biotype level using real-time PCR assay originally designed by Rudolf *et al.* (2013) with minor adaptations to primer concentrations. *Culex torrentium* DNA was detected using primers *CxTorr*F and *CxTorr*R and probe *CxTorr*P which were designed to amplify a segment of the ACE-2 gene. Primers *CxPip*F and *CxPip*R were used to amplify a segment of the CQ11 microsatellite marker for *Cx. pipiens* species whilst probe *CxPip*P confirmed species identity as *Cx. pipiens*. Probes *CxPipPip*P and *CxPipMol*P were used to differentiate the forms.

Reactions were performed in 10 µl reaction volume consisting of 5 µl TaqMan™ multiplex master mix (2x) (Applied Biosystems™, UK), 0.3 µM *CxPip*F, 0.4 µM *CxPip*R, 0.2 µM *CxPip*P, 0.2 µM *CxPipPip*P, 0.2 µM *CxPipMol*P, 0.15 µM *CxTorr*F, 0.15 µM *CxTorr*R R, 0.1 µM *CxTorr*P, 0.16 µl BSA (20 mg/ml) (Merck Life Science UK Limited, UK), 1.14 µl UltraPure™ water (Invitrogen™, UK) and 2 µl DNA extract. Sequences for the primers and probes are shown in Table 4.2. The thermal profile started with an

initial activation step of 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 second and 60°C for 1 minute using a QuantStudio™ 7 Flex Real-Time PCR machine (Applied Biosystems™, UK). All samples were run alongside pure *pipiens*, *molestus*, hybrid and *Cx. torrentium* DNA as positive controls as well as extraction and PCR negative controls.

Table 4.2. Primer and probe sequences for the simultaneous differentiation of *Culex pipiens* and *Culex torrentium* species through amplification of the ACE-2 gene as well as the *Culex pipiens* biotypes by the CQ11 microsatellite locus by real time PCR as described by Rudolf *et al.,* (2013). Letters in the primer/probe names identify whether they are forward (F) primers, reverse (R) primers or probes (P).

Primer/probe name	Primer/probe sequence
*CxPip*F	5'- GCGGCCAAATATTGAGACTT-3'
*CxPip*R	5'-CGTCCTCAAACATCCAGACA-3'
*CxTorr*F	5'-GACACAGGACGACAGAAA-3'
*CxTorr*R	5'-GCCTACGCAACTACTAAA-3'
*CxPip*P	5'-VIC- GGAACATGTTGAGCTTCGG-QSY-3'
*CxPipPip*P	5'-ABY-GCTTCGGTGAAGGTTTGTGT-QSY-3'
*CxPipMol*P	5'-JUN-TGAACCCTCCAGTAAGGTATCAACTAC-QSY-3'
*CxTorr*P	5'-FAM-CGATGATGCCTGTGCTACCA-QSY-3'

4.2.4.3.2 Biotype differentiation by conventional CQ11

A subset of samples were also analysed using the conventional PCR method also targeting the CQ11 microsatellite marker described by Bahnck and Fonseca (2006) to confirm identity. Samples were initially assigned to species level using the ACE-2 multiplex protocol, following which species identified as *Cx. pipiens* were differentiated by biotype by amplification of the CQ11 microsatellite locus.

Samples were assigned to species level using the ACE-2 multiplex protocol (Smith and Fonseca, 2004) with each reaction consisting of 2.5 µl PCR buffer (10X) (Invitrogen™, UK), 0.75 $MgCl_2$ (50 mM) (Invitrogen™, UK), 0.5 µl dNTP mix (10 mM) (Merck Life Science UK Limited, UK), 0.2 µl BSA (20 mg/ml) (Merck Life Science UK Limited, UK), 0.1 µl Platinum™ *Taq* DNA polymerase (Invitrogen™, UK), 0.1 µM forward primer ACEpip (5'-GGAAACAACGACGTATGTACT-3') and ACEtorr (5'-TGCCTGTGCTACCAGTGATGTT-3'), 0.2 µM reverse primer B1246s (5'TGGAGCCTCCTCTTCACGG-3'), 5 µl DNA made up to 25 µl with UltraPure™ DNase/RNase free distilled water (Invitrogen™, UK).

Biotype differentiation for *Cx. pipiens* was subsequently carried out through amplification of the CQ11 microsatellite locus (Bahnck and Fonseca, 2006). Each reaction consisted of 2.5 µl PCR buffer (10X) (Invitrogen™, UK), 0.75 µl $MgCl_2$ (50 mM) (Invitrogen™, UK), 0.5 µl dNTP mix (10mM) (Merck Life Science UK Limited, UK), 0.2 µl BSA (20 mg/ml), 0.1 µl Platinum™ *Taq* DNA polymerase (Invitrogen™,

UK), 0.24 µM forward primer CQ11F (5'-GATCCTAGCAAGCGAGAAC-3'), 0.32 µM reverse primer PipCQ11R (5'-CATGTTGAGCTTCGGTGAA-3'), 0.14 µM reverse primer MolCQ11R (5'CCCTCCAGTAAGGTATCAAC-3'), 5 µl DNA made up to 25 µl with UltraPure™ water (Invitrogen™, UK). A subset of samples were subsequently reamplified in two individual singleplex assays, each containing the universal forward primer (CQ11F) paired separately with each of the reverse primers. The volume of the second reverse primer was replaced with nuclease free water. The thermal cycling for both reactions consisted of 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, followed by a final elongation step of 72°C for 10 minutes. All reactions were carried out in an Applied Biosystems 96 well Veriti thermal cycler (Applied Biosystems™ by Thermo Fisher Scientific, UK).

Amplified PCR products were separated for 40 minutes at 100V on a 2% agarose gel stained with SYBR™ safe (Invitrogen™, UK) diluted 10,000-fold and visualised under ultraviolet (UV) light, using a TrackIt™ 100 bp DNA ladder (Invitrogen™, UK) as a size marker.

4.2.4.3.3 Assessment of HRM markers for biotype identification

Three previously reported HRM loci for biotype differentiation were tested for amplification in UK mosquitoes (Kang and Sim, 2013; Kim *et al.*, 2018a). Two of the markers failed to amplify and were redesigned using Primer3 version 0.4.0 (Untergasser *et al.*, 2012) to target the same segment of DNA as the original primer sets.

Reactions were performed in a final volume of 25 µl consisting of 2.5 µl PCR buffer (10X) (Invitrogen™, UK), 0.75 µl $MgCl_2$ (50 mM) (Invitrogen™, UK), 0.5 µl dNTP mix (10mM) (Merck Life Science UK Limited, UK), 0.2 µl BSA (20 mg/ml) (Merck Life Science UK Limited, UK), 0.1 µl Platinum™ *Taq* DNA polymerase (Invitrogen™, UK), 0.2 µM each primer and 5 µl DNA made up to 25 µl with UltraPure™ water (Invitrogen™, UK). The thermal cycling consisted of 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, followed by a final elongation step of 72°C for 10 minutes. All reactions were carried out in an Applied Biosystems 96 well Veriti thermal cycler (Applied Biosystems™, UK). Products were visualised by gel electrophoresis and prepared for sequencing as described above in Section 4.2.1. Primer sequences for each target are shown in Table 4.3.

Table 4.3. Forward and reverse primers sequences for three targets previously reported to be diagnostic for *Culex pipiens* biotype following high resolution melting analysis PCR.

Target	Forward primer (5'-3')	Reverse primer (5'-3')
CPIJ005487	ATGATCGTGTGGATGACCCG	CCGAGCGAAATCATCACCCT
CPIJ001674	TGTACGTGGAGCACAAGAGC	TCCGAGTAGACCGAGACCAG
ACE-2-HRM	TTTGGGTACCAACGAAGACC	AAGTCCTCATCGTCCTGGAA

4.2.4.3.4 Microsatellite analysis

Twelve microsatellite markers were chosen to characterise *Cx. pipiens* populations (Fonseca *et al.*, 1998; Keyghobadi *et al.*, 2004; Smith *et al.*, 2005a). Markers were amplified in a final reaction volume of 12.5 µl consisting of 1.25 µl PCR buffer (10X) (Invitrogen™, UK), 0.375 µl $MgCl_2$ (50 mM) (Invitrogen™, UK), 0.25 µl dNTP mix (10 mM) (Merck Life Science UK Limited, UK), 0.1 µl BSA (20 mg/ml) (Merck Life Science UK Limited, UK), 0.05 µl Platinum™ *Taq* DNA polymerase (Invitrogen™, UK), 0.25 µl forward and reverse primers, 2 µl DNA made up to 12.5 µl with UltraPure™ water (Invitrogen™, UK). For each of the loci, the forward primer was labelled with 6-FAM. The thermal cycling conditions consisted of 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 56-66°C for 30 seconds, 72°C for 30 seconds, followed by a final elongation step of 72°C for 10 minutes. Annealing temperatures and sequences for each of the primer sets is shown in Table 4.4. All reactions were carried out in an Applied Biosystems™ 96-well Veriti thermal cycler (Applied Biosystems™, UK).

Amplified PCR products were cleaned using the HT ExoSAP-IT™ High-Throughput PCR product Cleanup (Applied Biosystems™, UK) following the manufacturer's instructions. Briefly, 5 µl of ExoSAP-IT™ reagent were mixed with 12.5 µl of PCR product and incubated at 37°C for 15 minutes followed by a 15-minute incubation at 80°C. A subset of cleaned PCR products were separated on a 2% agarose gel stained with SYBR™ safe (Invitrogen™, UK) diluted 10,000-fold and visualised under ultraviolet light, using a TrackIt™ 100 bp DNA ladder (Invitrogen™, UK) as a size marker.

Products were sent for fragment analysis to the MRC Protein Phosphorylation and Ubiquitylation DNA sequencing unit within Dundee University. Samples were prepared by diluting 1 in 40 with the LIZ 500 ladder for size reference and separated by capillary electrophoresis. Subsequent data files were viewed, and sizing performed using Peak Scanner™ version 1.0.

A selection of five females testing as *Cx. pipiens* f. *pipiens,* according to CQ11, from each of the field sites from Sections 4.2.1 and 4.2.2 were included for microsatellite analysis (total of 8 sites as site "UR1" was common between the two). An additional 6 individuals from three of the sites were included for analysis which tested as *Cx. pipiens* f. *molestus* (2) or hybrid individuals (4) according to

the CQ11 microsatellite marker. Finally, five female individuals from each of the single biotype and hybrid colony lines as per Chapter 2, an autogenous *Cx. pipiens* f. *molestus* colony from Sweden and a *Cx. quinquefasciatus* colony were also included in the genetic analysis.

Table 4.4. Microsatellite markers for genetic analysis of *Culex pipiens* specimens.

Target	Repeat	Product size (bp)	Annealing temperature	Reference
CQ11	$(GT)_2ACTTC(GT)_9$	234-289	58°C	Fonseca *et al.* (1998)
CQ26	$(GTGTGTAT)_2+(GT)_{10}+(GT)_5$	178-220	66°C	Fonseca *et al.* (1998)
CQ41	$(GT)_{12}$	135-171	62°C	Fonseca *et al.* (1998)
CxpGT4	$(GT)_5(GTTT)_2GC(GT)_2CT(GT)_5$	139-153	64°C	Keyghobadi *et al.* (2004)
CxpGT9	$(GT)_{13}$	108-142	56°C	Keyghobadi *et al.* (2004)
CxpGT12	$(TG)_{14}$	144-178	62°C	Keyghobadi *et al.* (2004)
CxpGT20	$(TG)_{15}$	110-155	60°C	Keyghobadi *et al.* (2004)
CxpGT40	$(GT)_{15}$	169-231	62°C	Keyghobadi *et al.* (2004)
CxpGT46	$(TG)_{15}$	260-286	66°C	Keyghobadi *et al.* (2004)
CxpGT51	$(TG)_4CG(TG)_{15}$	108-174	60°C	Keyghobadi *et al.* (2004)
CxpGT53	$(TG)_{22}$	230-325	62°C	Keyghobadi *et al.* (2004)
CxqGT4	$(TG)_{22}$	152-156	64°C	Smith *et al.* (2005a)

Genetic diversity at each microsatellite locus was characterised by estimates of expected heterozygosity using Nei's unbiased estimator and inbreeding coefficient (F_{IS}). Departures from Hardy-Weinberg proportions were tested by exact tests available in FSTAT version 2.9.4 (Goudet, 1995). Genetic differentiation between groups was measured by estimated of the fixation index (F_{ST}) which were calculated according to Weir and Cockerham (Weir and Cockerham, 1984) using ARLEQUIN version 3.5.2.2 (Excoffier *et al.*, 2005).

Bayesian clustering analysis was used to infer population substructure and ancestry from the dataset without prior information of sampling groups, under the admixture model with correlated allele frequencies using STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000). Ten independent runs with 10^5 burn-in steps and 10^5 iterations were completed for each value of *K* (*K* = 1-10 clusters). The program STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt, 2012) was used to analyse these results and apply Evanno's DK (Evanno *et al.*, 2005) to determine the most likely number of clusters. Individual genetic assignment to clusters was based on a minimum posterior threshold (*Tq*) of 0.90, with individuals displaying $0.1 \leq qi \leq 0.9$ considered of admixed ancestry.

4.2.5 Statistical analysis

Variation of larval density between sites and habitat types was assessed using one-way ANOVA with Tukey correction for multiple comparison. Chi-square test with Bonferroni correction was used to compare rates (blood-feeding, egg production and oviposition) between locations and a one-way ANOVA with Tukey correction was used to study spatial variation in female fecundity. Environmental parameters (minimum, maximum and average temperature; average lux; BOD_5) between sites were investigated using one-way ANOVA with Tukey correction. Correlation between physiological and individual environmental factors were investigated with Pearson's correlation.

To assess the effect of different variables on the larval density of *Cx. pipiens* at each site, a generalised linear mixed model (GLMM) was fitted to the data. As the response variable (total larvae present) was in the form of count data, the initial model consisted of a Poisson GLMM with a log link function including average temperature, average lux, temperature fluctuation and biological oxygen demand as covariates, fitted by maximum likelihood with the Laplace approximation, with dipping replicate included as a random factor. The Poisson model indicated that the data were over dispersed (residual deviances > degrees of freedom) therefore a negative binomial GLMM was fitted to the data. The goodness of fit of the models to the data was assessed by comparison of Akaike information criterion (AIC) values, with the lower values indicating a better model fit. The final model was obtained by stepwise deletion of non-significant factors and variables based on AIC values. An AIC value change of ≤2 units indicated a particular factor or variable did not significantly explain the response variable.

To assess the effect of environmental factors on female fecundity, a Poisson GLMM was fitted to the data with a log link function. A further four models were constructed to assess how environmental factors influenced blood-feeding success, egg production, oviposition and adult survival. As the response variables were binary, the models consisted of a binomial GLMM with a logit link function. For all models, average temperature, average lux, temperature fluctuation, BOD_5 and larval density were included as covariates, fitted by maximum likelihood with Laplace approximation, with experimental cage included as a random factor.

To investigate the effect of larval diet on physiological parameters (development, fecundity, fertility) a one-way ANOVA with Tukey correction or Kruskal-Wallis H test where log transformation did not normalise data were used. To assess the influence of larval diet on rates (larval survival, sex ratio, blood-feeding, oviposition, biotype ratio) Chi-squared test with Bonferroni correction for multiple comparisons were used. For all tests the statistical significance level was set at 0.05 and was computed in R studio version 1.2 (RStudio Team, 2020).

4.3 Results

4.3.1 Biotype identity of *Culex pipiens* collected from inner London

In total, 303 adult host-seeking mosquitoes were collected from three Inner London sites between August and September 2019. The garden site was shown to have the highest abundance with 239 (78.88%) of the collected mosquitoes originating from this site. In contrast, 27 (8.91%) individuals from the total catch originated from the zoo site whilst the remaining 37 (12.21%) were collected from the allotment. All adults collected using the BG sentinel traps were morphologically identified as *Culex pipiens/torrentium.* Of the 303 adult mosquitoes caught, 112 (approximately 40%) were selected for molecular identification (Table 4.5). A total of 2 (1.79%) individuals were identified as *Cx. pipiens* f. *molestus*, 1 (0.89%) as a hybrid with the rest testing as *Cx. pipiens* f. *pipiens*. Adult hybrid individuals were only reported from the garden site, whilst *Cx. pipiens* f. *molestus* was only identified at two sites (the garden and zoo site).

Table 4.5. Biotype ratio of adult host-seeking *Culex pipiens,* as determined by the CQ11 assay, collected from BG sentinel traps baited with CO_2 from three inner London field sites representing different green spaces.

Site	Biotype		
	Culex pipiens* f. *pipiens	***Culex pipiens* f. *molestus***	**Hybrid**
Garden	46 (95.8%)	1 (2.08%)	1 (2.08%)
Zoo	26 (96.3%)	1 (3.70%)	0
Allotment	37 (100%)	0	0

Larvae were collected weekly from all sites and were reared to adults in an environmentally controlled insectary for morphological identification. A total of 4434 larvae were collected across all larval habitats throughout August and September 2019. Of the collected larvae, 4424 (99.77%) were morphologically identified as *Cx. pipiens*, with the remaining 12 (0.27%) identified as *Culiseta annulata* (Table 4.6). A subset of 150 individuals were chosen for molecular characterisation, which identified no individuals determined as *Cx. pipiens* f. *molestus*, 2 (1.34%) determined as hybrids and the remaining 148 (98.67%) determined as *Cx. pipiens* f. *pipiens*.

Table 4.6. Biotype ratio of larval *Culex pipiens* collected from permanent larval habitats from three inner London field sites representing different green spaces

Site	Biotype		
	Culex pipiens* f. *pipiens	***Culex pipiens* f. *molestus***	**Hybrid**
Garden	50 (100%)	0	0
Zoo	50 (100%)	0	0
Allotment	48 (98%)	0	2 (4%)

A total of 97 resting adult mosquitoes were collected across the three sites, with the London Zoo site yielding the highest collection of 80 (85.57%) mosquitoes. Collected mosquitoes were identified as either *Culex pipiens*/*torrentium* or *Culiseta annulata.* The resting box within the aviary at the zoo was shown to be the most productive collection point and was the only resting box to yield blood-fed mosquitoes. No resting blood-fed mosquitoes were collected from the allotment or garden site.

Of the 97 mosquitoes collected, 9 (9.28%) had recently taken a bloodmeal, of which successful PCR amplification and sequencing was achieved for 7 (77.78%). All blood-fed mosquitoes were assigned to the *pipiens* biotype. Sequence analysis of bloodmeal source is shown in Table 4.7. All females were shown to have recently acquired a bloodmeal from an avian host apart from one individual, which had recently fed on a dromedary camel (*Camelus dromedarius*). A total of three samples was not shown to closely align to any entries in Genbank, matching closest to the Indian roller (*Coracias benghalensis*) with a 90.53% identity. Animal inventory of species housed within the area suggest the sequence data most likely belong to the blue-bellied roller (*Coracias cyanogaster)* for which there are no current entries in Genbank for this locus. The remaining individuals were shown to have recently fed on the Eurasian magpie (*Pica pica*) which is widely present in the UK avifauna.

Table 4.7. Analysis of bloodmeal source from resting adult female *Culex pipiens* collected from an urban zoo in inner London.

ID number	Bloodmeal host	Query cover	% Identity	Accession no.
CxpR0001	*Pica pica*	99%	99.57%	GU571570.1
CxpR0002	*Coracias benghalensis*	97%	90.53%	KC439309.1
CxpR0003	*Pica pica*	97%	99.41%	GU571570.1
CxpR0004	*Coracias benghalensis*	97%	90.53%	KC439309.1
CxpR0005	*Pica pica*	97%	99.85%	GU571570.1
CxpR0006	*Coracias benghalensis*	97%	90.53%	KC439309.1
CxpR0007	No sequence data	-	-	-
CxpR0008	No sequence data	-	-	-
CxpR0009	*Camelus dromedarius*	97%	98.97%	MF598736.1

4.3.2 Assessment of environmental factors influencing reproductive traits of field collected *Culex pipiens*

Site characterisation

Daily maximum, minimum and average temperatures were shown to vary significantly between the sites (KW χ^2 = 33.019, df= 5, *P* = <0.001 and F= 7.96, df= 5, *P* <0.001; KW χ^2= 13.794, df= 5, *P* = 0.0170; Table 4.8, Figure 4.6), with site "PUR1" supporting the highest mean temperatures (average: 21.0 ± 3.0°C, maximum: 29.1 ± 6.77°C, minimum: 16.6 ± 2.66°C). In contrast site "UR1" experienced the lowest maximum and average temperatures (average: 18.2 ± 1.78°C, maximum: 20.0 ± 1.78°C), which was significantly lower than conditions experienced at site "PUR1" (*P* = 0.029). The lowest minimum temperatures were experienced at site "RU1" (12.5 ± 2.32°C), significantly lower than all other sites (*P* <0.05). Temperature fluctuations were also shown to significantly vary between the different sites (KW χ^2= 41.164, df= 5, *P* <0.001) with sites "RU1" and "RU2" experiencing the highest daily temperature fluctuations with average recordings of 16.0 ± 6.05 and 16.3 ± 8.05°C. In contrast sites "UR1" and "UR2" showed the lowest daily temperature fluctuations with average recordings of 4.34 ± 1.30 and 5.87 ± 2.32°C, which were significantly lower than all other sites (*P* <0.05).

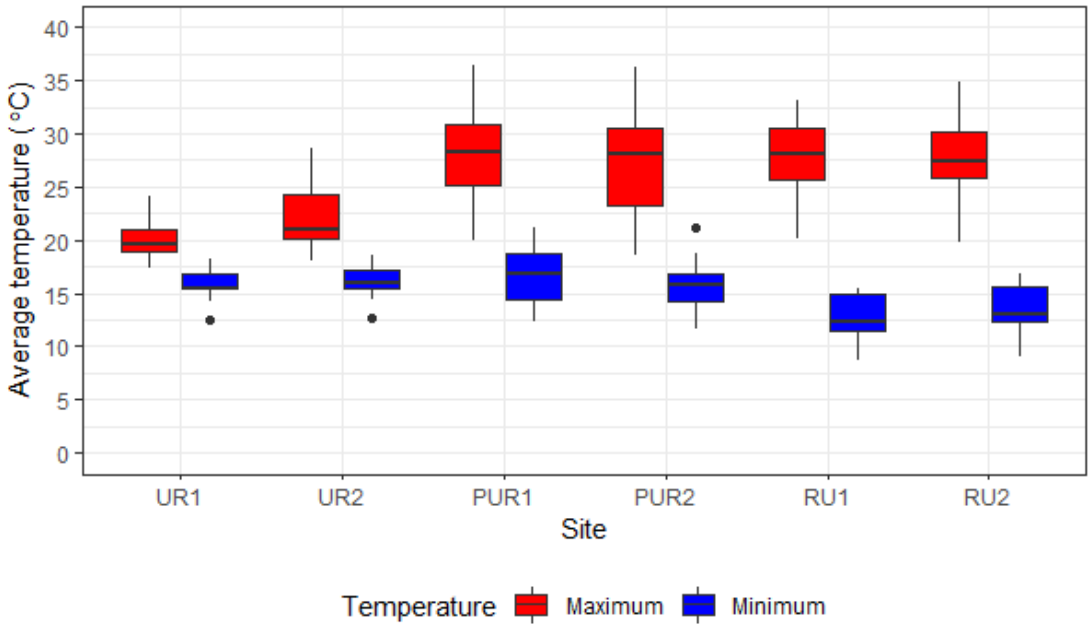

Figure 4.6. Minimum and maximum water temperatures recorded during the collection period at six sites across urban, peri-urban and rural habitats. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers.

Table 4.8. Monitoring of environmental conditions at six sites across urban, peri-urban and rural habitats. The letters following mean values indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

Site	Maximum temperature (°C)		Minimum temperature (°C)		Average temperature (°C)		Temperature fluctuation (°C)		BOD_5 (mg/L)		Light intensity (Lux)	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
UR1	20.0 **a**	1.78	15.8 **ac**	1.45	18.2 **a**	1.78	4.34 **a**	1.30	6.80 **a**	0.2	2238 **a**	-
UR2	21.8 **ab**	2.93	16.2 **a**	1.73	19.4 **ab**	2.25	5.87 **a**	2.32	6.40 **a**	0.1	1526 **a**	-
PUR1	29.1 **c**	6.77	16.6 **ac**	2.66	21.0 **b**	3.00	11.6 **b**	4.53	6.87 **a**	0.46	34911 **b**	-
PUR2	27.2 **bc**	5.15	15.7 **ac**	2.37	20.2 **ab**	2.51	11.5 **b**	5.06	6.70 **a**	0.2	5805 **cd**	-
RU1	27.1 **bc**	3.83	12.5 **b**	2.32	18.3 **ab**	1.33	16.0 **b**	6.05	6.37 **a**	0.06	15220 **bc**	-
RU2	26.5 **bc**	3.38	13.3 **bc**	2.48	19.1 **ab**	1.18	16.3 **b**	8.05	6.30 **a**	0.17	7956 **ad**	-

Biological oxygen demand was not shown to vary significantly between the sites (F= 3.218, df= 5, *P* = 0.0511), however, sites within peri-urban and urban areas were shown to have marginally higher BOD_5 recording than those in rural areas. The highest average BOD_5 measurement recoded from site "PUR1" with an average measurement of 6.87 mg/L with the lowest recording from site "RU1" with 6.3 mg/L. A significant positive correlation was noted between BOD_5 recording and average larval density (t= 3.29, df=16, *P* = 0.0046) however, BOD_5 was not shown to be significantly associated with any of the other variables.

Light intensity was shown to vary significantly between the larval habitats with the highest average intensities experienced at site "PUR1" with an average intensity of 34,910.82 lux. Contrastingly site "UR2" experience the lowest average light intensities with an average of 1526.15 lux.

Larval density

Larval habitats were left *in situ* for two weeks, following which larval densities were estimated. Larval densities were shown to vary significantly between the six sites (F= 10.27, df= 5, *P* <0.001; Figure 4.7) and by habitat classification (F= 12.31, df= 2, *P* <0.001). The larval habitat created at site "PUR1" was shown to have the greatest larval density (0.115 ± 0.189 larvae/L) which was significantly higher than all other sites (*P* >0.05) except for "PUR2" (0.0637 ± 0.0105; *P* = 0.237). Again, larval densities at site "PUR2" were not significantly higher than any of the other sites (*P* >0.05) except for "RU1" which had the lowest average larval density of 0.0239 ± 0.0115 larvae/L. Larval densities at site "RU1" were not shown to vary significantly from those measured at "RU2" (*P* = 0.269), "UR1" (*P* = 0.153) and "UR2" (*P* = 0.594). When considering the habitat classifications, the peri-urban sites were shown to have significantly higher larval densities compared with urban (*P* = 0.00667) and rural (*P* <0.001) sites. No significant difference in densities between urban and rural sites were detected (*P* = 0.513).

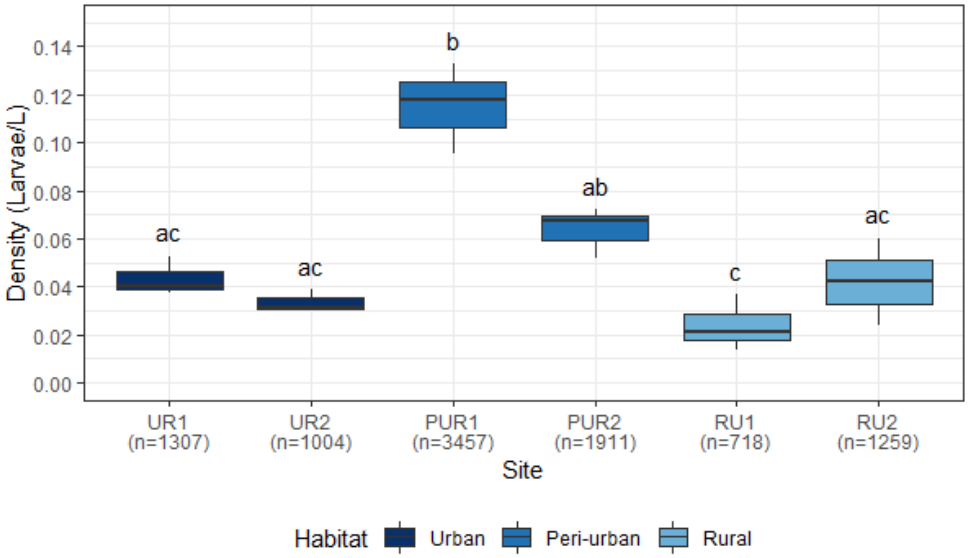

Figure 4.7. Larval density (larvae/L) recorded from six larval habitats across urban, peri-urban and rural habitats. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

Larval density at each site was shown to be positively correlated with average temperature (t= 3.4473, df=16, P = 0.00331) with an average temperature increase of 1^{o}C expected to lead to an estimated 121% increase in mosquitoes present in the larval habitats (P = 0.0086; Table 4.8). Furthermore, larval density was also shown to be positively correlated with minimum (t= 2.7581, df= 16, P = 0.014) and maximum temperatures (t= 2.306, df= 16, P = 0.0349). Biological oxygen demand measurements were positively correlated with larval density (t= 3.293, df= 16, P = 0.00459) with a 1 mg/L rise in BOD_5 would be expected to lead to approximately 283% more larvae present (Table 4.9). In contrast, no significant correlation was detected between temperature fluctuation and larval density (t= -0.917, df= 16, P = 0.373).

Table 4.9. Poisson GLMM regression coefficients, with 95% Wald confidence intervals and standard error. Predicted % difference is the (exponent x 100) of the value in the estimate column and gives the estimated change in larval density collected for a one-unit increase in meteorological variables. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Coefficients	Estimate (95% CI)	Predicted % difference	Std. error
(Intercept)	0.106 (0.0023; 4.80)	-	1.945
Average temperature	0.190 (1.064, 1.37) **	120.9	0.065
Average lux	0.065 (0.897, 1.27)	106.7	0.088
Temperature fluctuation	-0.014 (0.945, 1.03)	98.6	0.022
BOD_5	1.04 (1.31, 6.13) **	282.9	0.395

Blood-feeding

The percentage of adult females acquiring a bloodmeal was shown to vary significantly between the collection sites (χ^2 = 30.62, df= 5, *P* <0.001; Figure 4.8). The highest percentage of blood-feeding adults were seen at site "PUR1" with 50% of female mosquitoes successfully acquiring a bloodmeal. In contrast, site "UR1" recorded a significantly lower blood-feeding success (*P* < 0.001), with a total of 30 out of 150 (20%) adult females successfully taking a bloodmeal, the lowest recorded.

Average temperature experienced during larval development was shown to be positively correlated with proportion of female mosquitoes blood-feeding (t=7.865, df=4, *P* = 0.0014), with increased temperature levels having an increased chance of blood-feeding (odd ratio = 1.51, *P* <0.001; Table 4.10). Furthermore, larval density was also shown to be positively correlated with blood-feeding success (t= 3.086, df= 4, *P* = 0.037).

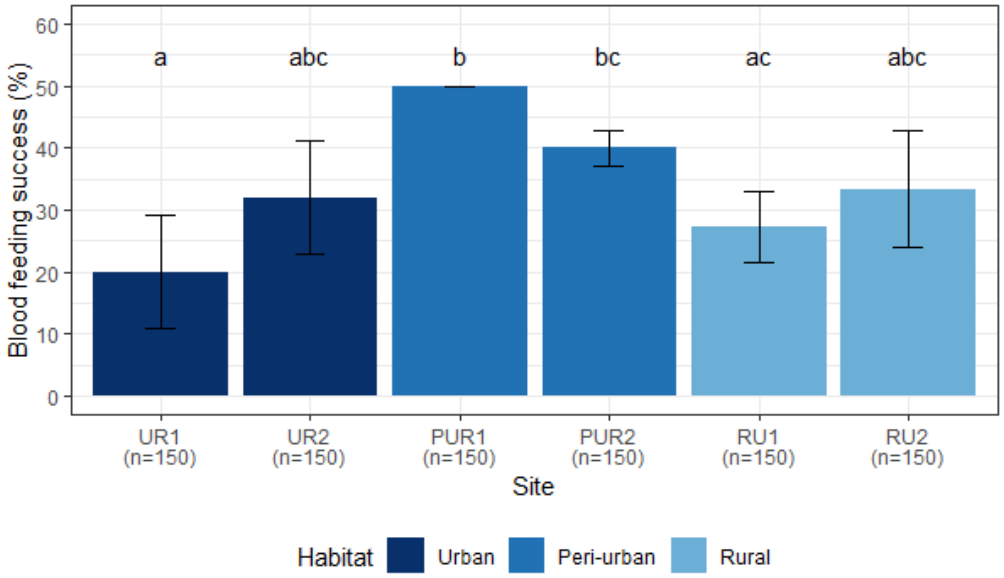

Figure 4.8. Blood-feeding success (%) of field collected *Culex pipiens* from six larval habitats. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly (*P* < 0.05) from one another. Specifically, groups which share a common letter are not significantly (*P* < 0.05) different from one another.

Table 4.10. Binomial GLMM regression coefficients, with 95% Wald confidence intervals and standard error, for the likelihood of successful bloodmeal acquisition. The odds ratios are the exponent of the values in the 'estimate' column and indicate the odds of successful feeding to repletion. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Coefficients	Estimate (95% CI)	Odds ratio	Std. error
(Intercept)	-8.055 ***	-	2.197
Average temperature	0.412 ***	1.51	0.120
Average lux	0.009	1.01	0.014
Temperature fluctuation	0.016	1.02	0.023
BOD_5	-0.182	0.833	0.321
Larval density	-0.01	0.99	0.021

Fecundity

A total of 78 egg rafts was assessed for number of eggs produced by females from each site. Female fecundity significantly varied between sites (F= 5.202, df= 5, P <0.001; Figure 4.9). The largest egg rafts were produced by females originating from site "UR1" with an average raft size of 109 ± 27.0 eggs/raft. In contrast the smallest egg rafts were produced by females from site "RU1" which produced an average of 53.2 ± 23.5 eggs/raft, significantly smaller than those produced by females from sites "UR1" (P <0.001), "UR2" (103 ± 45.0, P = 0.008) and "PUR2" (94 ± 27.6, P = 0.0207). However, these were not shown to differ significantly from egg rafts produced by females from sites "PUR1" (73.6 ± 33.2, P = 0.517) or "RU2" (84.2 ± 58.4, P = 0.461)

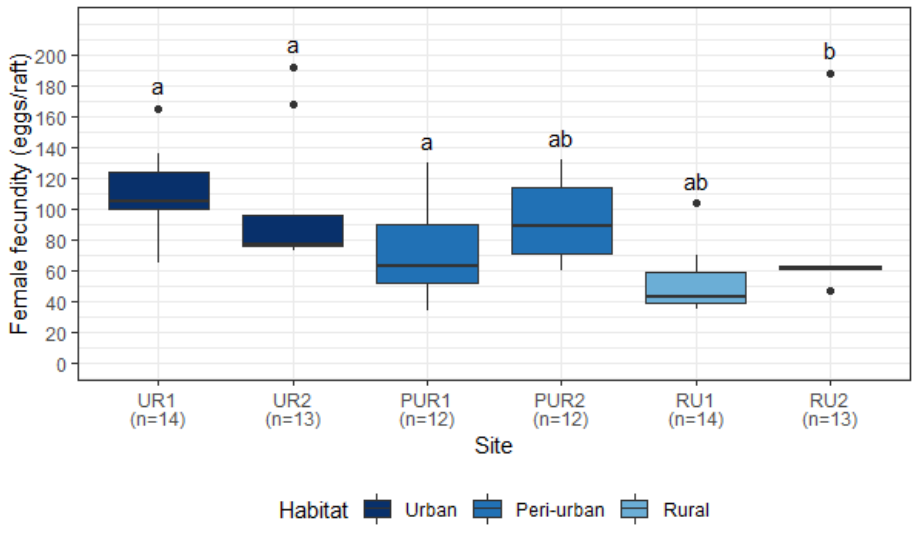

Figure 4.9. Fecundity (number of eggs/raft) of female *Culex pipiens* collected from six different larval habitats under disparate environmental conditions. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. The letters above the boxplots indicate which groups differ significantly (P < 0.05) from one another. Specifically, groups which share a common letter are not significantly (P < 0.05) different from one another.

Total egg raft size was also shown to be negatively correlated with average daily temperature fluctuations (t= -4.253, df= 53, P <0.001) with reduced egg raft size seen in individuals exposed to higher temperature fluctuations during larval development.

The covariates *average temperature* and *temperature fluctuation* were shown to significantly influence female fecundity (Table 4.11). A 1°C temperature increase during larval development would be predicted to lead to a 112.4% increase in egg raft size, while a 1°C increase in fluctuation between maximum and minimum temperatures would be expected to cause a 78.1% decrease in egg raft size.

Table 4.11. Poisson GLMM regression coefficients, with 95% Wald confidence intervals and standard error. Predicted % difference is the (exponent x 100) of the value in the estimate column and gives the estimated change in egg raft size for a one-unit increase in environmental variables. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Coefficients	Estimate (95% CI)	Predicted % difference	Std. error
(Intercept)	4.171 (49.514, 84.87) ***	-	0.137
Average temperature	0.126 (1.004, 1.28) *	112.4	0.062
Average lux	-0.189 (0.679, 1.01)	-82.8	0.101
Temperature fluctuation	-0.247 (0.678, 0.90) ***	-78.1	0.072
BOD_5	2.078 (0.285, 233.3)	798.4	1.700
Larval density	0.085 (0.917, 1.29)	108.9	0.087

Of the females that acquired a bloodmeal, approximately 34.94% produced egg rafts that were either laid or retained with no significant difference in the proportion of female producing eggs between the sites (χ^2= 9.358, df= 5, P = 0.0956). When considering proportion of gravid females laying eggs, a total of 46 egg rafts (58.97%) were oviposited, however this was not shown to be significantly different between the sites (χ^2= 4.695, df= 5, P = 0.454). None of the covariates were shown to significantly influence egg production (Table 4.12) or oviposition rate (Table 4.13).

Table 4.12. Binomial GLMM regression coefficients, with 95% Wald confidence intervals and standard error, for the likelihood of successful egg production. The odds ratios are the exponent of the values in the 'estimate' column and indicate the odds of successful egg production. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Coefficients	Estimate (95% CI)	Odds ratio	Std. error
(Intercept)	2.510 (0.003, 56682)	-	4.303
Average temperature	-0.478 (0.383, 1.00)	0.62	0.246
Average lux	-0.021 (0.927, 1.03)	0.98	0.028
Temperature fluctuation	0.071 (0.954, 1.21)	1.07	0.060
BOD_5	1.264 (0.947, 13.22)	3.54	0.673
Larval density	-0.020 (0.900, 1.08)	0.98	0.049

Table 4.13. Binomial GLMM regression coefficients, with 95% Wald confidence intervals and standard error, for the likelihood of successful oviposition. The odds ratios are the exponent of the values in the 'estimate' column and indicate the odds of successful ovipositing. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Coefficients	Estimate (95% CI)	Odds ratio	Std. error
(Intercept)	0.103 (0.00, 20462)	-	5.012
Average temperature	-0.244 (0.452, 1.33)	0.78	0.269
Average lux	-0.043 (0.893, 1.03)	0.96	0.036
Temperature fluctuation	0.031 (0.909, 1.17)	1.03	0.065
BOD_5	0.747 (0.466, 9.56)	2.11	0.771
Larval density	0.016 (0.794, 9.56)	1.02	0.126

Autogeny

A total of 900 adults collected from six larval habitat as late-stage larvae and pupae were assessed for the presence of autogeny. No occurrence of autogenous egg production was observed by monitoring of oviposition cups during this study, which was subsequently confirmed by dissection of females to monitor for egg retention.

Adult wing length

Adult wing length was shown to vary significantly between the sites (F = 11.11, df= 5, P <0.001; Figure 4.10). Females collected from site "UR1" had the greatest average wing length of 3.53 ± 0.21 mm whilst the smallest average wing lengths were recorded in adults from site "PUR1" (3.04 ± 0.28 mm). Average wing lengths of females collected from site "PUR1" were significantly smaller compared with those obtained from all other sites (P <0.001) with the exception of site "RU2" for which no statistically significant difference was detected (P = 0.105). Wing length was shown to be positively correlated with female fecundity (t= 2.53, df= 40, 0.016) with a greater number of eggs produced by larger females (Figure 4.11).

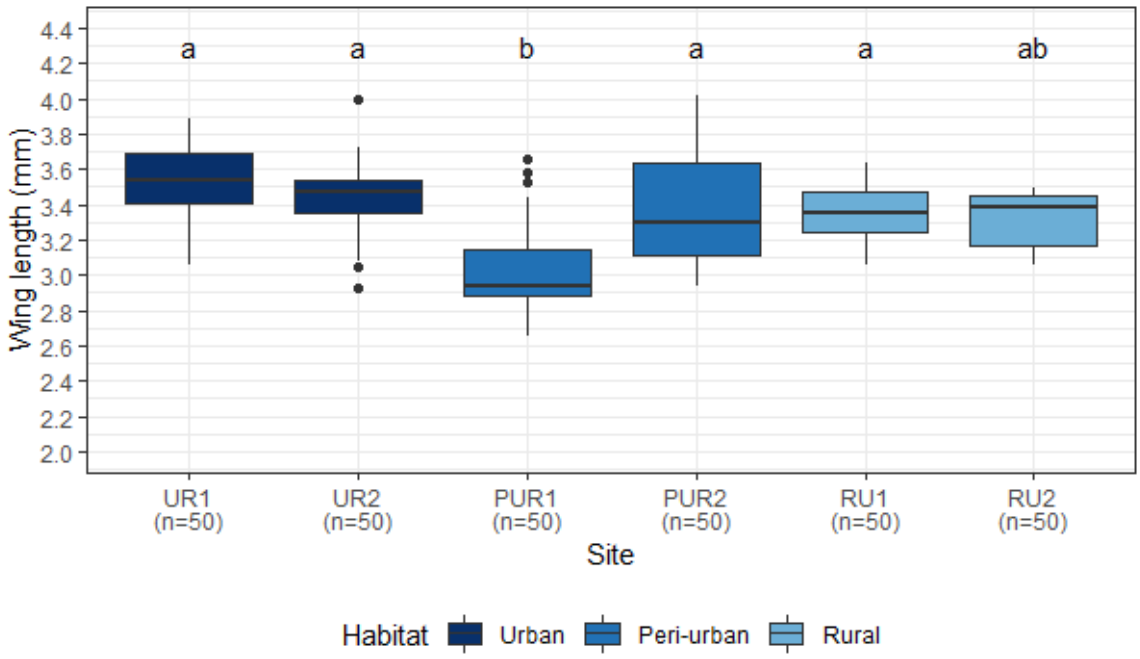

Figure 4.10. Wing length measurements (mm) of field collected female *Culex pipiens* collected from six sites across urban, peri-urban and rural habitats. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles. The letters above the boxplots indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

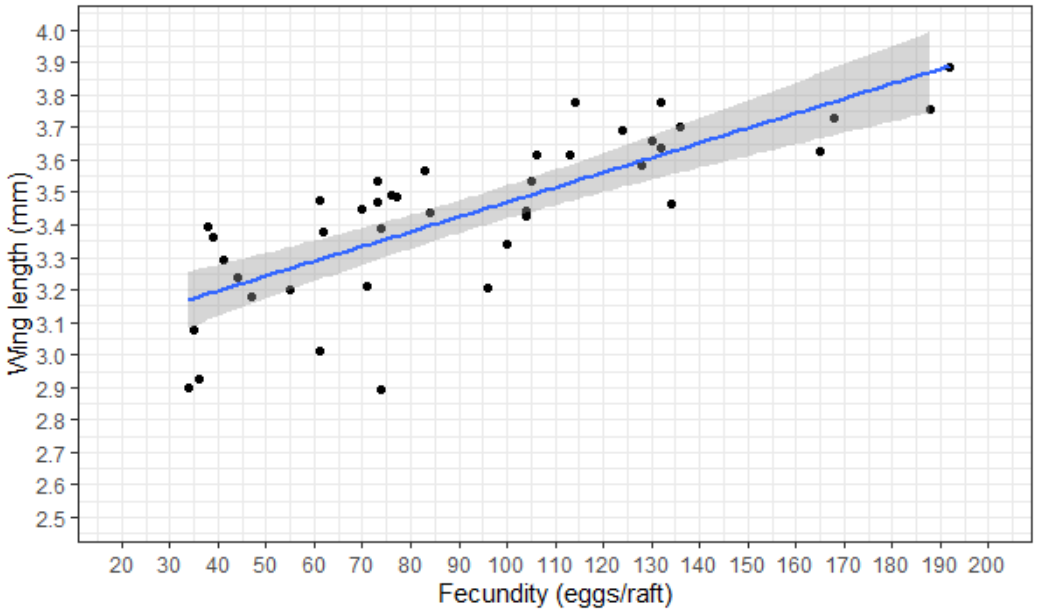

Figure 4.11. Wing length measurements (mm) of field collected female *Culex pipiens* collected from six sites across urban, peri-urban and rural habitats compared with corresponding female fecundity. Grey shaded areas indicate 95% confidence interval.

Adult Survival

Survival of adult mosquitoes was shown to vary significantly between the sites (χ^2 = 44.58, df = 5, *P* <0.001; Figure 4.12). The highest proportion of surviving adults were from site "UR1" with a total of 246 out of 300 (82 ± 7.21%) surviving the two-week observation period. This was significantly higher than those observed from all other sites (*P* <0.05) with the exception of sites "UR2" (*P* = 1.00) and "RU1" (*P* = 0.231). In contrast adults from site "PUR1" had the lowest proportion of adults surviving with only 156 of 300 adults surviving (52 ± 3.46%) which was significantly lower than adult survival from "UR1" (*P* <0.001), "UR2" (*P* = 0.001), and "RU1" (*P* = 0.046). Adult survival was also shown to be negatively correlated with average temperature (odd ratio = 0.68, *P* = 0.011; Table 4.14) and temperature fluctuation (odd ratio = 0.92, *P* <0.001).

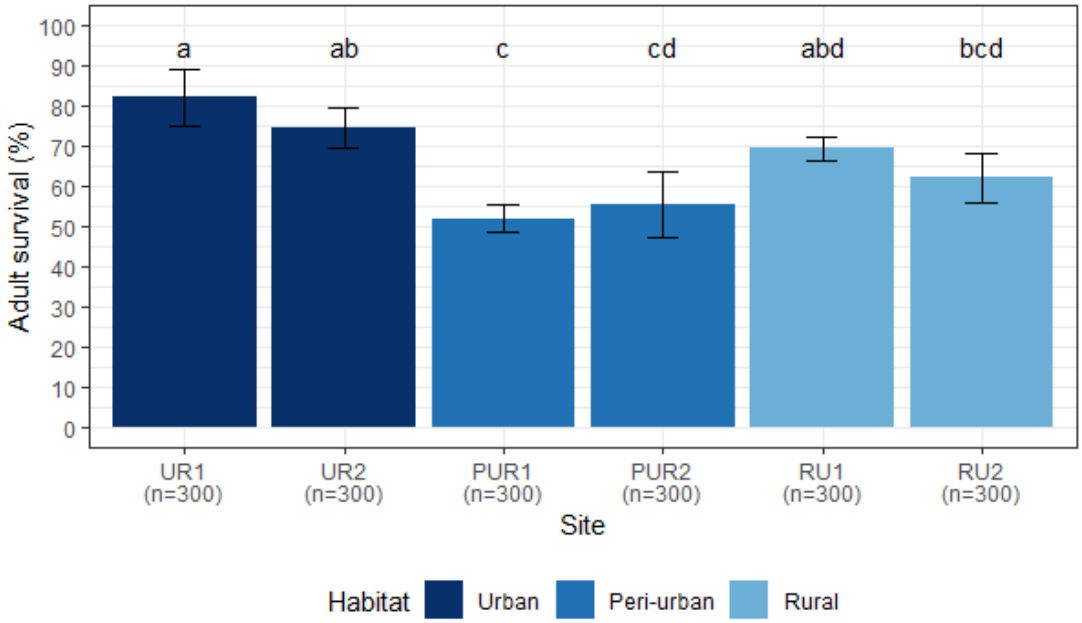

Figure 4.12. The percentage (%) of adults surviving over a two-week assessment period maintained under colony conditions of 25°C temperature with a relative humidity of 50%. Error bars indicate mean ± SD. The letters above the bars indicate which groups differ significantly (*P* < 0.05) from one another. Specifically, groups which share a common letter are not significantly (*P* < 0.05) different from one another.

Table 4.14. Binomial GLMM regression coefficients, with 95% Wald confidence intervals and standard error, for the likelihood of adult survival over a two-week period. The odds ratios are the exponent of the values in the 'estimate' column and indicate the odds of adult survival. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Coefficients	Estimate (95% CI)	Odds ratio	Std. error
(Intercept)	9.146 (0.00, 20462) **	-	2.824
Average temperature	-0.403 (0.491, 0.910) *	0.68	0.157
Temperature fluctuation	-0.062 (0.909, 0.972) ***	0.92	0.017
Larval density	0.005 (0.912, 1.11)	1.01	0.050

4.3.3 Influence of larval diet quantity on life history traits of field derived and colonised *Culex pipiens* populations

No significant difference between any of the parameters assessed was detected between the six field sites for each larval diet group, therefore, mosquitoes from all sites were pooled for further analysis.

Development time

Diet was shown to significantly influence larval development time in all populations (KW χ^2= 13.201, df=2, *P* = 0.00136; F= 38.3, df= 2, *P* =<0.001; KW χ^2= 7.2, df= 2, *P* =0.0273; KW χ^2= 7.36, df= 2, *P* = 0.0252 for field, *pipiens*, *molestus* and hybrid populations). Larvae maintained on a low protein diet were shown to have increased development times compared with the high and mid-protein diets (Table 4.15, Figure 4.13). Larvae reared under the high protein diets had the faster development times with larvae from the field mosquitoes reaching pupation within 8.01 ± 0.794 days, the fastest of all conditions and populations tested. In general, larvae reared on the low protein diets took significantly longer to develop when compared with the high (Z= -3.603, *P* <0.001; *P* = <0.001; Z= -2.683, *P* <0.001; Z= -2.711, *P* <0.001 for field, *pipiens, molestus* and hybrid lines) and mid-protein diet (Z= -3.011, *P* = 0.0078; Z=1.51, *P* =0.029; Z= 1.342, *P* <0.001; Z= 1.352, *P* <0.001).

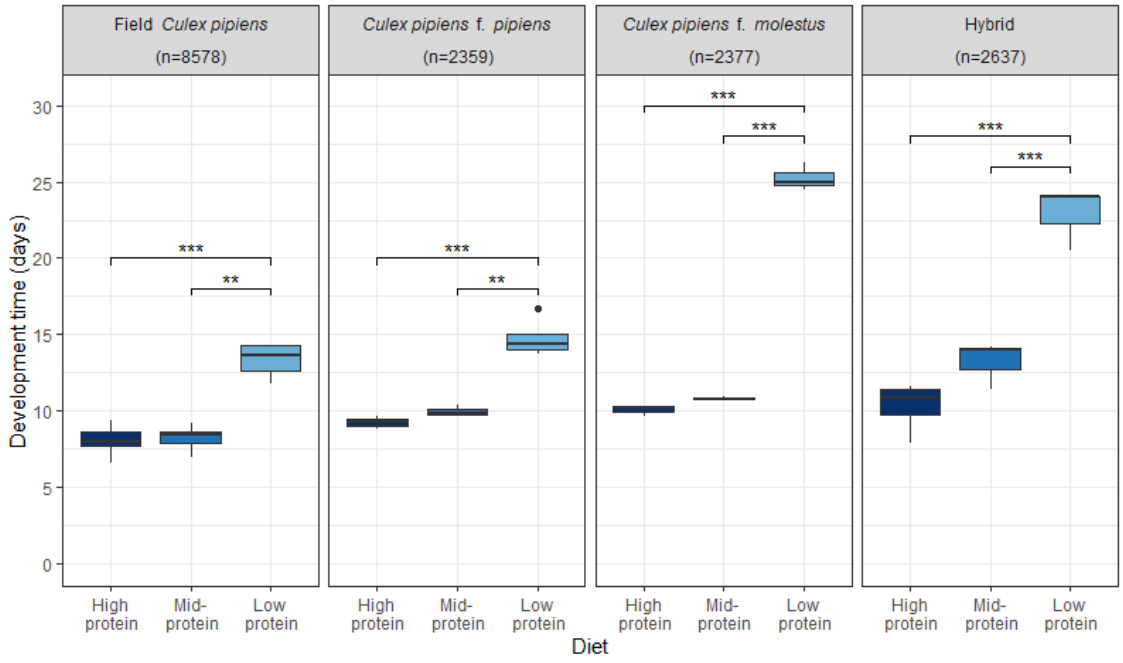

Figure 4.13. Development time (days) for 50% of larvae to reach pupation under three levels of larval nutrition for *Culex pipiens* from field derived populations as well as three colony lines. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

When considering differences between the populations, a statistically significant difference was detected within each treatment group (KW χ^2= 11.59, df= 3, P = 0.009; KW χ^2= 18.49, df= 3, P <0.0126; KW χ^2= 10.85, df= 3, P = <0.001; Table 4.15). Larvae from the *molestus* and hybrid colony lines had significantly longer development times compared with the field populations for all treatment groups (P <0.05). Development times of larvae from the *pipiens* colony were reduced significantly when reared on a low protein diet compared with the *molestus* (Z= 2.819, P = 0.0144) and hybrid (Z= 1.518, P = 0.0387) colony lines.

Larval survival

Larval nutrition was shown to significantly influence larval survival for each of the populations with the low protein diets suffering the highest mortalities (χ^2= 261.39, df= 2, P <0.001; χ^2= 142.03, df= 2, P <0.001; χ^2= 148.4, df= 2, P <0.001; χ^2= 117.6, df= 2, P <0.001 for the field, *pipiens*, *molestus* and hybrid populations respectively; Table 4.15, Figure 4.14). Larval survival in the low protein diets was significantly reduced compared with the high (P <0.001) and mid-protein (P <0.001) diets for all populations. In contrast the high protein diets had the greatest proportion of larvae surviving to pupation and was not shown to vary significantly from the mid-protein diets except for the hybrid

colony line (*P* <0.001). Hybrid colony line larvae fed on low protein diets suffered the highest mortality rates with only a 15.1 ± 5.58% chance of survival, the lowest of all treatment groups and populations.

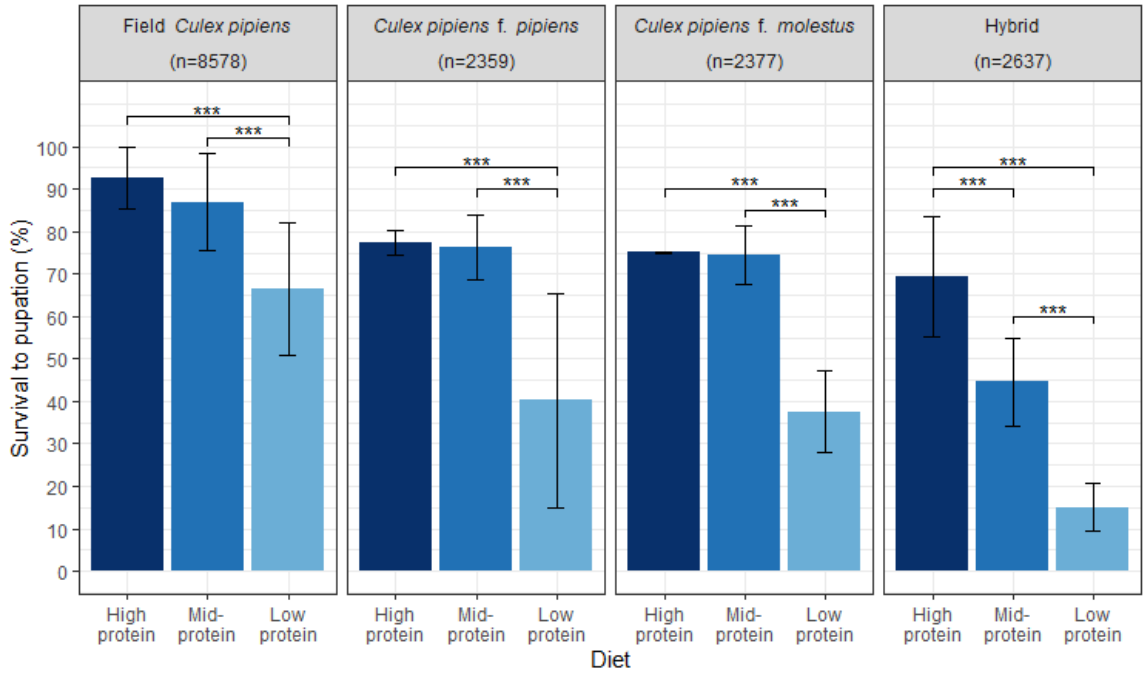

Figure 4.14. Mean larval survival (%) to pupation for four populations of *Culex pipiens*, originating from either field or colony, under vary levels of larval protein. Error bars indicate mean ± SD. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Response to larval nutrition was shown to vary significantly between the populations for each diet treatment group (χ^2= 213.21, df= 3, *P* <0.001; χ^2= 248.69, df= 2, *P* <0.001; χ^2= 162.48, df= 2, *P* <0.001 for the high, mid- and low protein diets respectively). Field derived larvae were shown to have a significantly higher larval survival compared with colony populations within each treatment group (*P* <0.001). Significant interactions between the colony lines were also noted, with the hybrid colony the most affected by depletion of larval nutrition. Larvae from the hybrid colony suffered significantly higher mortality rates within the mid- and low protein treatment groups when compared with the *pipiens* and *molestus* colony lines (*P* <0.001).

Eclosion success

Eclosion success was shown to decrease with reduced larval nutrition for all populations, although this association was only shown to be significant in the *molestus* colony line (χ^2 = 24.513, df= 2, *P* < 0.001; Figure 4.15, Table 4.15). Pupae from the low protein treatment group had a significantly reduced chance of completing eclosion compared with the high and the mid-protein groups (*P* <0.001) within the *molestus* colony line.

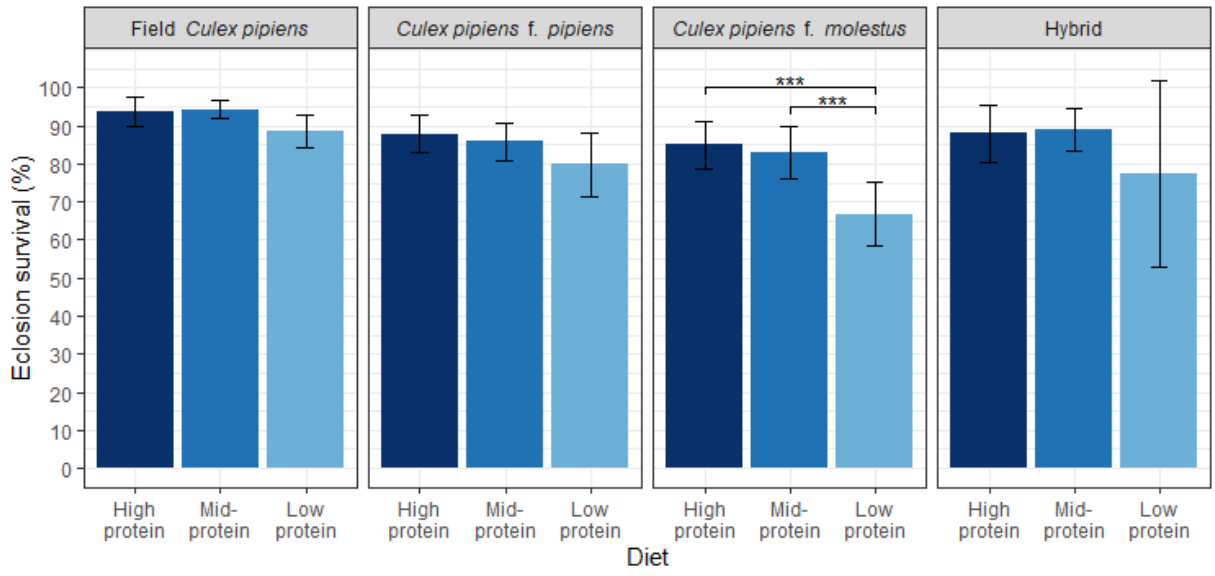

Figure 4.15. Average eclosion success of *Culex pipiens* under three levels of protein availability during larval development in three colony lines of *Culex pipiens*. Error bars indicate mean ± SD. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Pupation success was shown to be statistically different between the populations within the high (χ^2= 0.126, df= 2, P = 0.939), mid- (χ^2= 3.303, df= 2, P = 0.192) and low protein treatment groups (χ^2= 8.733, df= 2, P = 0.0127). Field populations has a significantly higher pupation success compared with the *molestus* colony for all treatment groups (P <0.001) and compared with the *pipiens* colony within the high (P = 0.0027) and mid-protein groups (P <0.001). Pupation success within the low protein group was also shown to be significantly higher in the *pipiens* compared with *molestus* colony (P = 0.0175).

Sex ratio

Sex ratio of pupae between the different treatment groups was not shown to vary significantly for the field (χ^2= 2.891, df= 2, P = 0.236), *pipiens* colony (χ^2= 0.776, df= 2, P = 0.679) or hybrid colony (χ^2= 0.711, df= 2, P = 0.701) populations, although a general trend of increased male:female ratio was observed with decreasing levels of larval nutrition for all three colony lines (Figure 4.16, Table 4.15). In contrast, this trend was shown to be significant in the *molestus* colony line (χ^2= 8.26, df= 2, P = 0.0161). The high protein diet was shown to have a 1:1.05 male:female ratio which was significantly different to the 1:0.59 ratio seen in the low protein diet (P = 0.0297).

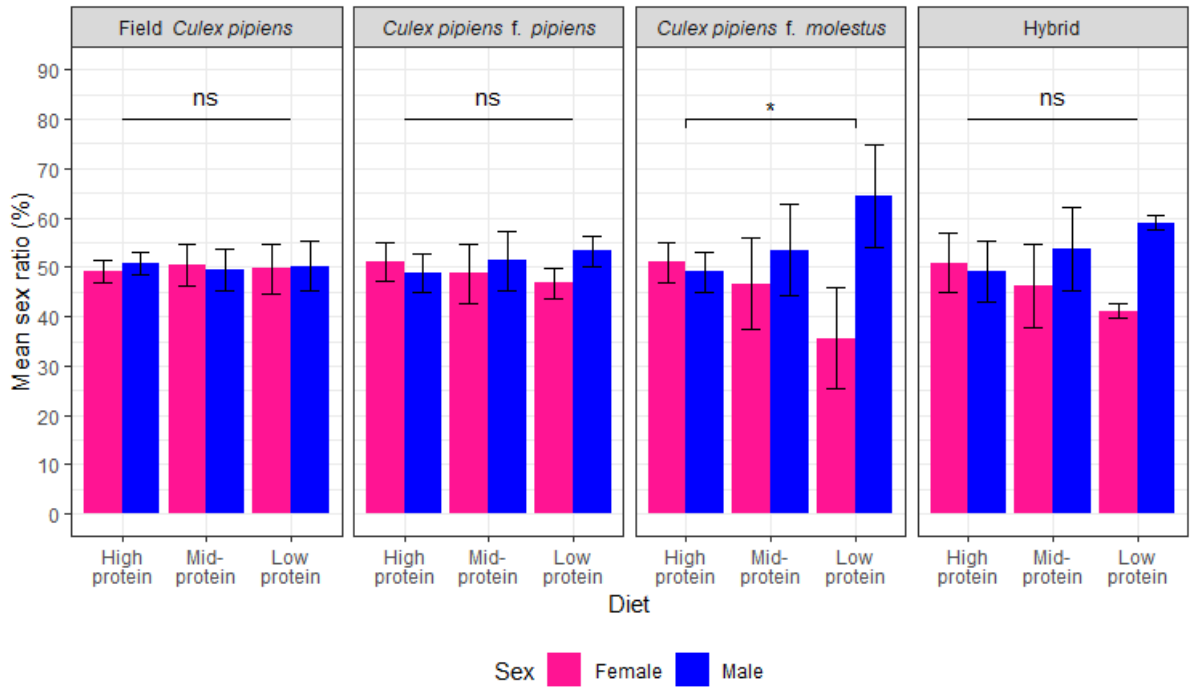

Figure 4.16. Average sex ratio (%) of male to females pupating under three larval diets of varying protein availability for three colony lines as well as field populations of *Culex pipiens*. Error bars indicate mean ± SD. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Table 4.15. The effect of larval nutrition of larval life history traits of field derived and colonised *Culex pipiens*. The letters following mean values indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

Origin	Diet	Larval development time (Days)		Larval survival (%)		Female proportion (%)		Eclosion success (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Field	High protein	8.01 **a**	0.794	92.8 **a**	7.26	49.18 **a**	2.29	93.8 **a**	3.80
	Mid-protein	8.27 **a**	0.561	86.9 **a**	11.5	50.50 **a**	4.33	94.3 **a**	2.23
	Low protein	13.3 **a**	1.22	66.6 **a**	15.6	49.73 **a**	4.99	88.7 **a**	4.22
Pipiens colony	High protein	9.22 a**b**	0.416	77.4 **b**	2.92	51.21 **a**	3.91	87.9 **b**	5.01
	Mid-protein	9.94 **ab**	0.362	76.3 **b**	7.60	48.69 **a**	6.12	85.9 **b**	4.93
	Low protein	14.8 **a**	1.34	40.2 **b**	25.2	46.76 **a**	3.00	79.8 **a**	8.37
Molestus colony	High protein	10.0 **b**	0.379	75.1 **b**	0.123	51.01 **a**	4.04	84.9 **b**	6.33
	Mid-protein	10.8 **b**	0.0855	74.4 **b**	6.94	46.57 **a**	9.26	82.9 **b**	6.90
	Low protein	25.2 **b**	0.892	37.6 **b**	9.61	35.56 **a**	10.26	66.8 **b**	8.25
Hybrid colony	High protein	10.3 **b**	1.70	69.3 **b**	14.2	50.84 **a**	6.04	87.9 **ab**	7.47
	Mid-protein	13.2 **b**	1.56	44.6 **c**	10.3	46.23 **a**	8.38	89.0 **ab**	5.66
	Low protein	22.8 **b**	2.02	15.1 **c**	5.58	41.05 **a**	1.49	77.4 **ab**	24.6

Proportion of adult blood-feeding

Female blood-feeding propensity was shown to vary significantly between the different diet groups for each population (χ^2= 10.578, df= 2, *P* =0.005; χ^2= 11.963, df= 2, *P* = 0.003; χ^2= 12.814, df= 2, *P* = 0.002; χ^2= 7.298, df= 2, *P* = 0.026; Figure 4.17). Females reared under the high protein diet had a greater probability of taking a bloodmeal compared with low protein diets, which was found to be statistically different in all populations except for the hybrid colony (*P* = 0.028, *P* = 0.005, *P* = 0.016 for the field, *pipiens* and *molestus* populations respectively). Females within the mid-protein group were also found to have a significantly higher chance of acquiring a bloodmeal compared with females reared on a low protein diet for the *pipiens* and *molestus* colony lines (*P* = 0.004 for both). In the field population, blood-feeding percentages were shown to be statistically greater in the high protein compared with the mid-protein group (*P* = 0.025).

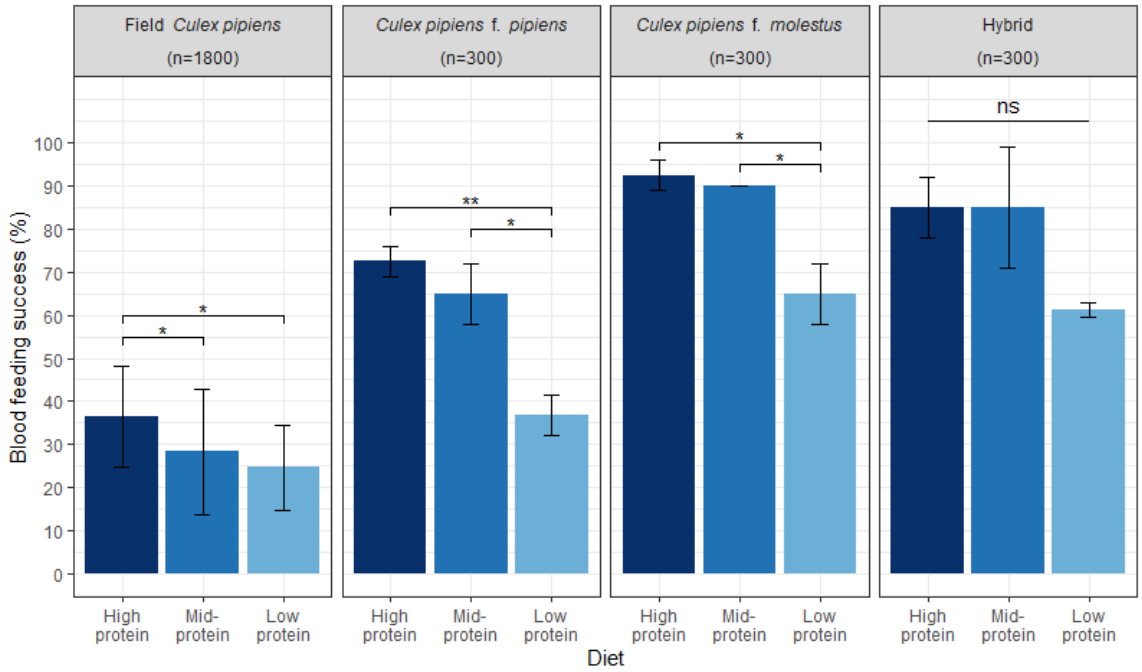

Figure 4.17. Mean blood-feeding success (%) of field collected and colonised female *Culex pipiens* reared under varying levels of nutritional availability. Error bars indicate mean ± SD. *** *P* ≤ 0.001, ** *P* ≤ 0.01, * *P* ≤ 0.05.

Colony populations were shown to be significantly more likely to acquire a bloodmeal compared with the field populations for all diet treatment groups (χ^2= 87.05, df= 3, *P* <0.001; χ^2= 114.86, df= 3, *P* <0.001; χ^2= 30.097, df= 3, *P* <0.001 for the high, mid- and low protein diets; Table 4.16). For all treatment groups field derived females were significantly less likely to successfully acquire a bloodmeal compared with colony females from the *molestus* and hybrid lines (*P* <0.001). When blood-feeding propensity of field derived females is compared with the *pipiens* colony, differences in

bloodmeal acquisition is significantly higher in the high and mid-protein groups (P <0.001) but not the low protein treatment group (P = 0.99).

Fecundity

A total of 265 egg rafts were assessed with an average size of 107.18 ± 37.62 eggs per raft. Combined, diet was shown to significantly influence the size of egg rafts. Females reared on the high protein diets produced the largest egg rafts (121 ± 39.1 eggs/raft; Figure 4.18) which was significantly higher than the 81.3 ± 29.9 eggs/raft produced by females reared on the low protein diet (P = 0.0118; P <0.001; P = 0.00149; P <0.001 for field, *pipiens*, *molestus* and hybrid colonies respectively). Females reared on the mid-protein diets produced an average of 103 ± 30.7 eggs/raft which were again significantly larger than those from the low protein diets for the three colony lines (P = 0.00535; P = 0.0226; P = 0.00334 for *pipiens*, *molestus* and hybrid lines respectively; Table 4.16). No significant difference was noted in egg raft size between the high and mid-protein diet for any of the colony lines (P = 0.344; P = 0.298; P = 0.631 for *pipiens*, *molestus* and hybrid lines respectively). However, for field derived populations, egg rafts produced by the mid-protein diet were shown to be significantly smaller compared with the high protein diet (P = 0.00105) whilst total eggs produced by the mid- and low proteins diets were not shown to differ significantly (Z= 0.135, P = 1.00).

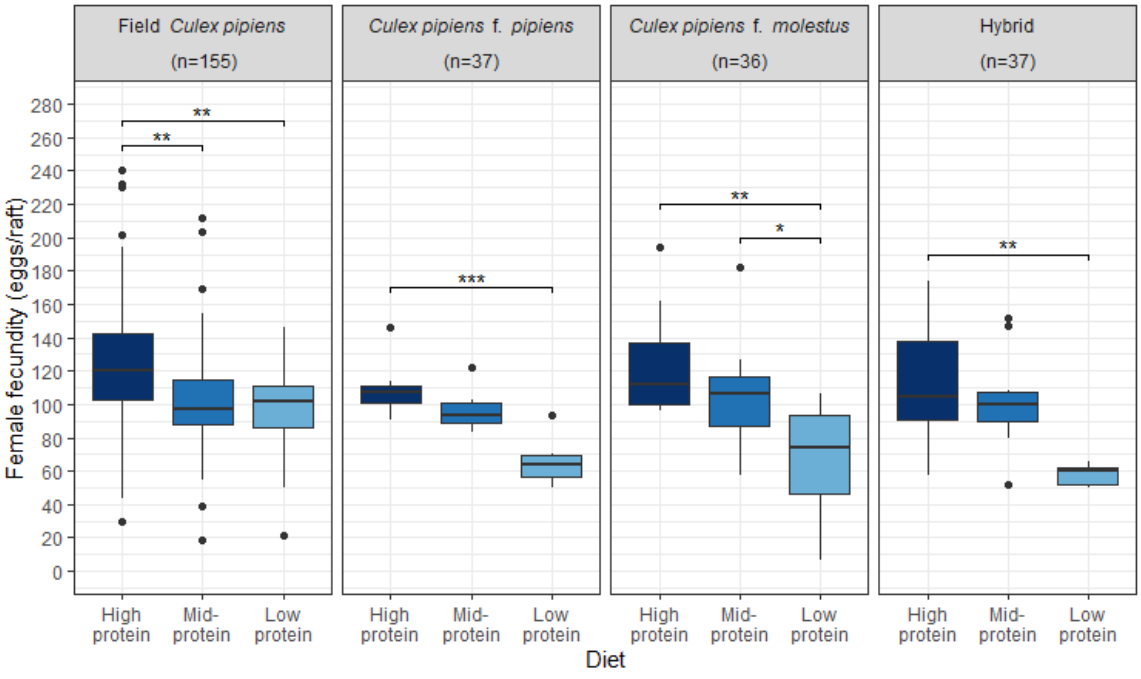

Figure 4.18. Female fecundity (number of eggs/raft) obtained from female mosquitoes reared under three larval diets of increasing protein availability for three colonies plus field derived populations of *Culex pipiens*. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles, closed dots indicate outliers. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Fertility, egg production and fecundity status

No hatching was observed from any egg rafts oviposited by field derived *Cx. pipiens*. From the colony populations a total of 60 of the 75 (80%) oviposited egg rafts hatched with an average of 71.5 ± 17.04% eggs/raft hatching and a significant difference seen between the treatment groups (KW χ^2 = 13.004, df= 2, *P* = 0.0015). Egg rafts collected from females reared on the high protein diet had an average proportion of 77.3 ± 15.4% eggs/raft hatching which were slightly higher than those collected from females reared on mid-protein diets although this difference was not shown to be statistically significant (*P* = 0.600). Egg rafts laid by females fed on the low protein diet were shown to have a proportion of 54.6 ± 16.7% eggs/raft hatching which was significantly different from those reared under mid- (72.5 ± 14.9%; *P* = 0.0234) or high protein diets (*P* <0.001).

For all populations and treatment groups combined, a total of 164 eggs were laid whilst 101 (38.11%) were retained. The proportion of egg retention was shown to increase as larval diet decreased for all colony lines combined (Figure 4.19). This ratio was shown to differ significantly when considering the *pipiens* and hybrid lines (Fisher's exact: *P* = 0.0325 and *P* = 0.00428 for *pipiens* and hybrid; Table 4.16). Females reared under the low protein diet within the *pipiens* and hybrid lines were significantly more likely to retain their egg rafts compared with the high (*P* = 0.0303 for *pipiens* and *P* = 0.0254 for hybrid lines) and mid-protein (*P* = 0.021 for *pipiens* and *P* = 0.0226 for hybrid lines) diets. Proportion of retained egg rafts in the field population was shown to be the highest with 66 of 155 (42.58%) eggs rafts retained.

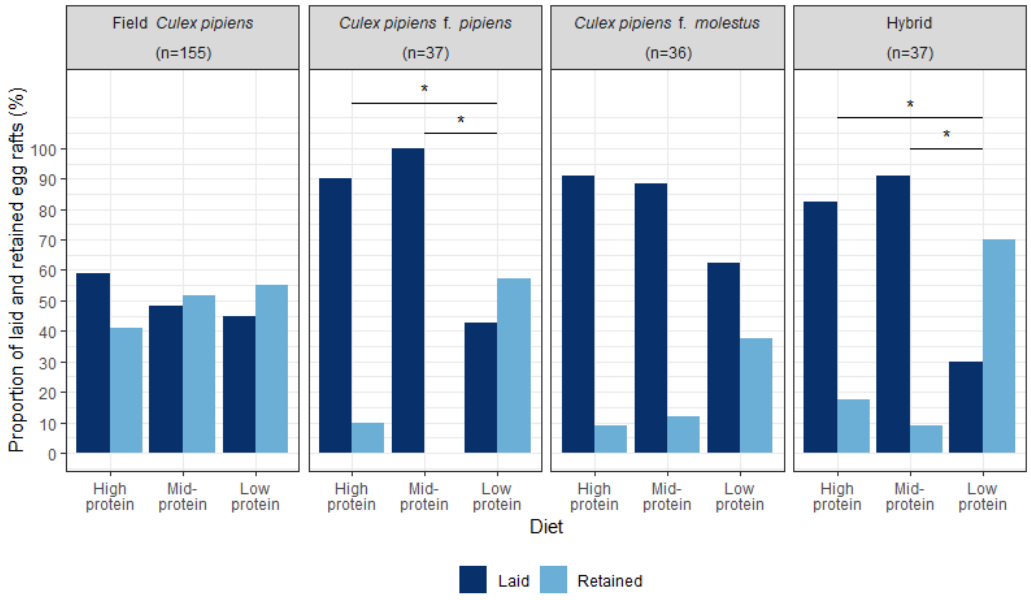

Figure 4.19. Proportions of laid and retained egg rafts produced by field derived female *Culex pipiens* as well as from three colony lines when reared under varying levels of nutritional availability. *** *P* ≤ 0.001, ** *P* ≤ 0.01, * *P* ≤ 0.05.

Mating success

Mating success was shown to be influenced by larval diet for all populations (Fisher's Exact P = 0.00496; χ^2= 8.784, df= 2, P = 0.0124; χ^2= 7.934, df= 2, P = 0.0189; χ^2= 6.136, df= 2, P = 0.0465 for field, *pipiens*, *molestus* and hybrid populations) with the high protein groups found to have the highest mating successes, which subsequently decreased with larval nutrition (Figure 4.20). Females from the high protein treatment group were shown to have a significantly higher mating success compared with the low protein group for all populations (P = 0.005; P = 0.0245; P = 0.0288; P = 0.039).

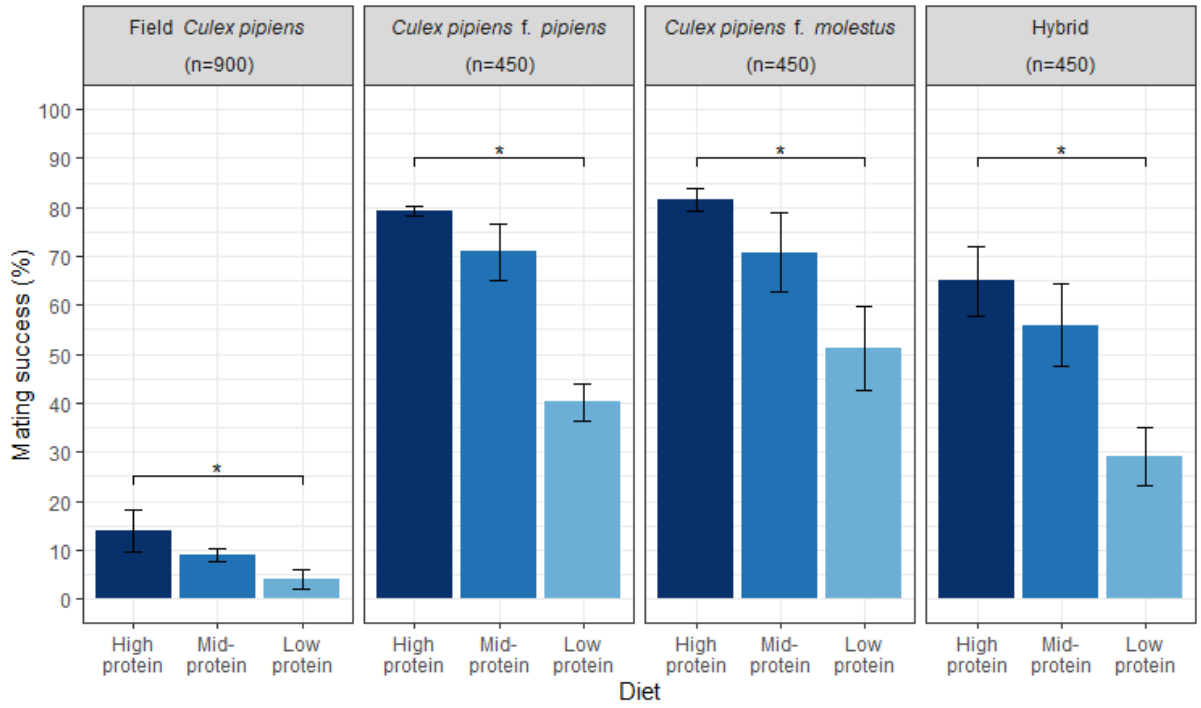

Figure 4.20. Mating success of females from three colony lines plus field derived populations of *Culex pipiens* under varying levels of larval nutrition. A successful mating event was determined by the presence of sperm stored in the female spermathecae. Error bars indicate mean ± SD. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Mating success between the populations was shown to vary significantly within each treatment group (χ^2= 112.22, df= 3, P <0.001; χ^2= 71.843, df= 3, P <0.001; χ^2= 56.03, df= 3, P <0.001 for high, mid- and low protein diets respectively; Table 4.16). Field populations were shown to have a significantly reduced mating success compared with colony populations within each treatment group (P <0.001) under colony conditions.

Adult wing length

Diet significantly influenced adult wing length within all populations tested (KW χ^2 = 16.93, df= 2, *P* <0.001; KW χ^2 = 22.38, df= 2, *P* <0.001; KW χ^2 = 10.66, df= 2, *P* =0.005; KW χ^2 = 18.05, df= 2, *P* <0.001 for field, *pipiens*, *molestus* and hybrid populations; Figure 4.21). Females reared on the low protein diet were shown to have the smallest wing length measurements (3.70 ± 0.23 mm) which was significantly smaller than females reared on the high protein diet (3.93 ± 0.16 mm; Z= 4.09, *P* <0.001; Z= 4.70, *P* <0.001; Z= 2.03, *P* = 0.007; Z= 4.19, *P* <0.001). Females reared on the mid-protein diet had an average wing length of 3.90 ± 0.18 mm which was also significantly greater than females reared on a low protein diet for each of the populations tested (Z= -2.87, *P* =0.008; Z= -3.21, *P* = 0.007; Z= -2.87, *P* = 0.008; Z= -2.61, *P* = 0.018 for field, *pipiens*, *molestus* and hybrid populations respectively.

Wing length was also revealed to vary significantly between the populations within each treatment group (KW χ^2 = 59.24, df= 3, *P* <0.001; KW χ^2 = 50.22, df= 3, *P* <0.001; KW χ^2 = 21.40, df= 3, *P* <0.001 for the high, mid- and low protein groups). In general field collected females had the greatest wing length (3.98 ± 0.16 mm) which was significantly larger for all treatment groups compared with the *pipiens* colony (Z= 10.57, *P* <0.001) which had the smallest wing length (3.73 ± 0.16 mm). Wing length of field individuals were also shown to be significantly greater compared with hybrid females for each of the treatment groups (Z= 6.68, *P* <0.001). Females from the *molestus* line were also shown to have larger wing length compared with the *pipiens* colony (Z= 6.31, *P* <0.001) for all treatment groups combined. *Molestus* colony females were also demonstrated to have greater wing length than females from the hybrid colony within the high (Z= -2.27, *P* = 0.047) and mid-protein (Z= -3.25, *P* =0.004). However, *molestus* females were shown to have a reduced wing length compared with field populations although this was only significant in the high protein group (Z= 2.64, *P* = 0.025). Adult wing length was positively correlated with female fecundity (t= 4.96, *P* <0.001) with larger egg rafts produced by females with greater wing lengths.

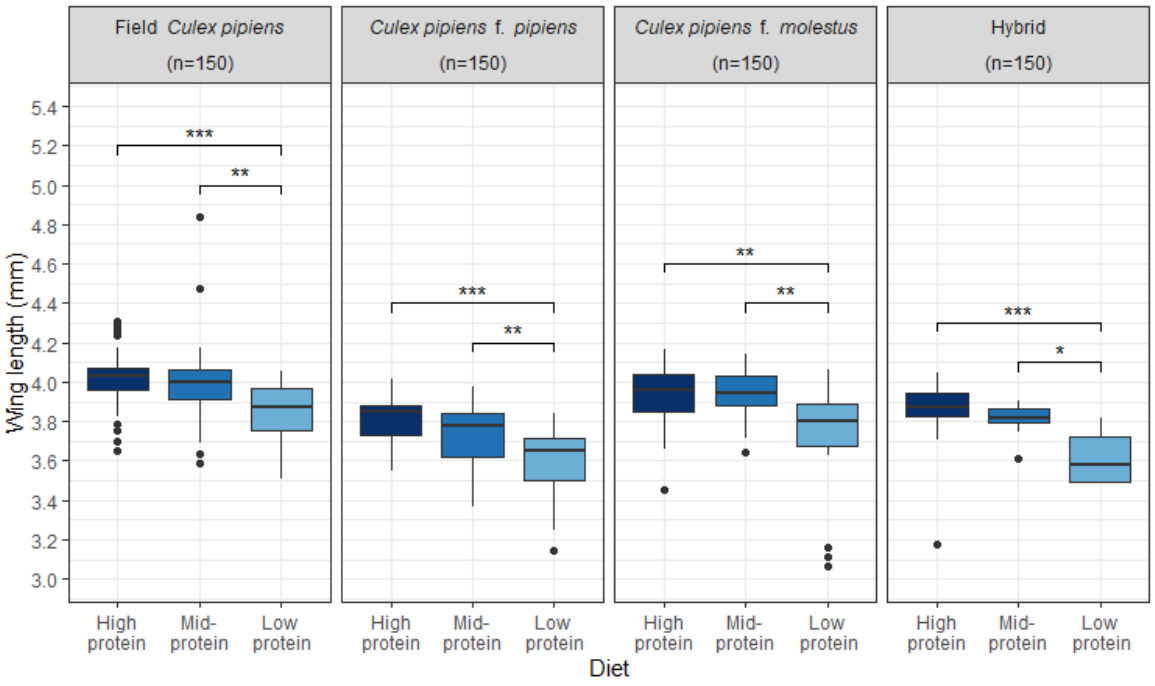

Figure 4.21. Wing length measurements (mm) of *Culex pipiens* from field populations compared with *pipiens, molestus* and hybrid colony lines reared under three levels of larval nutrition availability. Horizontal black lines indicate median, 25th and 75th percentiles, whiskers extend to the largest and smallest values within 1.5 times the interquartile range from the 25th and 75th percentiles. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Autogeny

A total of 600 adults reared under varying levels of larval nutritional availability from different populations were assessed for the presence of autogeny. No occurrence of autogenous egg production was observed by monitoring of oviposition cups during this study, which was subsequently confirmed by dissection of females to monitor for egg retention.

Biotype identification

Ratios of mosquito biotypes surviving eclosion were assessed within the hybrid colony line to determine if larval diet significantly influenced population dynamics through differential survival. All three treatment groups were shown to be dominated by the hybrid form, making up approximately 60% of the population under all three treatments, with the *pipiens* and *molestus* forms each accounting for approximately 20% of the population (Figure 4.22). Therefore, larval diet was not shown to have a significant influence on the biotype ratio between the treatment groups (χ^2= 0.172, df= 4, P = 0.997).

A total of 40 field collected individuals from each site (a combination of individuals from Sections 4.2.2 and 4.2.3) were identified to biotype level. Of the 240 mosquitoes tested, only one (0.42%) hybrid individual was detected whilst no *molestus* forms were identified.

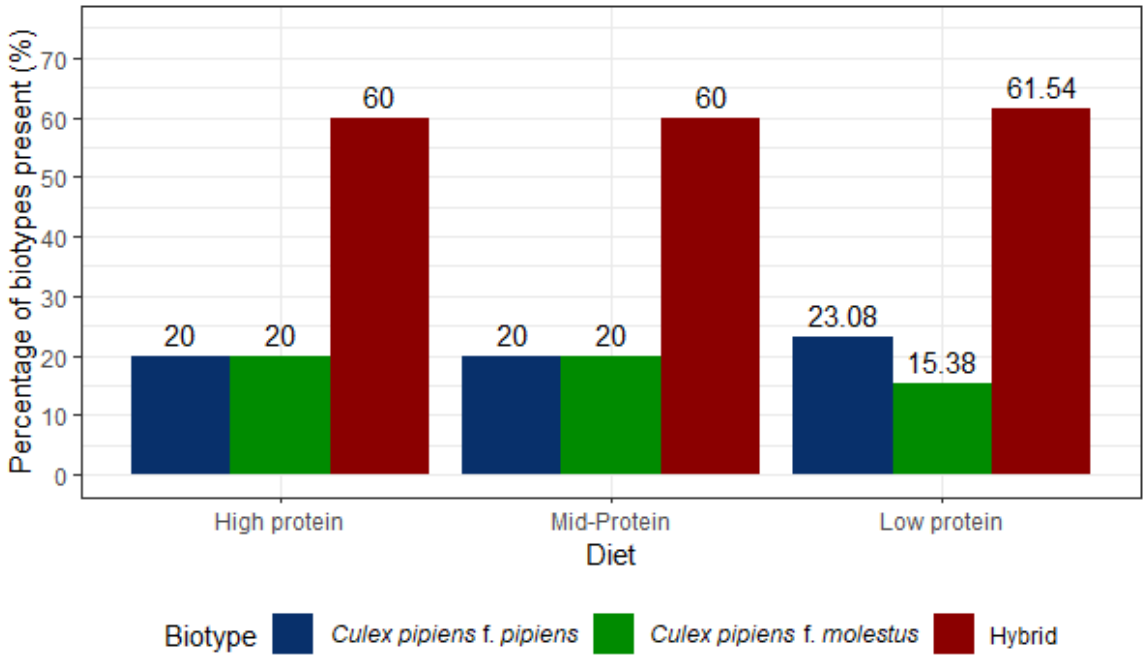

Figure 4.22. Proportions (%) of surviving adult biotypes for *Culex pipiens* reared under varying levels of nutritional availability within the hybrid colony line.

Table 4.16. The effect of larval nutrition on reproductive traits of field derived and colonised *Culex pipiens*. The letters in bold following mean values indicate which groups differ significantly ($P < 0.05$) from one another. Specifically, groups which share a common letter are not significantly ($P < 0.05$) different from one another.

Origin	Diet	Blood-feeding proportion (%)		Fecundity (Eggs/raft)		Fertility (%)		Retention (%)		Mating success (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Field	High protein	36.4 **a**	11.8	123.0 **a**	48.7	-	-	41.0 **a**	-	14.0 **a**	4.32
	Mid-protein	28.4 **a**	14.6	94.1 **a**	22.9	-	-	51.8 **a**	-	9.00 **a**	1.42
	Low protein	24.7 **a**	9.87	95.5 **a**	28.0	-	-	55.2 **a**	-	2.00 **a**	2.00
Pipiens colony	High protein	72.5 **b**	3.54	108 **a**	15.4	81.4 **a**	0.044	10.0 **b**	-	79.3 **b**	1.01
	Mid-protein	65.0 **b**	7.07	97 **a**	14.1	77.0 **a**	0.0599	0.00 **b**	-	70.8 **b**	5.89
	Low protein	36.7 **ab**	4.71	66 **ab**	15.1	53.8 **a**	0.196	57.1 **a**	-	40.2 **b**	3.79
Molestus colony	High protein	92.5 **b**	3.54	124 **a**	31.7	77.7 **a**	0.115	9.09 **b**	-	81.7 **b**	2.36
	Mid-protein	90.0 **b**	0.00	105 **a**	34.2	72.6 **a**	0.133	11.8 **b**	-	70.7 **b**	8.11
	Low protein	65.0 **b**	7.07	68.2 **ab**	29.0	60.7 **a**	0.203	37.5 **a**	-	51.1 **b**	8.59
Hybrid colony	High protein	85.0 **b**	7.07	112 **a**	31.3	72.6 **a**	0.244	17.6 **b**	-	65.0 **b**	7.07
	Mid-protein	85.0 **b**	14.1	102 **a**	28.2	69.5 **a**	0.210	9.09 **b**	-	54.8 **b**	9.09
	Low protein	61.2 **b**	1.77	57.9 **b**	6.06	44.9 **a**	0.244	70.0 **a**	-	29.2 **b**	5.89

4.3.4 Biotype identification of field collected *Culex pipiens*

A subset of samples collected during the field season in 2019 was amplified using the conventional CQ11 PCR assay (Bahnck and Fonseca, 2006), of which 5 (10%) showed variable results compared with the real time PCR assay, whilst 29 (58%) failed to amplify. Reamplification of these samples identified mis-priming with the *pipiens* reverse primer, producing a double banding pattern, usually indicative of a hybrid individual (Figure 4.23B; Lanes 2 and 6). Differentiation of the forms by this assay relies on disparity in banding pattern, with the *pipiens* form producing a band of approximately 180 base pairs whilst the *molestus* form amplified a product of approximately 210 base pairs. Amplification in singleplex revealed variation in banding size of the *pipiens* form, which could result in misidentification of biotypes.

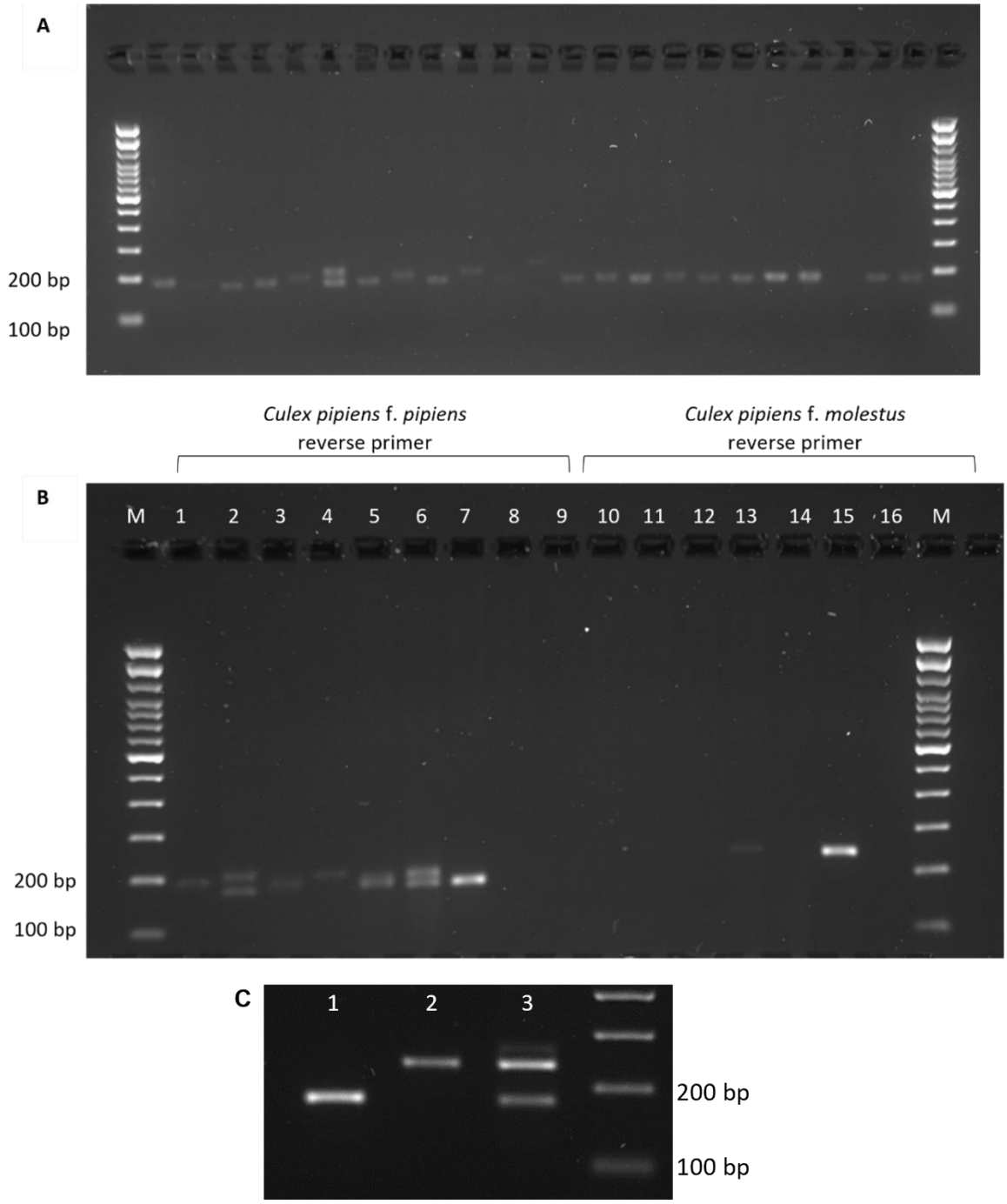

Figure 4.23. Gel electrophoresis of CQ11 amplification products of field collected adult mosquitoes, separated on a 2% agarose gel. "M" indicated marker lane, with size reference carried out compared with the TrackIt™ 100 bp ladder. **A.** Amplification of a subset of field collected *Culex pipiens* f. *pipiens* as determined by amplification of the CQ11 locus by real time PCR (Rudolf *et al.*, 2013). **B.** Amplification products of the CQ11 locus by conventional PCR with primers amplified in singleplex for a subset of samples previously designated as *pipiens* biotypes by real time PCR. Lanes 1-9 show amplification products for the universal forward primer (CQ11F) when paired with the *pipiens* specific reverse primer (PipCQ11R) whilst lanes 10-16 show amplification products for when CQ11 is paired with the *molestus* specific reverse primer (MolCQ11R). **C.** Positive control samples, the biotypes produce differentially sized amplicons with *pipiens* forms producing a product of approximately 180 base pairs (Lane 1) whilst *molestus* forms product an amplicon of approximately 250 base pairs (Lane 2). Hybrid specimens are identified by a double banding pattern (Lane 3).

Three HRM loci previously described to differentiate between the *Cx. pipiens* biotypes by accumulation of SNPs, were tested for differences in DNA sequence between UK field derived *Cx. pipiens* f. *pipiens*, colonised *Cx. pipiens* f. *molestus* from Sweden and colonised *Cx. quinquefasciatus*. All samples produced good quality sequences yet revealed no consistent differences within and between forms in DNA sequence. Markers were successfully able to differentiate the *Cx. pipiens* biotypes from *Cx. quinquefasciatus* as shown by sequence alignment of the ACE-2 gene shown in Figure 4.24 as an example.

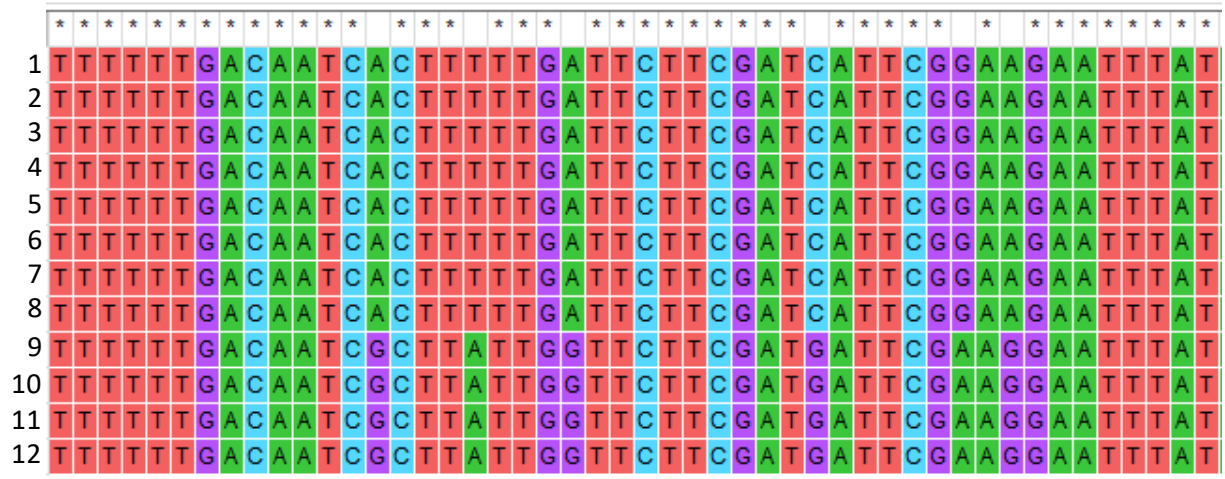

Figure 4.24. Sequence alignment of consensus sequences for PCR products amplified using primers targeting a segment of the ACE-2 gene. Samples 1-4: *Culex pipiens* f. *pipiens*; samples 5-8: *Culex pipiens* f. *molestus*; samples 9-12: *Culex quinquefasciatus*. Asterix (*) indicate common bases between all samples

4.3.5 Genetic analysis of *Culex pipiens* samples

The highest genetic divergence was seen between the four colony lines included in the analysis with *Cx. quinquefasciatus* being the most diverged group. Fixation indices demonstrated that individuals from the *pipiens* colony line were most closely related to field collected *Cx. pipiens* from the UK designated as *pipiens* biotype by the CQ11 microsatellite (Figure 4.25 and Figure 4.26). Interestingly, F_{ST} also indicated that individuals from the *molestus* colony were more closely related to *pipiens* field populations than to *molestus* individuals collected from the UK as well as colony samples from Sweden. Field collected *Cx. pipiens* mosquitoes, designated as the *molestus* biotype according to the CQ11 microsatellite were also more closely related to field *Cx. pipiens* f. *pipiens* compared with colony *Cx. pipiens* f. *molestus* from Sweden.

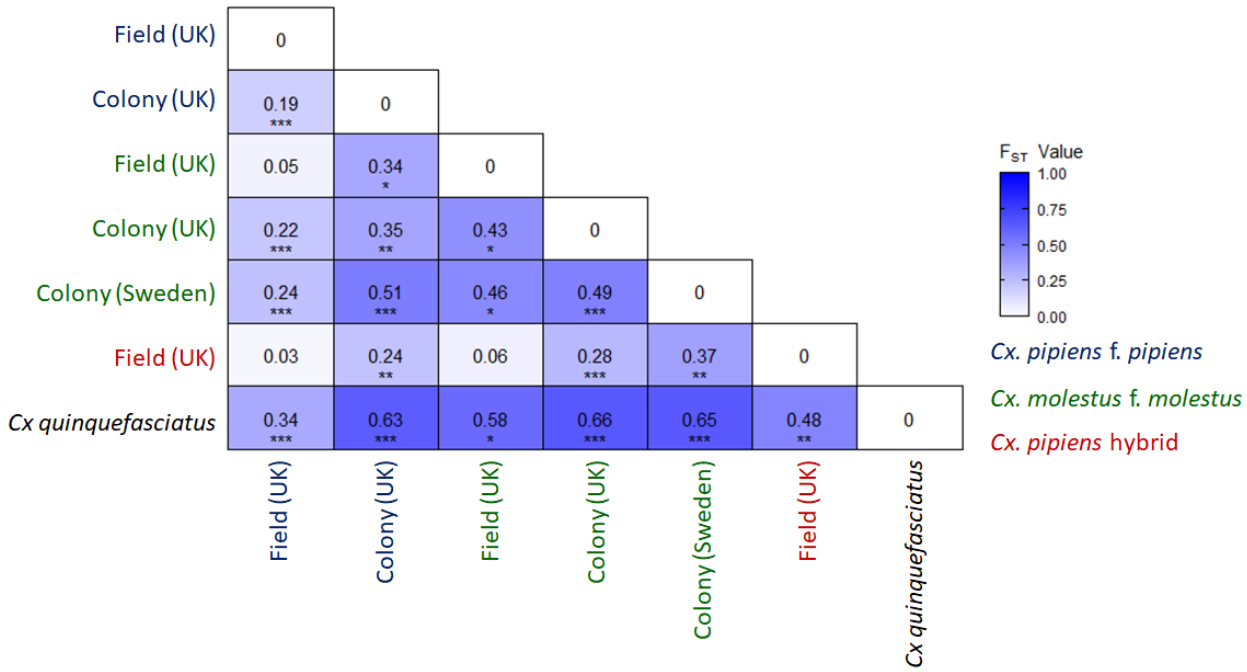

Figure 4.25. F_{ST} matrix between the studied species/locations, populations grouped by geographic location. (*) indicate signficance of species/population divergence. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Bayesian cluster analysis implemented by STRUCTURE (Pritchard *et al.*, 2000) for colony populations revealed 3 ($K = 3$) genetically distinct ancestry clusters (Figure 4.27A) with a Delta K value of 139.54. Cluster 1 grouped colony specimens from the pure *pipiens* and *molestus* lines established in Chapter 2, whilst clusters 2 and 3 were represented by individuals from *Cx. pipiens* f. *molestus* colonies from Sweden and *Cx. quinquefasciatus* lines. When $K = 4$, a fourth cluster differentiates the *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* colony lines from Chapter 2 based on their CQ11 genotype (Figure 4.27B).

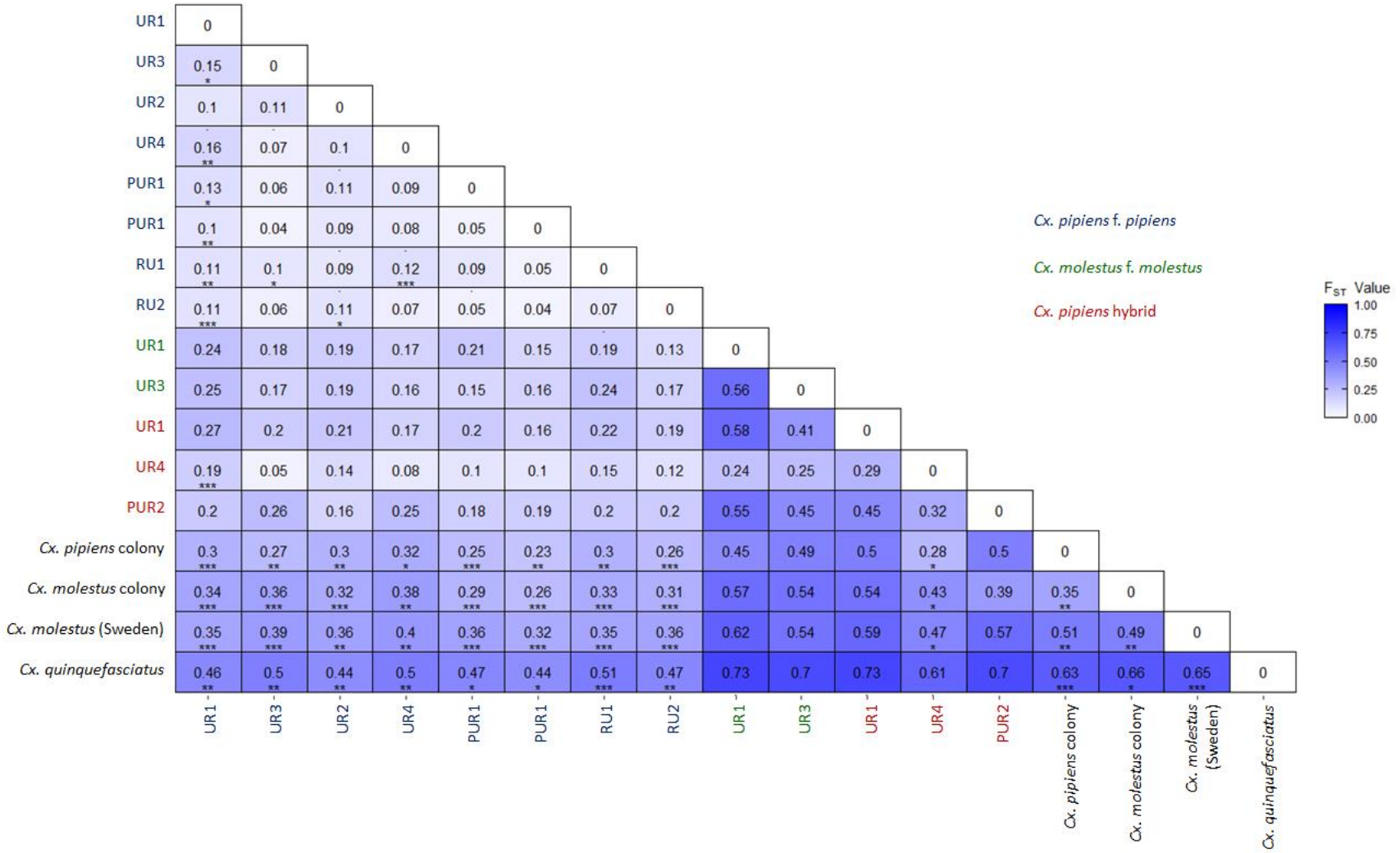

Figure 4.26. F_{ST} matrix between the studied species/locations. Asterix (*) indicate signficance of species/population divergence. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

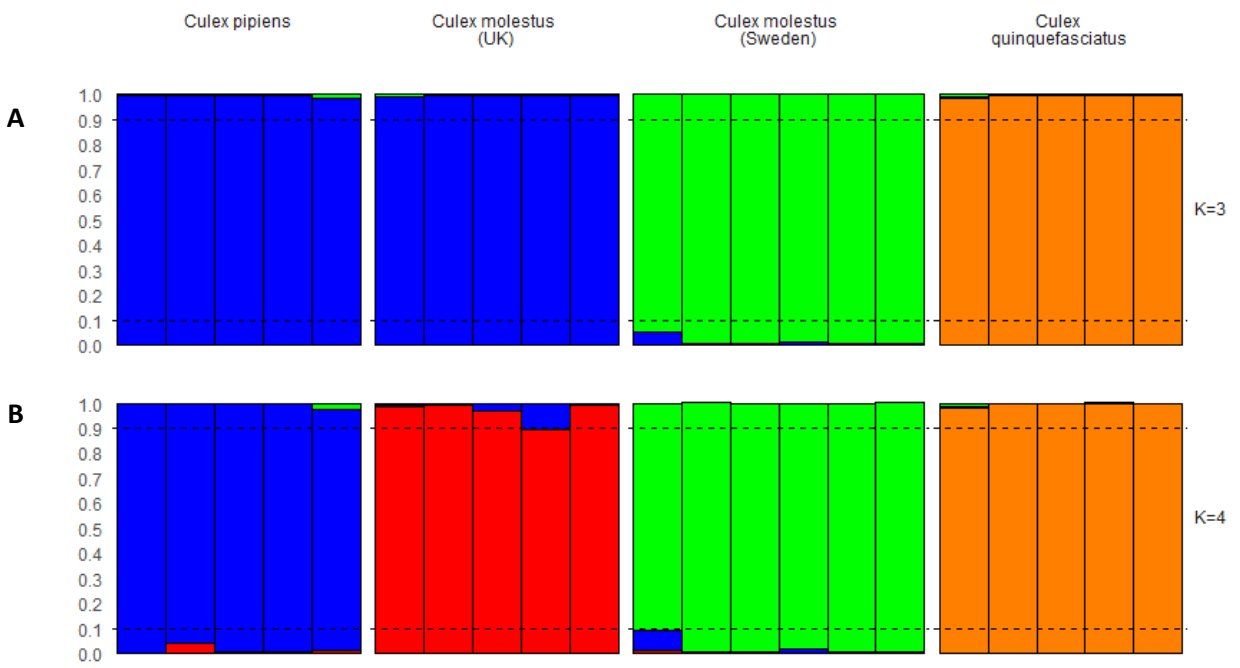

Figure 4.27. Inferred genetic clustering by Bayesian analysis as implemented by STRUCTURE for colony populations of *Culex pipiens*. Each column represents an individual analysed and is partitioned into colours according to probably of assignment to each cluster. Dashed lines highlight the probability thresholds (Tq) used to assign individuals to clusters. **(A)** clustering of colony lines when K = 3. **(B)** clustering of colony lines when K = 4.

When considering field populations, Bayesian clustering revealed 2 (*K* = 2) genetically distinct ancestry clusters (Figure 4.28A) with a delta *K* value of 89.80, with UK *Cx. pipiens* forming a single cluster, regardless of biotype identity, separate from Swedish *Cx. pipiens* f. *molestus*. Increased differentiation between UK *Cx. pipiens* was observed when *K* was increased (*K* = 3), although clustering did not appear to conform to CQ11 genotyping (delta *K* = 8.08; Figure 4.28B).

When *K* = 3, cluster 1 grouped 10 specimens all of which had homozygous CQ11 genotype specific for *Cx. pipiens* f. *pipiens*. In contrast, cluster 2 was comprised the remaining *pipiens* form samples, with 24 (88.89%) out of 27 specimens assigned presenting a CQ11 homozygous genotype. Interestingly, cluster 2 also contained both specimens testing as *Cx. pipiens* f. *molestus* and one individual with a heterozygote phenotype, when assigned to biotype using the CQ11 real-time PCR assay.

There were eight (17.78%) individuals of the total UK samples (n= 45) exhibiting an admixed ancestry, of these, only three (37.50%) had a heterozygote CQ11 genotype while the remaining five (62.50%) samples were homozygous for *Cx. pipiens* f. *pipiens*.

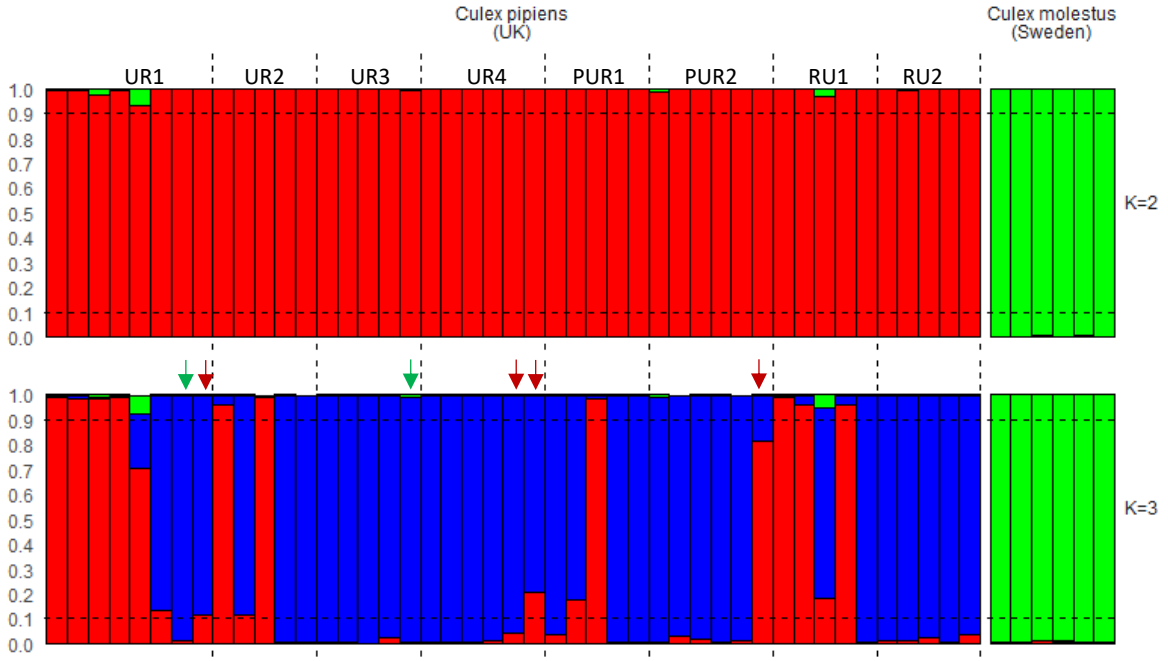

Figure 4.28. Inferred clustering of field collected *Culex pipiens* by Bayesian analysis as implemented by STRUCTURE. Each column represents an individual analysed and is partitioned into colours according to probably of assignment to each cluster. Horizontal dashed lines highlight the probability thresholds (Tq) used to assign individuals to clusters. Arrows indicate individuals testing as *Culex pipiens* f. *molestus* (green) or hybrid (red) according to the CQ11 microsatellite locus (**A**) Clustering when K = 2. **(B)** Clustering when K = 3.

4.4 Discussion

Results from this study demonstrated plasticity in life history traits between *Cx. pipiens* from different larval habitats as well as under variable levels of nutritional availability at the larval stage. This study also provides a comparison of the effect of larval nutrition on both field derived and colonised *Cx. pipiens*. Preliminary field sampling of adult and larval *Cx. pipiens* populations identified very low occurrence of *molestus* and hybrid individuals as characterised by the CQ11 microsatellite locus. Further genetic analysis demonstrated genetic separation of UK *Cx. pipiens* from *Cx. pipiens* f. *molestus* samples from Sweden, whilst no clear sub-structuring of British *Cx. pipiens* according to biotype designation, as determined by traditional assays, could be identified. Results presented here demonstrate the significant influence of larval nutrition on a variety of life history traits in the *Cx. pipiens* species, whilst highlighting both population and colonisation as additional factors influencing phenotype and individual susceptibility to shifts in nutritional availability. Likewise, environmental factors experienced in the larval habitat including temperature and density were shown to influence the presence of adult phenotypes. Combined, these results indicate the complexity of the assessment of the ability of *Cx. pipiens* species to act as a disease vector in the UK.

Determination of *Culex pipiens* biotype from UK populations

In the current study, microsatellite analysis demonstrated the limitations of the use of single molecular markers such as CQ11, revealing variation within and between populations cannot be detected with a single marker and are not necessarily indicative of phenotype. This was demonstrated by significant genetic distance between individuals testing as homozygous for the *molestus* allele from the UK and Sweden following microsatellite analysis, despite unequivocal results within the CQ11 assay. The genetic variation may differentially influence an individual's response to selection pressures in nature or through colonisation or fluctuation in environmental conditions, inhibiting broad inference of responses to external factors of the forms. Rather, a more detailed assessment of local populations is required to accurately evaluate the potential role species could contribute to disease transmission through either maintenance within an avian population or by facilitating spill over events into a susceptible mammalian population.

Two hypotheses have been proposed to explain the origin of *Cx. pipiens* f. *molestus* which either consider the form as an eco-physiological variant (Byrne and Nichols, 1999; Kothera *et al.*, 2010) or an evolutionary distinct entity (Yurchenko *et al.*, 2020) of *Cx. pipiens*, with recent literature supporting both arguments. Genetic analysis of 'pure' colony lines created during this thesis (Chapter 2) revealed genetic separation of UK *Cx. pipiens*, irrespective of CQ11 determined biotype, independent from

autogenous *Cx. pipiens* f. *molestus* from Sweden. This was also reflected in field collected samples where UK field individuals testing homozygous for the *molestus* allele, as designated by the CQ11 microsatellite, formed a distinct cluster with field *Cx. pipiens* f. *pipiens*, separate from Swedish *Cx. pipiens* f. *molestus*. This was confirmed by the assessment of F_{ST} values which demonstrated that UK field and colony *Cx. pipiens*, homozygous for the *molestus* form, were more closely related to UK *Cx. pipiens* f. *pipiens* compared with autogenous *molestus* samples from Sweden.

These findings contradict recent studies using whole genome analysis which supports the hypothesis that *Cx. pipiens* f. *molestus* is genetically distinct from *Cx. pipiens* f. *pipiens* and has a single origin (Yurchenko *et al.*, 2020). However, analysis in the current study was limited due to the low sample size of individuals testing as *Cx. pipiens* f. *molestus* according to CQ11 assays. Moreover, due to the lack of *molestus* individuals found during larval sampling, determination of phenotypic characteristics could not be assessed for field collected *molestus*, rather, assessment of phenotypes was limited to use of colonised mosquitoes which had undergone several generations of introgression prior to separation by genotype (Chapter 2). Results from the genetic analysis conducted during this study combined with the lack of expression of *molestus* specific phenotypes such as autogeny, despite the provision of sufficient nutrition indicates that UK *molestus* samples tested during this study are likely not be representative of true *molestus* forms, and instead are expected to have arisen from a hybridisation or backcrossing event. To fully assess the genetic structure of UK *molestus* populations, more representative samples sizes of each *Cx. pipiens* form, complemented with characterisation of phenotypes, would be required.

The present data highlight concerns regarding the reliability of single molecular markers for accurate biotype delineation under conditions of continued hybridisation and backcrossing, with hybrid ancestry proven to be underestimated when using CQ11 in isolation. This is demonstrated by the characterisation of the hybrid colony line in Section 4.3.3, where approximately 40% of the population tested as homozygous for either the *pipiens* or *molestus* CQ11 allele despite a known hybrid ancestry. Results from studies using a single molecular marker to characterise *Cx. pipiens* populations should be interpreted with caution due to this potential underestimation of hybridisation within a population, as has been previously reported (Gomes et al 2009). Moreover, this also raises concerns regarding broad inference of expected population phenotypes from single molecular marker assays. To aid in biotype identification, three additional loci have been suggested as diagnostic for the forms by HRM assays, exploiting SNPs between the biotypes of *Cx. pipiens* sampled from America (Kang and Sim, 2013; Kim *et al.*, 2018a). However, in the current study when British field samples and colony derived Swedish mosquitoes were tested at the three loci, consistent differentiation of the two forms could

not be achieved, similar to results obtained when testing one of the loci in field collected specimens from Sweden (Luande *et al.*, 2020). However, these targets should be tested in a larger sample size of field derived individuals which have been fully characterised by phenotype as well as genetic analysis. The differences in the life history traits of populations and lack of conformity according to single molecular markers demonstrates differentiation of biotypes by simple molecular means over broad scales is currently unresolved and requires further characterisation.

Assessment of adult and larval forms of *Cx. pipiens* revealed that aboveground populations sampled in the current study were dominated by the *pipiens* form, with very few *molestus* and hybrid individuals identified, according to CQ11 assays. The *molestus* biotype was absent from all larval populations despite collection of adult *molestus* females in some sampling locations, similar to previous results of a nationwide study of *Cx. pipiens* from the Netherlands (Vogels *et al.*, 2015). In the UK, identification of *molestus* and hybrid forms aboveground has been sporadic, with some studies finding all three forms in sympatry in larval habitats from a range of locations across the south of England (Byrne and Nichols, 1999; Danabalan *et al.*, 2012; Manley *et al.*, 2015). However, an assessment of a range of habitats from London found only the occasional occurrence of *molestus* larval individuals, whilst identification of *molestus* adults was concentrated at a single site, with no identification of hybrids throughout the studies (Curtotti, 2009). Moreover, an assessment of mosquito populations present on farms in the south of England failed to identify either *molestus* or hybrid individuals in the adult populations through resting box collections (Brugman *et al.*, 2017b) or human landing catches (Brugman *et al.*, 2017a). These studies together suggest low levels of sympatry for *Cx. pipiens* in the UK, which may also act to limit hybridisation.

All studies of field populations here employed a real time PCR assay for biotype differentiation which also targets the CQ11 microsatellite assay (Rudolf *et al.*, 2013), similar to the conventional assay which is widely implemented (Bahnck and Fonseca, 2006). Reamplification of a subset of samples with the conventional assay identified incongruence in results obtained between the two. Upon further investigation, the conventional assay was shown to overestimate the amount of hybrid and *molestus* individuals through mis-priming of the *pipiens* specific reverse primer, as amplification of a double banding pattern was observed when only amplifying with a single primer set. This assay has previously been suggested to overestimate hybrid presence through the cross amplification with *Cx. torrentium* species, however, assignment to the *Cx. pipiens* species had already been confirmed through the incorporation of the ACE-2 assay, thus cannot account for this overestimation (Danabalan *et al.*, 2012). Therefore, as previously reported, studies should exercise caution interpreting results from a single

genetic marker, considering results at a population level, rather than individual level (Gomes *et al.*, 2009; Danabalan *et al.*, 2012).

The effect of larval diet on life history traits of *Cx. pipiens*

Despite the lack of genetic differentiation of the colony lines created during Chapter 2, differences in response to larval conditions according to genotype, as determined by the CQ11 marker, were noted yet autogeny did not occur despite the potential for larval food storage. As autogeny would be predicted to be selected for under the laboratory rearing conditions, it is likely that this is an inherited trait that is not present in these populations. Larval diet has previously been closely linked to autogeny, with higher protein diets increasing autogenous egg batch sizes and the proportion of individuals expressing autogeny in *Cx. pipiens* f. *molestus* in Australia (Kassim *et al.*, 2012b). Likewise, other studies have identified larval nutrition as a key parameter influencing the penetrance and expressivity of alleles for autogeny in *Cx. pipiens*, *Aedes togoi* (Theobald, 1907) and *Wyeomia smithii* (Coquillett, 1901) (Lounibos *et al.*, 1982). Genetic analysis demonstrated clustering of autogenous *molestus* samples from Sweden, separate to UK colony *molestus* samples thus the lack of autogeny within this population is unsurprising.

Plasticity of life history traits was demonstrated in both field and colony mosquitoes when exposed to variable rates of larval nutrition. In concordance with previous studies in *Cx. pipiens* f. *molestus* from Australia, results here demonstrate a positive correlation between larval nutrition, development rate and larval survival (Kassim *et al.*, 2012b), indicating that increasing protein availability at the larval stage could lead to a more rapid proliferation of *Culex* populations. In contrast to previous studies, differences in development rates between the high and mid-protein groups were not detected despite provision of double the quantity of nutrition to the high protein treatment group, suggesting protein was not limiting in the mid-protein group. As diet proportions or protein source were not varied between the studies, discrepancies could be attributed to genetic variation between populations, meaning UK *Cx. pipiens* reach capacity for larval nutrition uptake earlier, supporting the hypothesis of significant population-level phenotypic variation.

Moreover, results from the current study also demonstrated a positive correlation between adult fitness and larval nutrition with elevated female fecundity and mating success observed. Egg retention rates were also shown to increase as nutritional availability decreased, although this interaction was not significant, most likely due to the low frequency of retention, hence a larger sample size would be required to investigate this further. These factors demonstrate that increased larval nutrition has the potential to increase female reproductive output, which can influence natural population densities. Reproductive output of females has previously been shown to be correlated with larval nutrition in

Cx. pipiens f. *molestus* (Kassim *et al.*, 2012b) although a significant decline in mating success was only noted when males reared on a low protein diet were mated with females reared on a high protein diet (Kassim *et al.*, 2012a). Female fecundity was shown to be lower in the previous study for the respective treatment groups although egg rafts collected were laid by autogenous females, which previous work has demonstrated produce smaller egg rafts than those receiving a bloodmeal (Korba *et al.*, 2016) hindering direct comparison with the current study.

Although larval nutrition was responsible for much of the variation in life history traits measured, important population differences were also identified. Similarly, disparity in fitness levels between field and colonised mosquito populations has previously been reported in *Culex* and *Anopheles* species (Tabachnick, 2003; Ciota *et al.*, 2014). Among the most distinct differences detected in the current study was the sensitivity of colonised larvae to variable levels of nutrition, as opposed to the robustness of field *Cx. pipiens*, for which little effect of diet on larval survival and sex ratio were noted. In addition, survival of immature stages was significantly lower for colonised populations and overall blood-feeding and egg production were generally higher relative to field populations. Lower blood-feeding success of field derived females compared with colony individuals is likely attributed to the use of horse blood throughout experiments as this is not a natural bloodmeal host of *pipiens* species, whereas colony lines have been selected over several generations for mammalian feeding through regular colony maintenance (Manley *et al.*, 2015).

Previously, temperature was shown to have a similar effect on both field derived and colonised *Cx. pipiens* and *Cx. quinquefasciatus* (Ciota *et al.*, 2014). These differences are likely a reflection of selection events resulting from long-term colonisation and rearing at generally constant conditions, indicating that the use of colonies to measure the effect of some environmental parameters on life history traits may at times provide imprecise representation of natural populations. Despite these differences, it is unsurprising that the response seen in the *pipiens* colony lines to variation in larval nutrition was closest to those observed from field populations, as field collections were predominantly made up of the *pipiens* form, as determined by the real-time CQ11 assay. Although differences in the susceptibility of populations to larval nutrition depletion between field and colony mosquitoes were noted, the same general trends were identified. Thus, despite a higher robustness for field derived individuals to environmental stressors when exposed for a single generation, colony populations may have limited utility to inform likely patterns that could emerge in natural populations when exposed to external pressures over several generations.

Response to variation in nutritional availability during larval development were also shown to differ between the colony lines. As an example, rearing under a protein deficient diet was shown to have a

much larger effect on development time for the *molestus* and hybrid lines, taking significantly longer than the *pipiens* colony. Although variation between the lines is likely an artefact of colonisation it is an interesting concept that in natural populations the forms may respond to external stressors differently. Disparity in response to environmental factors such as larval nutrition between the forms may influence their preference for ecological niches and their ability to adapt and survive under different constraints. Further work should be conducted to test this interesting theory in natural populations of both *pipiens* and *molestus* forms which have undergone thorough phenotypic and genetic characterisation.

The effect of larval habitat on adult reproductive traits

In agreement with previous studies, larval density was positively correlated with average water temperature. Adult activity has been shown to be affected by elevated temperatures, by increasing the number of females host-seeking and ovipositing, which could, in part, account for the greater larval densities in these areas. The highest maximum temperatures, and densities, were recorded at the peri-urban sites. These sites were on average 1.8^{o}C higher than those recorded in the urban and rural locations. Interestingly, no differences were seen between maximum or average temperatures experienced between the urban and rural sites, contradictory to previous findings which noted a 1.2^{o}C difference between urban and rural gardens in Reading (Townroe and Callaghan, 2014). Differences could be due to the types of containers used plus definition used to categorise urban and rural between the two studies.

Biological oxygen demand is the amount of DO required by aerobic biological organisms to break down organic material present in water and is often used as a proxy for the degree of organic pollution of water. Larval densities were shown to be positively correlated with BOD_5 measurements, contradictory to previous findings from Algeria (Korba *et al.*, 2016) which demonstrated the opposite relationship. However, previous research has revealed tolerance of *Cx. quinquefasciatus* to variable levels of organic pollution within a range suitable to support mosquito development, with abundance demonstrated to increase with amount of organic waste. Despite this relationship, BOD_5 recordings were not shown to vary significantly between the sites, which is unsurprising given the larval habitats were all created with water from the same source, as well as seeding each bucket with the same amount of organic matter.

Adults experiencing elevated temperatures during larval development in the current study had an increased blood-feeding success, as was previously reported in another study (Ciota *et al.*, 2014), but, contrastingly, increased blood-feeding was not correlated with elevated oviposition rates (Ciota *et al.*,

2014). Moreover, increased average temperatures, temperature fluctuations, and larval densities were all negatively correlated with female fecundity during the present study, which is in agreement with other studies where increased larval density (Alto *et al.*, 2012) and temperature fluctuations (Paaijmans *et al.*, 2010) reduced female adult fitness and reproductive output as indicated by their smaller wing lengths. Thus, despite an increase in blood-feeding success for females experiencing higher average temperatures during larval development, greater densities impacted adult fitness, resulting in an overall decrease in female reproductive output. In contrast, despite recorded variation in larval densities and average temperatures between rural and urban habitats in Reading and Berkshire, differences in fecundity rates of females collected from gravid traps was not observed (Townroe and Callaghan, 2015). Whilst collection of females from gravid traps provides a more natural representation of female fecundity in field-derived adults, it also precludes the ability to control the bloodmeal source which can also influence fecundity rates of females (Richards *et al.*, 2012).

Longevity and rate of autogeny are important factors when considering vectorial capacity of populations. Successful transmission requires individuals to survive long enough to acquire an infectious bloodmeal, complete the extrinsic incubation period and finally acquire a secondary bloodmeal to achieve transmission. For autogenous individuals, longevity is even more vital as production of an autogenous egg batch delays the age at which adult females acquire their first bloodmeal, thus potentially reducing their vectoral capacity. During the present study, environmental conditions experienced during larval development were shown to influence adult longevity with greater temperatures, densities and temperature fluctuations associated with higher mortality rates, in concordance with previous findings (Paaijmans *et al.*, 2010). Moreover, the present study found no evidence of autogeny in any field or colony populations tested across either experiment. Despite this lack of autogeny, reduced adult longevity associated with higher temperatures could reduce overall risk of virus transmission by reducing adult survival and limiting pathogen proliferation within a population.

Bloodmeal analysis revealed a single *Cx. pipiens* f. *pipiens* individual feeding on a mammalian host. Although few blood-fed individuals were collected, the collection of an individual that fed on a mammal from a site with an extensive choice of avian and mammalian hosts demonstrates that the host choice of *Cx. pipiens* f. *pipiens* as determined by the CQ11 marker is not restricted to birds. Moreover, although blood-feeding rates in field individuals were lower compared with colony specimens, approximately 35% of field-derived females acquired a bloodmeal when offered defibrinated horse blood, despite not being a natural host for the *pipiens* form. Similarly, previous research has reported collection of the *pipiens* form during human landing catches, although the

specimens were collected prior to bloodmeal acquisition, precluding confirmation of human feeding by bloodmeal analysis (Brugman *et al.*, 2017a). Further work conducted at Chester Zoo identified humans as a significant bloodmeal host in *Cx. pipiens* f. *pipiens* mosquitoes collected from resting habitats. These studies were however limited to biotype assignment by CQ11 (Hernandez-Colina *et al.*, 2021). Microsatellite analysis of blood-fed *Cx. pipiens* has suggested a link between mammalian feeding in the *pipiens* form, as designated by CQ11, with high levels of introgression from *molestus* populations (Huang *et al.*, 2009). Further work is required to establish any link between host use and genetic ancestry within the UK, however the wide scale collections over several seasons required to assess the potential for both biotypes to act as bridge vectors were beyond the scope of the current project.

To conclude, this study demonstrates variation in life history traits under varying levels of larval nutrition availability in field and colony populations as well as between adult mosquitoes collected from different larval sites. Phenotypic variation can be important drivers of disease transmission, for example, decreased development times and increased larval survival, as seen under conditions of ample larval nutrition can lead to more rapid proliferation of populations. Moreover, correlations between rising temperatures with increased blood-feeding propensity can increase an individual's vectorial capacity. Despite identification of traits that may increase *Cx. pipiens* abundance and reproductive success, genetic analysis of UK field populations revealed low levels of admixture which overall may limit the ability of *Cx. pipiens* to bridge viral transmission into the human population in the UK if indicative of an ornithophilic phenotype. However, molecular analysis also highlighted discrepancies in biotype identification between different methods for estimating hybridisation rates and admixed ancestry within a population. Therefore, further assessment of UK populations should be undertaken to investigate the risk posed by UK species to act as bridging vectors between avian hosts and the human population.

Chapter 5 – General discussion

5.1. Introduction

The *Culex pipiens* species are considered the primary vector of arboviruses such as West Nile virus (WNV) and Usutu virus (USUV) due to their ability to exploit a wide range of larval habitats coupled with their close association with humans. Plasticity in life history traits in response to environmental changes and habitat niches can influence their vectorial capacity which can have implications when considering the risk of disease transmission. The present study explored the genetic structure of aboveground UK *Cx. pipiens* in the field and those in colony. These populations were separable and selectable using the CQ11 marker for biotype, and yet individuals homozygous for *molestus* allele and heterozygous for both alleles did not exhibit autogeny, a phenotypic trait associated with the *molestus* biotype. In turn, *Cx. pipiens* f. *pipiens* individuals and selected lines were shown to mate under close confinement and exhibited mammalian blood-feeding. These data suggest a plasticity of life history parameters within both biotypes, rather than a rigid phenotypic differentiation, according to single molecular markers. Thus, genotype obtained using molecular markers such as CQ11 do not directly correlate with phenotype. Comparison with colonised *molestus* from outside of the UK using microsatellite analysis, showed a greater similarity between UK forms, as determined by CQ11, than between UK and Swedish *molestus* colony lines, further questioning the utility of single locus markers for biotype delineation. Incomplete mating barriers between the forms were identified within colony conditions, with mating success determined by biotype in paired and grouped mating conditions. Perturbation of larval nutrition affected the three colony lines differentially with the *molestus* colony exhibiting a greater development time in a low protein diet, and yet high larval protein did not facilitate the expression of autogeny. Exploration of the life history traits of the biotypes and hybrids, according to CQ11, was only possible through the development on a non-invasive method of molecular determination of biotype of individuals using the pupal exuviae.

5.2. Significance of main findings

The ability of a mosquito population to act as a vector by successfully transmitting disease is contingent on the complex interplay between genetics and environmental conditions acting at both the larval and adult stage. These factors combined define the vectorial capacity of a species or population, thus understanding how these factors interact is vital for future assessments of the role a species may play in the transmission cycle and the overall risk this poses to human and animal health. The factors explored in this thesis and their impact on life history traits are summarised in Figure 5.1.

In the larval phase, environmental conditions such as temperature or larval nutrition availability may alter development times or larval survival rates as well as influencing resulting adult traits. In the adult phase, factors such as diet, temperature and pathogen status can determine traits such as longevity, host preference and vector competence. Predictions of how changes in these traits may alter vectorial capacity of the populations tested are also presented. As an example, although increased temperatures and nutritional availability experienced during larval development were shown to increase blood-feeding propensity of eclosing *Cx. pipiens* f. *pipiens* females (Chapter 4), high levels of hybridisation may offset this, as results presented in Chapter 2 demonstrated a reduced frequency of bloodmeal acquisition in females mating with heterospecific males. Understanding how these factors relate to field populations is imperative for quantifying disease risk.

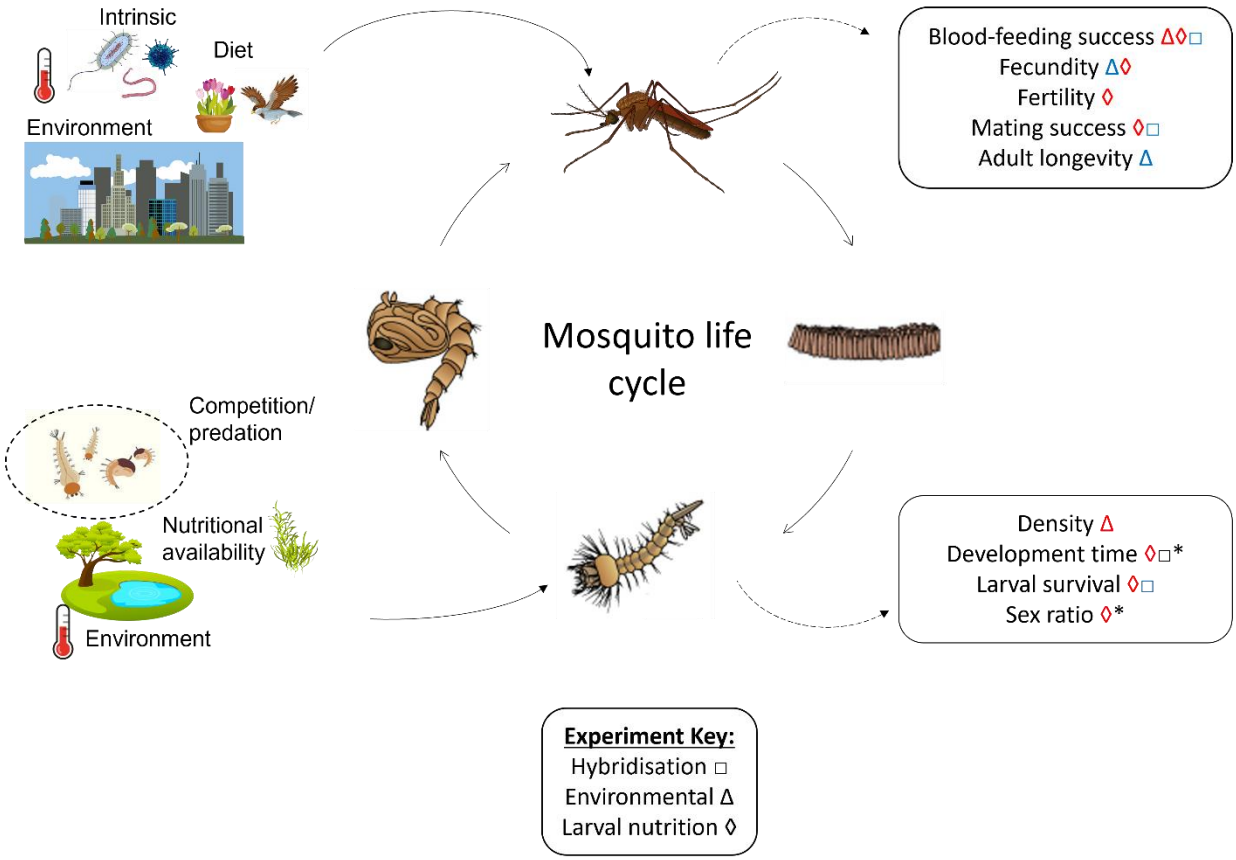

Figure 5.1. Schematic summary of some intrinsic and extrinsic factors acting on both the larval and adult life stages and the resulting physiological traits they can influence. Specific responses to hybridisation (Chapter 2; □), environmental conditions experience in the larval habitat (Chapter 4; Δ) and increasing levels of larval nutrition (Chapter 4; Φ) are demonstrated with an estimate of whether this change in behaviour/trait would either increase (red) or decrease (blue) the vectorial capacity of the populations tested. Asterix (*) indicate a significant difference was only recorded for one biotype.

5.3. Utility of a single locus marker for biotype differentiation

Field sampling of larval populations across London and Surrey in Chapter 4 demonstrated that *Cx. pipiens* larvae are widely present. Analysis of biotype distribution, as designated by CQ11, determined that *Cx. pipiens* f. *pipiens* is the predominant form breeding in aboveground container habitats (99%), with only a few hybrid individuals identified. Screening of larvae from eight sites between 2019 and 2020 failed to detect the presence of *Cx. pipiens* f. *molestus* larvae in aboveground container habitats, despite previous identification of sympatric populations in the UK from these habitats (Danabalan *et al.*, 2012; Manley *et al.*, 2015). Indeed, during adult collections, only two host-seeking adult *molestus* individuals were collected from Inner London compared with 300 *Cx. pipiens* f. *pipiens* and one hybrid.

Sympatric populations have previously been identified from northern Europe, yet hybridisation rates vary significantly between studies, despite the implementation of the same differentiation assays across most studies. Many studies, including those conducted in this thesis (Chapter 4), report low levels of hybrid specimens according to the use of single molecular markers (Rudolf *et al.*, 2013; Vogels *et al.*, 2015). The dominance of *Cx. pipiens* f. *pipiens* observed in Chapter 4, aligns with a shift in increasing proportions of *Cx. pipiens* f. *pipiens* previously observed from southern to northern latitudes in Europe (Vogels *et al.*, 2016). The high abundance of *Cx. pipiens* f. *pipiens* species may pose an increased risk for arbovirus establishment, providing these populations are competent vectors, by facilitating amplification of arbovirus transmission within the avian reservoir. Therefore, the observed disparity in WNV circulation across Europe cannot be attributed to biotype composition alone. High levels of arbovirus circulation within a natural reservoir may increase the risk of virus spill-over. This could be particularly important in urban areas, such as London, which consist of multiple green spaces providing ideal breeding sites for vector and avian hosts in areas with high human population density.

Screening of UK field collected samples conducted in Chapter 4 highlighted incongruence between biotype designations obtained from the two CQ11 assays (Bahnck and Fonseca, 2006; Rudolf *et al.*, 2013). Further investigation demonstrated mis-priming of the *pipiens* specific reverse primer in a small number of samples resulting in a double banding pattern, indicative of hybrid individuals, for *pipiens* biotypes. Despite this, the conventional CQ11 assay is the most widely applied for biotype delineation in lieu of superior methods. Moreover, DNA sequencing of three high resolution melt (HRM) analysis targets, previously reported to differentiate the *Cx. pipiens* forms, found no consistent differences between the biotypes when tested in UK field populations or well characterised *molestus* samples from Sweden, a sentiment that has been echoed by research conducted in Sweden (Luande *et al.*, 2020). Further work would be required to test these markers in *Cx. pipiens* forms from the UK which have been fully characterised by phenotype and genetic analysis and therefore represent pure forms.

Accurate biotype identification is important for quantifying the risk of disease transmission within a population. Thus, advances in genetic characterisation of the *Cx. pipiens* forms as well as genome sequencing of geographically diverse populations of the species will hopefully facilitate development of superior molecular markers for biotype delineation.

Genetic analysis undertaken in the current study demonstrated significant genetic differentiation of UK colony and field individuals testing homozygous for the *molestus* allele from specimens obtained from an autogenous *molestus* colony from Sweden. Recent genetic analysis has suggested that true *molestus* forms share a common ancestor (Yurchenko *et al.*, 2020), with a recent review of over two centuries of literature supporting the hypothesis that *molestus* is a separate genetic entity which likely originated from Egypt (Haba and McBride, 2022). A third hypothesis has however been suggested that broadly supports the notion that *pipiens* and *molestus* are genetically distinct but hypothesised that Old World and New World *molestus* potentially have independent evolutionary origins, arising from *Cx. pipiens* f. *pipiens* separately in each region (Aardema *et al.*, 2020).

Moreover, genetic clustering of colony *pipiens* and *molestus* samples, distinct from Swedish samples indicates a shared genetic ancestry between the UK colony lines, representing the several generations of inbreeding this population underwent prior to separation by CQ11 genotype in Chapter 2. This is unsurprising given results from Chapter 2 (characterisation of colonies after 10 generations) and Chapter 4 (hybrid colony characterisation) demonstrate that many of the individuals used to create the first generation of 'pure' colonies may have in fact arisen from hybridisation or backcrossing events and are therefore not representative of pure forms of *Cx. pipiens*. Under these conditions, recombination between loci can result in hybrids losing diagnostic bands, adding further complexity to the molecular identification of the *Cx. pipiens* biotypes when using a single molecular marker (Bahnck and Fonseca, 2006; Gomes *et al.*, 2009). Despite this, variation in life history traits (Chapter 3) and differential responses to environmental conditions (Chapter 4) were noted in studies undertaken as part of this thesis, a result that is surprising given the shared ancestry of the individuals used to create these lines. Additionally, colonisation causes significant genetic bottlenecks within a population, as has been demonstrated to reduce genetic diversity in colony lines compared with field populations (Yurchenko *et al.*, 2020). This would be expected to homogenise the population and their response to stressors, such as larval nutrition depletion and competition, selecting for individuals with traits that would promote their survival under colony conditions. It is therefore surprising that the Brookwood colony line maintained significant variation, which could be broadly attributed to the different forms according to CQ11.

Current evidence indicates that individuals tested during this thesis from the UK which were homozygous for the *molestus* allele are not representative of pure *molestus* forms. Rather, these individuals are more closely related to *Cx. pipiens* f. *pipiens* specimens. Overall, this demonstrates that genotype, as designated by single molecular markers, such as the CQ11, should not be relied upon to predict phenotype. Likewise, previous research in the UK has identified individuals testing as homozygous for the *molestus* allele, according to CQ11, from aboveground populations, yet when tested in assays shown to separate the forms in other countries (Shaikevich, 2007), such as Russia, these were found to be ineffective (Danabalan *et al.*, 2012). Interestingly, only one UK sample from one study was accurately separated, a sample of a typical form *molestus* specimen collected from the London Underground and displaying characteristic *molestus* traits such as autogeny. Further work should be undertaken to determine the utility of this assay for identifying true *molestus* isolated from the UK, which possess characteristic traits of the form. If this assay is proven efficient at distinguishing characteristic *molestus* forms, incorporation of this assay into standard biotype testing alongside the CQ11 assay could be beneficial to add robustness to biotype identification in lieu of full microsatellite analysis or more accurate single-locus markers. As yet, the genetic signature of characteristic *molestus* forms from the UK still remains unresolved. To fully elucidate this, a broader sample size, complemented by assessment of physiological traits such as autogeny, would be required.

The use of single molecular markers are favoured over full genetic analysis by many studies due to the reduced cost and processing time as well as the comparative ease of methodology and analysis. However, to fully elucidate the link between phenotype and genotype, future studies should focus on full genetic analysis. Samples collected during this project have been contributed to a population genomics project (https://mcbridelab.princton.edu/pippop/) aiming to compile a global database of *Cx. pipiens* genomes to broaden our understanding of the evolutionary history of this species.

5.4. Development of a non-invasive molecular method to determine biotype

Results from Chapter 2 demonstrate that mosquito pupal exuviae can be successfully used as a source of trace DNA for biotype delineation in *Cx. pipiens*. Data obtained prove that this method is capable of isolating sufficient DNA yield to permit successful PCR amplification for up to twenty-four hours post-eclosion, whilst samples collected within twelve hours of eclosion demonstrate very high levels of amplification success (100%). Versatility of this method was demonstrated by successful PCR amplification following processing with a range of extraction methods as well as direct PCR which all varied in cost, time, and skill level required. Therefore, the method is accessible to a range of projects with differing resources and requirements. Moreover, successful application under field conditions

provides opportunities for preliminary field screening of populations without affecting population dynamics through the removal of large numbers of adults or larvae.

One of the major constraints for experiments utilising cryptic taxa is the ability to identify individuals without impacting adult behaviour or longevity. This is particularly prominent in behavioural studies of the *Cx. pipiens* biotypes, with many studies omitting biotype analysis. Preliminary experiments conducted in this thesis (Chapter 2) highlight logistical challenges of working with a mixed population lacking in morphologically distinguishing features. Implementation of the non-invasive method developed in Chapter 2, was used to select live individuals of known biotype to establish pure colony lines from the existing mixed population. This facilitated work undertaken assessing life history traits in subsequent studies (Chapters 3 and 4), that logistically would not have been possible using a mixed colony or field population. This technique would be of particular benefit to further understand biotype specific responses to various factors, including environmental stressors, and for studies in other cryptic taxa investigating individual species or form response in sympatric species. Current identification of other cryptic taxa of mosquitoes and insects of interest predominantly relies on the use of end-point PCR methods; therefore, the lower DNA yield obtained from pupal exuviae may limit adaptation to other insect species. Further assessments of PCR success using end-point PCR assays combined with development of real time PCR techniques would enable use of this method for other arthropod taxa.

5.5. Barriers to gene flow between the *Culex pipiens* biotypes and the effect of hybridisation on life history traits

Experiments conducted in Chapter 3 revealed several barriers to gene flow between the 'pure' colony lines created as part of Chapter 2, resulting in reduced fitness of hybrids, although these barriers appear to be incomplete. Mating experiments highlighted noteworthy behavioural barriers to hybridisation with a significantly reduced mating success of the *pipiens* colony under single-pair mating conditions. However, this barrier appears to, in part, be overcome when switching from single-pair to grouped mating experiments, despite still being confined to colony cages. Similarly, behavioural barriers were previously highlighted as a significant factor limiting hybridisation (Kim *et al.*, 2018b).

Females of all colony lines were demonstrated to have a lower mating success when paired with non-conspecific males compared with homologous crosses, in concordance with previous literature (Kim *et al.*, 2018b). Although previous studies reported greater disparity between homologous and

reciprocal mating frequencies, these experiments relied on the use of genetically pure colony lines which cross mated to create first generation hybrids (Kim *et al.*, 2018b). When second generation hybrid crosses were assessed hybrid fitness was improved (Kim *et al.*, 2018b), with results aligning with those reported in the current study, which utilised a population that had undergone several generations of hybridisation prior to separation according to genotype. First-generation hybrids have been shown to suffer reduced fitness compared with individuals exposed to continuous conditions of hybridisation, which likely accounts for differences seen between previous studies and data presented in this thesis. Whilst behavioural differences between the colony lines were observed, genetic analysis revealed a shared ancestry between the forms (Chapters 2 and 4). This likely facilitated the enhanced levels of mating success recorded in the current studies compared with previous results plus the process of colonisation would have selected for individuals with traits beneficial to survival in colony and are therefore not truly representative of natural populations (Kim *et al.*, 2018b). This is also reflected in larval survival as although reduced fitness of hybrid progeny was demonstrated (Chapter 3), larval survival rates were superior to previous findings (Kim *et al.*, 2018b).

Given the shared ancestry of the lines, this poses an interesting question that although an adaptive shift by one biotype to occupy the same habitat as the other form may initially lead to a significant reduction in mating frequency and survival of hybrid progeny, continued introgression between the forms may diminish these barriers. Further work should focus on testing these factors in local but isolated populations of the two forms to further elucidate this relationship. However, despite this shared ancestry of the lines, data presented in Chapter 3 demonstrated that females preferentially mated with males testing homozygous for the same allele, therefore it would be interesting to investigate whether this is reflected in natural populations.

Inter-form mating was also shown to alter blood-feeding success of *Cx. pipiens* females, demonstrating a reduction in bloodmeal acquisition in response to mating with non-conspecific males as opposed to homologous mating. The most prominent response was seen in the *pipiens* colony females which reduced bloodmeal acquisition by approximately 30% when paired with non-conspecifics compared with *pipiens* males (Chapter 3). The effect of male accessory gland (MAG) extract on blood-feeding behaviour of mosquitoes has previously been reported to increase blood-feeding propensity (Villarreal *et al.*, 2018) and bloodmeal digestion rate in *Aedes aegypti* (Downe, 1975). It is interesting that these differences were observed given the lack of genetic differentiation and ancestral introgression between the colony lines. Although the individual colony lines used within these studies are not representative of field populations of *Cx. pipiens* forms displaying the typical behavioural and physiological characters, the results of Chapter 3 pose an interesting question of how

levels of hybridisation within a population could influence blood-feeding propensity, especially in the *pipiens* form. If this is shown to be reflected in natural populations of typical *Cx. pipiens* forms this may act to reduce circulation of arboviruses such as WNV in the avian population by reducing the levels of vector-host contact. Further work should investigate the effects of MAG extract on blood-feeding behaviours of the forms and explore how these behavioural responses are affected by interform mating.

5.6. Plasticity of life history traits in *Culex pipiens* in response to different environmental stressors experienced during larval development

The studies presented in this thesis demonstrate the plasticity of *Cx. pipiens* in response to different environmental pressures, highlighting key differences not only between colony and field populations, but also between the *Cx. pipiens* colony lines created as part of Chapter 2. Field studies conducted during Chapter 4 demonstrate the positive effect that elevated average temperatures and decreased temperature fluctuations exhibit on colonisation of larval habitats as well as mosquito biology including increased proportion of female mosquitoes blood-feeding. The highest larval densities (0.12 larvae/L) were recorded in areas experiencing the highest average daily temperatures, which in the present study were associated with peri-urban environments. Elevated water temperatures during larval development were also shown to positively influence adult willingness to blood-feed, with a 1^{o}C temperature rise predicted to result in 112.4% increase in number of eggs per raft. However, this would be countered by a predicted 78.1% decrease in female fecundity if temperature fluctuations were to increase by the same increment.

Experiments using field and colony mosquitoes demonstrated the positive effect of increased provision of larval nutrition on a range of life history traits including decreased larval development times and increased blood-feeding success, fecundity, oviposition, and mating in adults. Together, these factors have the potential to influence adult population dynamics by resulting in a more rapid proliferation of *Culex* populations compared with those in nutritionally poor environments. This could be of particular importance in urban environments which have been shown to support higher average temperatures (Townroe and Callaghan, 2014), with reduced fluctuation between day and night-time conditions (Chapter 4) which facilitates greater larval densities, coupled with a closer association of mosquito habitats with avian and human hosts.

However, these experiments also revealed that elevated average temperature and temperature fluctuations during development negatively impact adult survival in concordance with previous

research (Ciota *et al.*, 2014). Moreover, assessment of field and colony mosquitoes exposed to variable levels of larval nutrition failed to detect autogenous egg production in aboveground UK populations (Chapter 4). The expression of autogeny was previously shown to fluctuate with larval and adult nutrition (Kassim *et al.*, 2012b). In contrast, results presented in Chapter 4 demonstrate that the UK populations tested appear to be incapable of autogenic expression, despite sufficient provision of larval nutrition, which suggests that these species are genetically anautogenous. This is an unsurprising result given that aboveground larval populations appear to be dominated by the *pipiens* form according to characterisation by the CQ11 microsatellite. This was reinforced by microsatellite analysis of UK samples, which demonstrated genetic differentiation between individuals homozygous for the *molestus* allele from the UK compared with autogenous colonised *molestus* populations from Sweden indicating that *molestus* colony forms in the UK are not representative of true *molestus* forms (Chapter 4). Rather, typical form *molestus* populations, isolated from underground environments, such as the London Underground system, should be assessed under the same conditions to test the potential influence of varying larval nutrition availability on the penetrance of autogenic expression in UK populations.

The expression of autogeny and decreased adult longevity are key factors determining the vectorial capacity of a species, reducing the probability of successful arbovirus transmission. Production of autogenous egg batches delays the age at which females have the potential to acquire an infectious bloodmeal, thereby reducing the time available for onward transmission. This is especially important in obligatory autogenous populations such as *Cx. pipiens* f. *molestus* from Australia (Kassim *et al.*, 2012b). Thus, the presence of anautogenous *molestus* and hybrid populations may enhance risk of disease transmission by increasing the number of bloodmeals acquired by a population, the time that they are infectious and the probability of onward transmission.

To understand the impact of climate change on vectorial capacity and patterns of disease transmission, quantification of the relationship between environment and life history traits is required. A major limitation of the current study was the small number of sites sampled; however, these relationships will likely vary across geographical regions and between genetically diverse populations. Nonetheless, a larger scale study would be beneficial to understand how environmental changes may influence mosquito populations in different ecological niches. Further studies would also benefit from inclusion of above- and belowground populations to determine whether the divergent populations are differentially affected by changing environmental factors.

5.7. Environmental factors affecting life history trait plasticity of the *Culex pipiens* biotypes

Data presented in Chapters 3 and 4 reveal key differences in life history traits between the colony lines as well as differential responses to environmental stressors. Although experiments in Chapter 4 show similar trends in response to nutritional availability, differences were observed such as the increased susceptibility to depleted larval nutrition of the *molestus* and hybrid lines, as evidenced by elevated development times and reduced survival. Moreover, important differences between life history traits of the colony lines, including differential development times under standard rearing conditions, have been observed (Chapter 3).

Experiments highlight significantly extended development times in the *Cx. pipiens* f. *molestus* compared with the *Cx. pipiens* f. *pipiens* colony line (Chapter 3) which is particularly evident under conditions of depleted larval nutrition availability (Chapter 4). Differential development times have previously been reported in other insect species and are hypothesised to limit hybridisation under natural conditions (Matute and Coyne, 2010). A reduced survival rate of larvae with elongated development times is predicted through exposure to elevated risk of nutrition depletion, predation, and desiccation. If these patterns are shown to translate to field populations, disparity in development time could reduce biotype interactions under natural conditions through asynchrony, limiting hybridisation between the forms even in areas of sympatry. This combined with a preference to mate with conspecific males (Chapter 3) could overall reduce the levels of hybridisation within a population. Reduced development times in some species of *Anopheles* have previously been linked to ecological niche and associated with species utilising transient larval habitats (Gimnig *et al.*, 2001), as well as those with high levels of larval competition (Knight *et al.*, 2004). Traditionally, the two *Cx. pipiens* forms have been associated with distinct ecological niches, with the *molestus* form associated with underground, stable environments with relatively low chance of predation therefore it would be interesting to test this hypothesis with a typical *molestus* population. Hence, further work with natural populations of the different forms, displaying characteristic phenotypes, should be used to test this hypothesis.

Moreover, disparity in larval survival rates between the colony lines under varying levels of larval nutrition have also been demonstrated (Chapter 4) with hybrid specimens more susceptible to larval nutrition depletion than *pipiens* or *molestus*. Although temperature has previously been shown to influence life history traits of *Cx. pipiens* (Ciota *et al.*, 2014; Chapter 4), the specific response by each of the biotypes has not been assessed. Increasing temperatures could be important in regulating population dynamics in high population density areas exposed to urban heat island (UHI) effect. A

significant limitation of the present study is the use of a mixed population, with a high degree of introgression prior to experiments (discussed in Section 5.3), meaning the 'pure' colony lines were unlikely to be representative of the typical forms found in the field. Therefore, future work should focus on further differentiating the responses to environmental factors between the forms and how this may influence their adaptation to different ecological niches.

5.8. Plasticity of behavioural and physiological traits of *Culex pipiens*

Deviations from traditional behavioural or physiological definitions of the forms, as designated by the CQ11 marker, were highlighted as part of the studies conducted during Chapters 3 and 4. These results revealed a lack of autogeny in UK *molestus* populations in colony and the ability of *Cx. pipiens* f. *pipiens* to mate, at least to some degree, under confined conditions, as discussed in Section 5.5. Moreover, identification of mammalian blood-feeding by a field collected engorged *Cx. pipiens* f. *pipiens* individual despite the presence of ample avian hosts indicates a plasticity of blood-feeding behaviour within the *pipiens* form. This is reinforced by the willingness of field collected *pipiens* to acquire a bloodmeal under colony conditions, despite experiments utilising horse blood, a non-natural host of this form. Previous evidence has revealed adaptability of feeding preference of the forms to limited host availability (Fritz *et al.*, 2015), whilst other studies have exposed a broader preference of bloodmeal source for the forms (Gomes *et al.*, 2013). In addition, a recent study investigating mosquito bloodmeal hosts in Chester Zoo identified 497 blood-fed *Cx. pipiens* f. *pipiens*, with humans found to be the bloodmeal source in 47.5% of cases (Hernandez-Colina *et al.*, 2021).

Despite this, many studies, including results from Chapter 4, demonstrate that birds are still a major bloodmeal host of *Cx. pipiens* f. *pipiens*. However, mammalian feeding by the *pipiens* form may be more common than initially thought, although much wider scale surveys would be required to test this hypothesis. Collection of blood-fed mosquitoes can be challenging as these individuals are poorly attracted to commonly used mosquito traps using light or host-derived scents as bait (Thiemann and Reisen, 2012). Collection of sufficient numbers is problematic, usually relying on manual aspiration which can be time consuming and still yield low numbers of blood-fed individuals (Brugman, 2016). Therefore, this was not within the scope of the current project, but further studies could incorporate wide scale collections with genetic analysis to determine the extent of bloodmeal hosts for each form.

Within colony populations tested, the lack of autogeny and the ability to mate under confined conditions display plasticity in typical behaviours. These are likely attributed to populations bottlenecks experienced during colonisation as well as the selection of individuals which possess traits beneficial to survival in colony. Moreover, mammalian blood-feeding by these colony individuals is

unsurprising given the several generations of selection for mammalian feeding through regular colony maintenance (Manley *et al.*, 2015). Overall, results have demonstrated that genotype using single molecular markers, such as CQ11, does not necessarily inform phenotype. This was demonstrated with individuals testing as homozygous for the *molestus* allele in colony failing to display characteristic signs of *molestus* form such as autogeny, despite the provision of sufficient larval nutrition (Chapter 4). Further genetic analysis indicated that in fact these individuals were not representative of true *molestus* forms, despite unambiguously and consistently testing as homozygous for the *molestus* allele by traditional CQ11 assays.

Data presented in this thesis have added to mounting evidence of phenotypic plasticity within each of the forms, as identified by CQ11, contradictory to the rigid definitions typically associated with results from these assays. Rather, data indicate that a continuum of life history traits and phenotypes exist, likely influenced by the degree of introgression with other forms with a population, with the typical forms displaying the extremes. Given the within form variation, it is unsurprising that single molecular markers are not always able to accurately differentiate the forms. Consideration of the flexibility of these traits should be considered when assessing risk of disease transmission and the threat posed to animal and human health within a population.

5.9. Risk of arboviral disease transmission in the UK by *Culex pipiens*

The epidemic potential of WNV in a naive population has been illustrated in North America where after initial introduction, WNV spread rapidly across the continent (Lanciotti *et al.*, 1999; Marfin *et al.*, 2001). Despite the presence of competent vectors and susceptible hosts coupled with the close endemic circulation in southern Europe, outbreaks of WNV in Northern Europe do not regularly occur. This lack of circulation in northern Europe is likely to limit the risk of introduction to the UK under current conditions. However, in 2018, a particularly hot summer with sustained above average temperatures spanning several months, facilitated the spread of WNV into Germany (Ziegler *et al.*, 2019), considerably further north than reported in previous years. A recent risk assessment by the Department for Environment, Food and Rural Affairs (Defra) concluded that the greatest risk of WNV incursion to the UK is through migratory birds, although the probability of migratory birds arriving in the UK with high enough levels of viraemia to support onwards transmission was considered unlikely (Defra, 2012). However, detection of WNV in more northern latitudes in Europe may facilitate spread to the UK by providing a source of infection for migratory birds to acquire during their stopover, which would increase the probability of hosts still being viraemic upon arrival to the UK.

High density populations of *Cx. pipiens* species driven by elevated summer temperatures could facilitate establishment and enzootic transmission of WNV within the avian population in the event of incursion. Current evidence in the UK suggests that the *Cx. pipiens* forms predominantly occupy distinct niches (Byrne and Nichols, 1999), however, data presented during this thesis has demonstrated high levels of plasticity for *Cx. pipiens*. Therefore, should the *molestus* form undergo an adaptive shift to facilitate colonisation in sympatry with the *pipiens* form, incomplete barriers to gene flow may facilitate higher levels of hybridisation which could drive transmission to humans.

5.10. Future research directions

5.10.1. What factors lead to successful arbovirus transmission by populations of *Culex pipiens*?

Despite a near ubiquitous distribution of vector species, circulation of *Culex*-transmitted arboviruses is not uniform. Following introduction to North America, *Cx. pipiens* facilitated the rapid spread of WNV across the country, with no apparent geographical barriers. Interestingly, despite the presence of competent vector species WNV outbreaks in northern Europe are limited. Previous studies have demonstrated that vector competence can vary on a spatial and seasonal scale (Kilpatrick *et al.*, 2010). Until recently, most vector competence studies for WNV were conducted with American mosquito populations, which therefore cannot be assumed to be directly applicable to European populations. Although recent studies have aimed to address this, still relatively few studies have explored specific differences in vector competence between the *Cx. pipiens* biotypes (Vogels *et al.*, 2017a; Holicki *et al.*, 2020). Whilst vector competence is an essential component, other life history traits may determine overall vectorial capacity. As arboviruses such as WNV or USUV are maintained in primary transmission cycles between the avian host and maintenance vector, identifying areas of high vector-bird interaction, and where these coincide with high human habitation are vital for identifying areas of high risk in the event of incursion. These areas are increasingly represented by urban areas surrounded by green spaces. Further studies are required to determine the geographical distribution of the biotypes and hybrids and how these relate to current arbovirus distribution patterns.

Several studies have now focused on biotype composition in different European countries. Thus far, most studies consider the average population composition over the whole period when adult vectors are active, therefore potentially overlooking seasonal patterns in biotype abundance. As peaks in WNV transmission typically follow seasonal peaks in mosquito abundance (Andreadis *et al.*, 2004), it is important to further investigate seasonal changes in the *Cx. pipiens* biotype composition. Moreover, as migratory birds are thought to play a key role in the spread and introduction of arboviruses such as

WNV to new areas, aligning biotype abundance and composition with temporal migration patterns would be informative of transmission risk.

Finally, the ability of adult *Cx. pipiens* to overwinter is considered an important factor facilitating maintenance of transmission in temperate regions between years, with WNV RNA previously isolated from overwintering females in the Czech Republic (Rudolf *et al.*, 2017). Increased average temperatures, especially in areas affected by an UHI effect, could significantly affect the seasonal dynamics of *Cx. pipiens* populations. Milder autumn and/or winter months could significantly increase the abundance and survival of overwintering populations whilst this combined with warmer spring months may extend the activity period of adult mosquitoes, thereby increasing the virus transmission period (Ciota *et al.*, 2011). Therefore, further studies addressing the overwintering capability of *Cx. pipiens* in the UK should include assessments of overwintering survival and emergence of biotypes in response to rising temperatures.

5.10.2. What are the factors influencing the rate of hybridisation between the *Culex pipiens* biotypes?

The variable rates of hybridisation in sympatric *Cx. pipiens* populations alludes to the existence of multiple barriers to gene flow between the forms that act beyond ecological isolation. Work undertaken in this thesis has demonstrated variation in life history traits between different crosses of colony populations which may influence their ability to hybridise under natural populations. One of the key aspects of the mosquito mating sequence is the recognition of conspecific mates through wing beat frequencies. Data from this thesis demonstrated significant variation in wing length between the three colony lines (Chapter 3) as well as in response to environmental stressors (Chapter 4). Equivocal results have been presented as to the influence of wing length on wing beat frequency (Villarreal *et al.*, 2017; de Nadai *et al.*, 2021), thus this may act to influence the rate of hybridisation within a population by interfering with conspecific mate recognition. Moreover, disparity in wing morphology has been attributed to ecological isolation between populations of the South American fruit fly (Vera *et al.*, 2006) and may also potentially play a role in mating interference within the *Cx. pipiens* species. Therefore, further work should include assessment of disparity in wing beat frequencies between the forms and the potential impact these may exert on mate recognition.

Intrinsic factors, such as those mediated by commensal *Wolbachia* strains and cytoplasmic incompatibility are also hypothesised to decrease hybridisation between the *Cx. pipiens* forms. *Wolbachia* has been widely reported from *Cx. pipiens* (Hertig, 1936) and was proven to be present in UK colony lines (Bell-Sakyi *et al.*, 2021). Yet, the extent to which this contributes to limiting

hybridisation events is unknown at present. Further work is required to assess the distribution and contribution of *Wolbachia* infection to limiting hybridisation within this species.

Whilst studies presented in this thesis demonstrate a reduction in mating frequency between reciprocal crosses the reasons for this were not explored further due to time constraints. Research in *Aedes* mosquitoes have revealed satyrization as a method of mating interference between *Aedes aegypti* (Linnaeus, 1762) and *Aedes albopictus* (Skuse, 1894) where reciprocal mating between the two induces refractoriness of females to further mating with conspecific males, despite a lack of sperm retention from the initial mating event (Carrasquilla and Lounibos, 2015). Studies such as these utilise fluorescent dyes to detect occurrence of mating events without the retention of sperm, that may otherwise go undetected. Evidence for this intriguing behaviour between *Cx. pipiens* forms would represent further elucidation of the maintenance of *Cx. pipiens* forms and hybrids within different populations. Finally, further investigation of the integrity of these barriers in response to other environmental conditions, such as elevated temperature, would also be valuable.

5.10.3. What are the potential impacts of a changing environment and further urbanisation?

The effect of climate change on arthropods is well established and the potential influences on arthropod-borne pathogens have been explored (Rocklov and Dubrow, 2020; Colon-Gonzalez *et al.*, 2021). Moreover, anthropogenic changes which alter the landscape, such as the creation of urban wetlands and the provision of additional container habitats associated with urbanisation, could facilitate proliferation of mosquito populations whilst also amplifying vector-host contact. These changes may also facilitate adaptive shifts by other species to utilise urban habitats, as evidenced by an increasing urban population of *Anopheles plumbeis* (Stephens, 1828) in areas of the UK (Townroe and Callaghan, 2014). Increasing average temperatures and UHI effects have previously been correlated with increasing mosquito densities (Townroe and Callaghan, 2014). Indeed, work conducted as part of this thesis (Chapter 4) found that increased water temperatures were correlated with higher larval densities. Further, rising temperatures have been demonstrated to affect abundance (Vogels *et al.*, 2016) and vector competence (Vogels *et al.*, 2017a) of *Cx. pipiens*. Therefore, lower temperatures have been proposed as an important factor which may explain the absence of WNV outbreaks in northern Europe despite the presence of competent vectors. One proposed mechanism for this is through extension of the extrinsic incubation period under milder conditions which subsequently decreases the infectious lifetime of a mosquito (Vogels *et al.*, 2017a). Warmer and extended summers as a consequence of climate change may favour the northward movement of diseases such as WNV in Europe. During the summer of 2020, unusually high temperatures were

experienced across the UK, which also coincided with the first detection of USUV in the British avifauna (Folly *et al.*, 2020). Given the similar ecology of WNV and USUV, it is, therefore, not implausible that further temperature rises could facilitate the incursion of WNV into the UK.

Artificial lights at night (ALAN) are proven to significantly influence mosquito biology, with sex-specific responses at the cellular level reported (Honnen *et al.*, 2016). Physiology of female mosquitoes has also been demonstrated to be influenced by ALAN, specifically extension of female host-seeking period and increased fecundity have been reported (Honnen *et al.*, 2019; Fyie *et al.*, 2021). Furthermore, exposure of the common house sparrow (*Passer domesticus*), a widespread urban-dwelling garden bird, to ALAN increase the period in which transmissible viral titres were detected without affecting individual survival (Kernbach *et al.*, 2019). The authors postulated that in North America this could increase WNV outbreak potential by up to 41%.

Further understanding the effects ALAN can have on mosquito and host biology is imperative to understand disease risk in urban areas. For example, if ALAN were shown to increase transmission rates in species hybrids, this coupled with an increase in vector populations, period of host-seeking and increased viraemic period of the reservoir host together could have serious implications for disease transmission. These factors together could facilitate transmission and outbreak amplification, not only within the avian reservoir, but also increase the risk of virus spill-over into the human population by increasing the opportunity for females to acquire an infectious bloodmeal and to transmit disease. Hence future work should expand upon the existing research to establish the physiological effects ALAN imposes on not only the vector but also on avian host species. These studies should include explicit comparison of the effect of ALAN on the individual *Cx. pipiens* biotypes to determine any differential responses of the forms which may determine the role they play in disease transmission.

5.10.4. What is the plasticity of feeding behaviour for the *Culex pipiens* biotypes?

Current evidence continues to demonstrate the importance of birds as a major bloodmeal host for *Cx. pipiens*. However, there is considerable evidence to support the plasticity of host preference for the *pipiens* form to also acquire bloodmeals from mammals and humans as demonstrated through bloodmeal and host-baited studies (Gomes *et al.*, 2013; Brugman *et al.*, 2017a; Hernandez-Colina *et al.*, 2021) which was alluded to by data presented in the current thesis (Chapter 4). Additionally, previous data has revealed bird feeding by the *molestus* form (Gomes *et al.*, 2013), to the same degree as the *pipiens* biotype. Considering this, it is therefore necessary to consider the potential for both forms to be capable of enzootic amplification of disease whilst also potentially acting as bridging

vectors for medically important arboviruses. Much larger scale surveys incorporating full genetic analysis of mosquito species over several seasons would be required to investigate this further.

5.11. Conclusions

Understanding vector ecology and factors that may influence colonisation of habitats by different species as well as vector-host contact is essential to understanding the risk of nuisance biting and the risk of the establishment, or re-establishment, of pathogens in the UK. Response to environmental conditions can be highly plastic in many insect species, providing a mechanism by which adult fitness is maximised in a fluctuating environment. Alteration in adult traits as a consequence of environmental stressors can have crucial implications for disease transmission through changes in epidemiologically important traits such as longevity, flight capabilities and vector competence. This thesis provides key data on how hybridisation and larval habitat influence life history traits of *Culex pipiens,* specifically identifying variation between populations in response to external stressors. Field studies highlighted that environmental factors experienced within the larval habitat during development can influence life history traits, with increased average water temperature predicted to increase *Cx. pipiens* reproductive output and population proliferation. However, explicit assessment of how the individual *Cx. pipiens* forms would respond under these conditions was not assessed due to the lack of *molestus* populations found during larval sampling. Moreover, life history traits of both field and colony mosquitoes were shown to be positively influenced by increased provision of larval nutrition availability. Although disparity in the degree to which these two sets of populations responded to larval nutrition was noted, with the field collected individuals shown to be more robust to nutritional fluctuation, the same general trends were identified. Thus, although colony lines tested as part of this thesis are not representative of field populations, experiments utilising colonised mosquitoes can be utilised to indicate patterns likely to emerge in natural populations although it is important to remember that results cannot be broadly applied. Genetic analysis of field collected and colonised mosquitoes highlights concerns regarding the accuracy of current biotyping techniques, particularly under conditions of continued hybridisation and backcrossing. Further, genetic analysis questions the status of *Culex pipiens* f. *molestus* from aboveground populations, indicating that these are more closely representative of the *pipiens* form rather than characteristic *molestus*. Overall, these results also demonstrate that genotype according to these single marker techniques cannot necessarily predict phenotype. The data presented here highlight the degree of plasticity of life history traits of UK *Cx. pipiens* and the caution required when considering transmission potential of *Cx. pipiens* populations based on differentiation using single locus markers.

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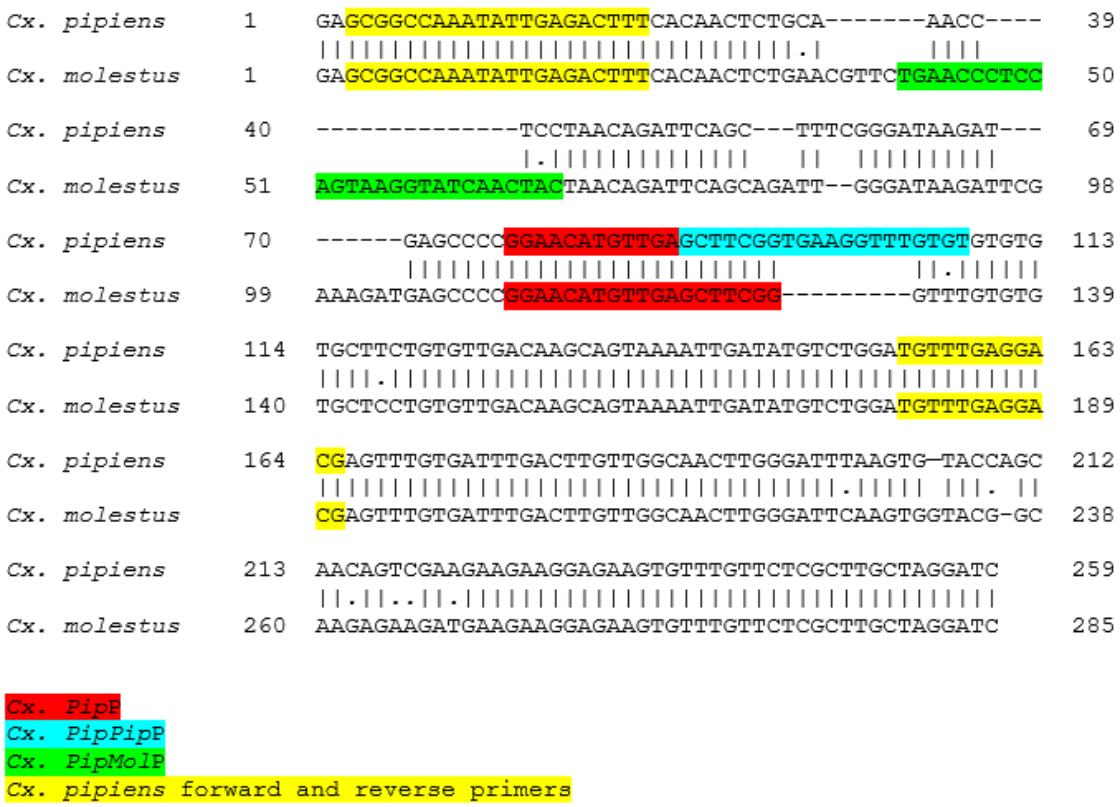
Appendices

Appendix 1. Multiplex PCR optimisation and validation

This protocol was designed to simultaneously differentiate *Cx. pipiens* from *Cx. torrentium* as well as distinguishing between the two biotypes of *Cx. pipiens.* Primers *CxPip*F (5'-GCGGCCAAATATTGAGACTT-3') and *CxPip*R (5'-CGTCCTCAAACATCCAGACA-3') amplify a segment of the CQ11 microsatellite locus of all *Cx. pipiens* species. Three probes, *CxPip*P (5'-VIC-GGAACATGTTGAGCTTCGG-QSY-3'), *CxPipPipP* (5'-ABY-GCTTCGGTGAAGGTTTGTGT-QSY-3') and *CxPipMol*P (5'-JOE-TGAACCCTCCAGTAAGGTATCAACTAC-QSY-3') were then used to differentiate between the two (binding of primers and probes shown in Appendix 1.1). *Culex torrentium* DNA was detected using the primers *CxTorr*F (5'-GACACAGGACGACAGAAA-3') and *CxTorr*R (5'-GCCTACGCAACTACTAAA-3') and the probe *CxTorr*P (5'-FAM-CGATGATGCCTGTGCTACCA-QSY-3') which binds to the nucleotide positions 112 to 131 of the ACE-2 gene.



Appendix 1.1. Primer and probe alignment with the CQ11 microsatellite sequence of *Culex pipiens* f. *pipiens* and *Culex pipiens* f. *molestus* from the protocol designed by Rudolf *et al.,* (2013).

To optimise primer concentrations and to confirm amplification of a single product for each primer pair, melt curve analysis using SYBR™ green was performed. Briefly, 10 µl reaction mixtures consisted of 5 µl PowerUp™ SYBR® green master mix (2X), 0.1 µl BSA, 2 µl DNA, 0.9 µl UltraPure™ water and 1 µl of each of the primer dilutions. Primer concentrations trialled for *Cx. pipiens* primers were 0.1 µM, 0.2 µM, 0.3 µM, 0.4 µM and for *Cx. torrentium* were 0.05 µM, 0.1 µM, 0.15 µM, 0.2 µM and 0.3 µM. All amplifications were performed in duplicate.

Quantitative cycle (Cq) values were assessed between singleplex and multiplex for each primer/probe pair combination. Standard curves were constructed from a 1 in 10 serial dilution of 6 logs of DNA template to assess assay performance and range. Gradients of standard curves were subsequently used to calculate PCR efficiency. Reaction mixtures for singleplex and multiplex reactions are shown in Appendix 1.2 with all reactions performed in triplicate.

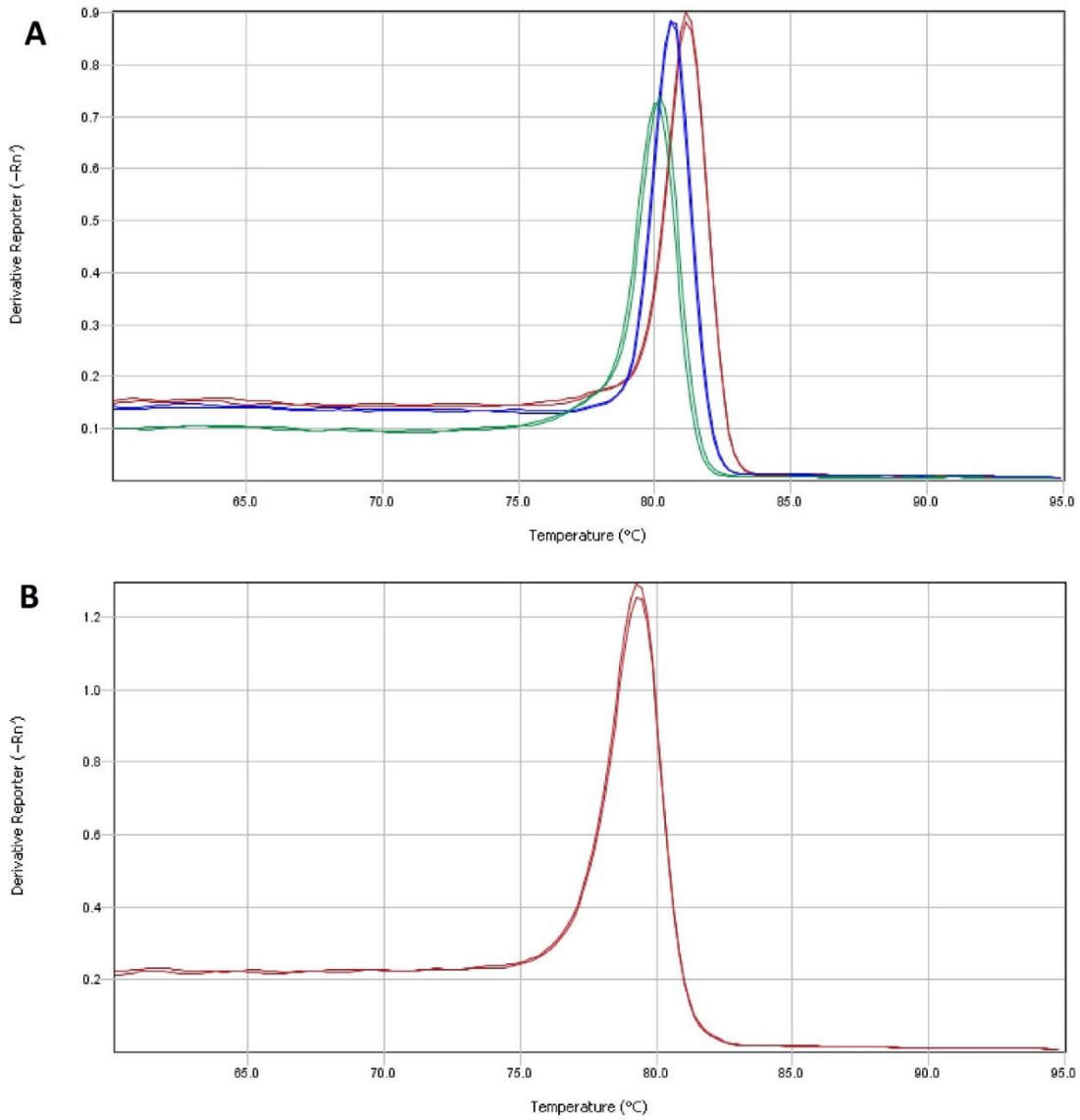
Appendix 1.2. Reaction mixtures of singleplex and multiplex reaction used to make standard curves

	***CxPip*P singleplex**	***CxPipPip*P singleplex**	***CxPipMol*P singleplex**	***Cxtorr*P singleplex**	**Multiplex**
Taqman master mix	10 µl	10 µl	10 µl	10 µl	10 µl
BSA	0.15 µl	0.15 µl	0.15 µl	0.15 µl	0.15 µl
***Cx*F**	0.6 µl	0.6 µl	0.6 µl	-	0.6 µl
***Cx*R**	0.8 µl	0.8 µl	0.8 µl	-	0.8 µl
***CxPip*P**	0.4 µl	-	-	-	0.4 µl
***CxPipPip*P**	-	0.4 µl	-	-	0.4 µl
***CxPipMol*P**	-	-	0.4 µl	-	0.4 µl
***Cxtorr*F**	-	-	-	0.3 µl	0.3 µl
***Cxtorr*R**	-	-	-	0.3 µl	0.3 µl
***Cxtorr*P**	-	-	-	0.2 µl	0.2 µl
DNA	2 µl	2 µl	2 µl	2 µl	2 µl
H_2O	6.05 µl	6.05 µl	6.05 µl	7.25 µl	4.65 µl

Results

Primer concentrations and annealing temperatures were optimised to give final concentrations in each reaction of 0.3 µM, 0.4 µM and 0.15 µM for *Cxpip*F, *Cxpip*R and *Cxtorr*F and CxtorrR, with an annealing temperature of 60°C. Melt curve analysis with SYBR™ green confirmed amplification of single products for each primer pair (Appendix 1.3), confirming specificity of the primers. *Culex torrentium* is not kept in colony at The Pirbright Institute and was not identified in field collected samples from a water container in Guildford, Surrey. Therefore, to optimise the *Cx. torrentium*

primer/probe pair, the expected amplified sequence was synthesised (Sigma-Aldrich, USA) and a 1:100,000 dilution was used for all optimisation, validation and positive controls.



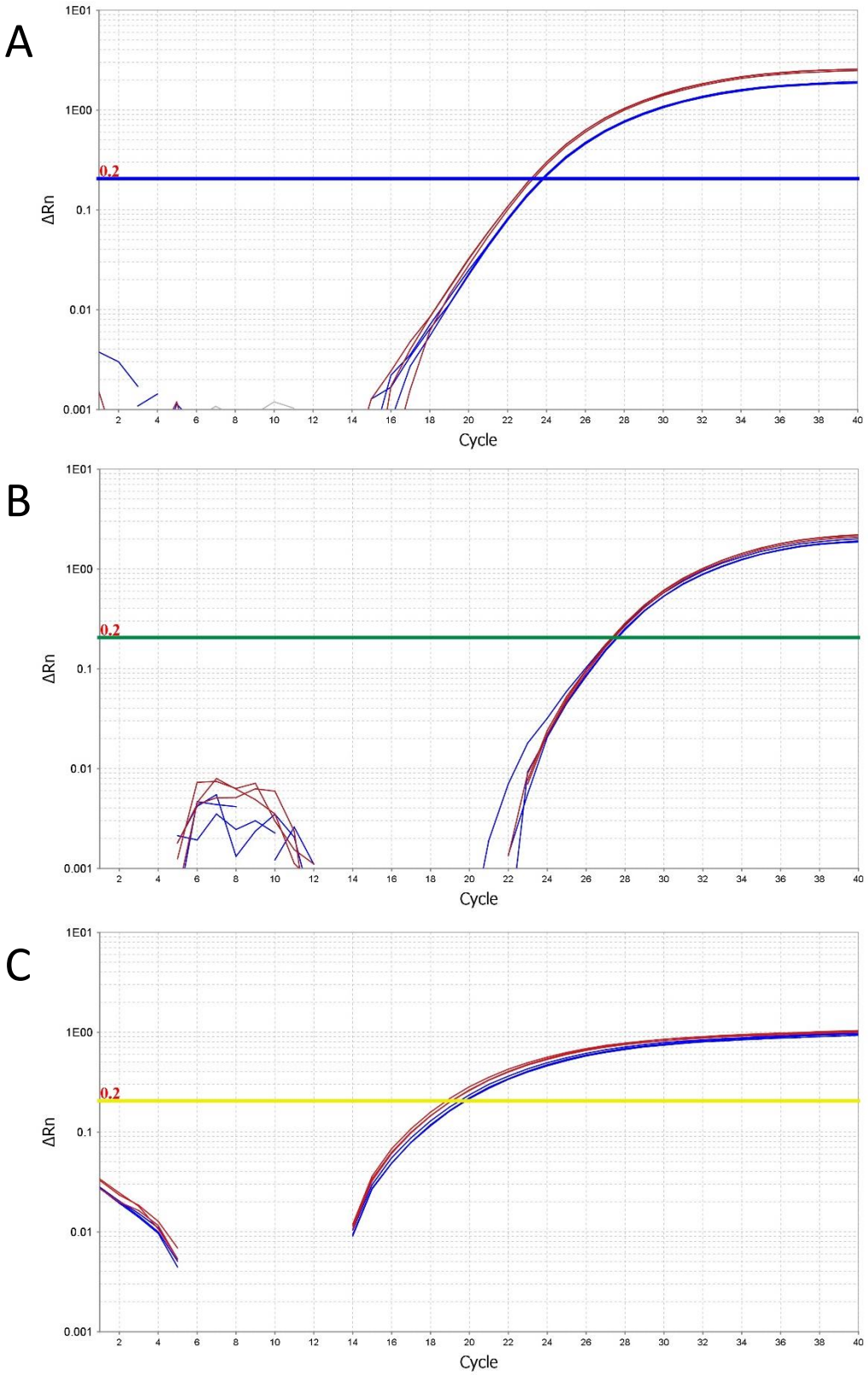
Appendix 1.3. Melt curve amplification plots of each product. A: *Culex pipiens* f. *pipiens* (Red), *Culex pipiens* f. *molestus* (Blue) and hybrid DNA (Green); B: *Culex torrentium* DNA.

Following optimisation, Cq values for each probe was compared between singleplex and multiplex to determine whether a multiplex reaction caused a loss in amplification efficiency. Samples were amplified in triplicate with the mean Cq values ± standard deviation presented in Appendix 1.4. The assay was found to be highly reproducible with standard deviation of technical replicates less than 0.167 for all samples. Little variation in Cq values from singleplex to multiplex were observed for all probes with the exception of *CxPip*P (Appendix 1.4; Appendix 1.5; Appendix 1.6). When comparing Cq values of *CxPip*P for different DNA templates, DNA of *Cx. pipiens* f. *molestus* samples were not observed to cause an increase in Cq values however, an increase from singleplex to multiplex was

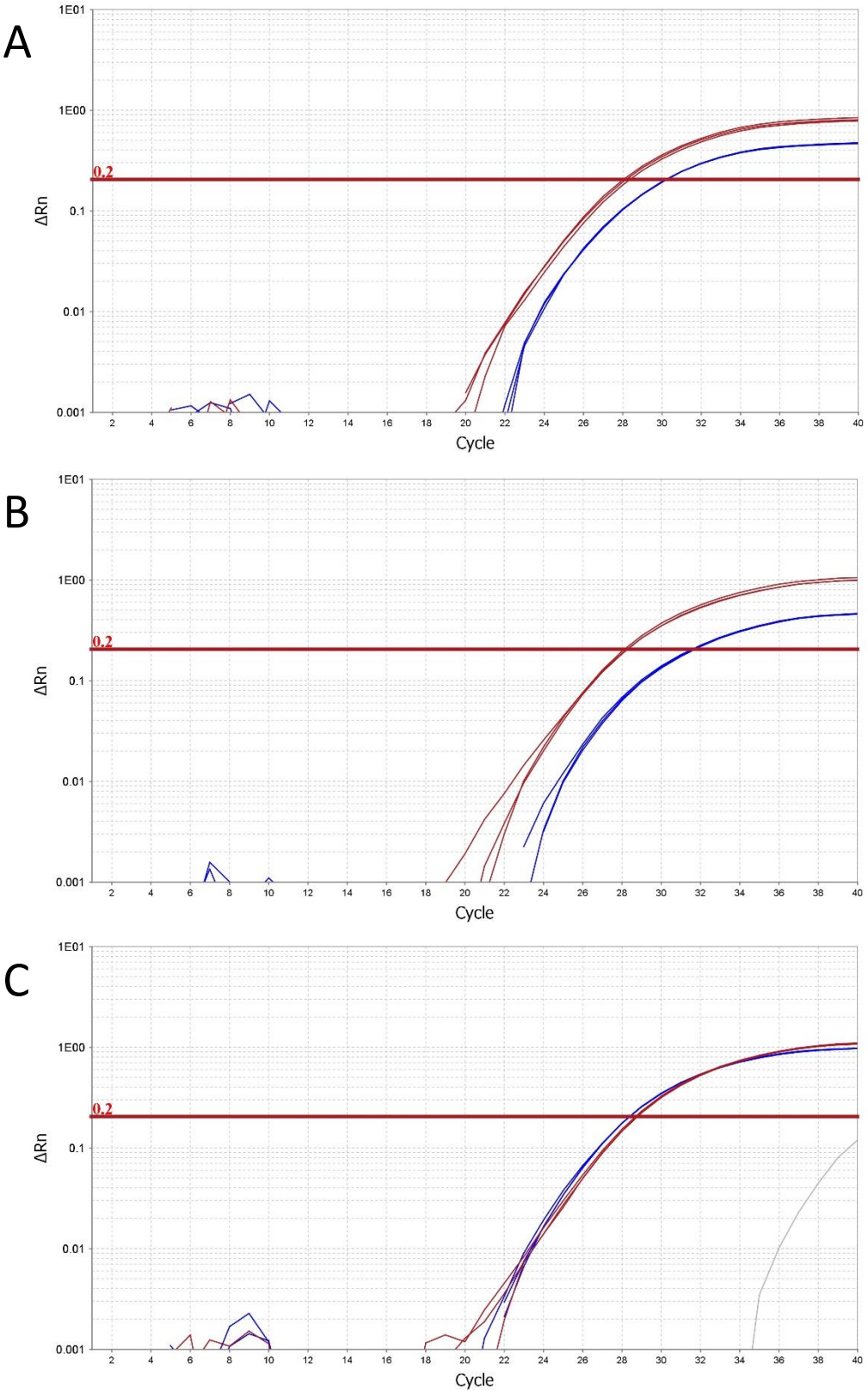
observed for *Cx. pipiens* f. *pipiens* and hybrid DNA samples (Appendix 1.6, panel C compared to A and B).

Appendix 1.4. Comparison of mean Cq values ± Standard deviation for each probe between singleplex and multiplex.

Probe	Sample	Mean Cq values (± Standard deviation)	
		Singleplex	**Multiplex**
***CxPip*P**	*Culex pipiens* f. *pipiens*	28.136 ± 0.081	31.502 ± 0.076
	Culex pipiens f. *molestus*	28.671 ± 0.061	28.303 ± 0.016
	Hybrid	28.107 ± 0.161	30.106 ± 0.028
***CxPipPip*P**	1	25.055 ± 0.018	25.525 ± 0.032
	2	23.161 ± 0.068	23.757 ± 0.046
***CxPipMol*P**	1	26.501 ± 0.134	26.711 ± 0.068
	2	27.298 ± 0.066	27.433 ± 0.146
***Cxtorr*P**	1	18.907 ± 0.155	19.589 ± 0.183
	2	18.982 ± 0.071	19.492 ± 0.113

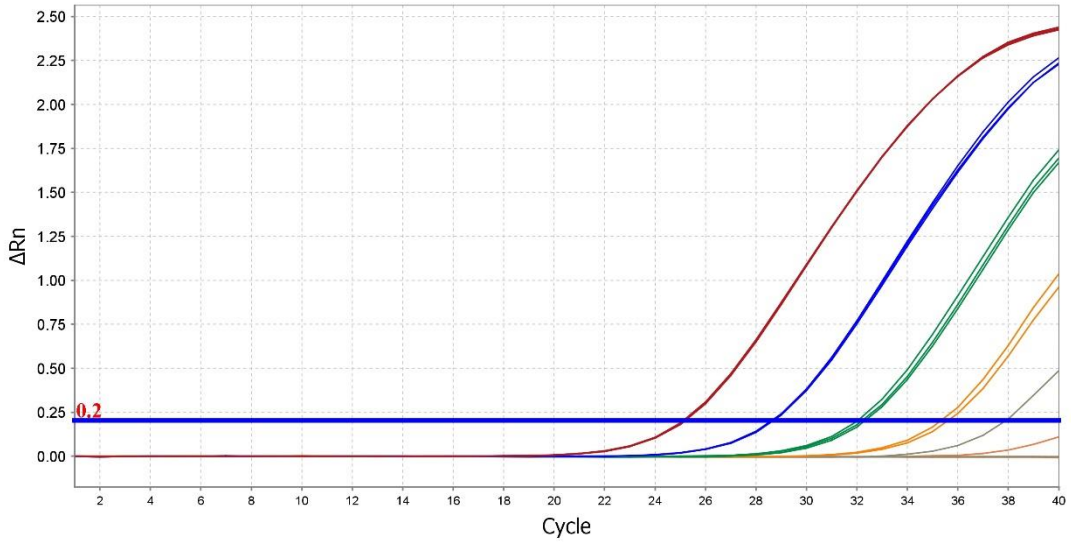


Appendix 1.5. Amplification curves of *CxPipPip*P (A), *CxPipMol*P (B) and *CxTorr*P (C) probes. Singleplex reactions are shown in red and multiplex reactions in blue.



Appendix 1.6. Amplification curves of *CxPip*P probe with *Culex pipiens* f. *pipiens* (A), hybrid (B) and *Culex pipiens* f. *molestus* (C) DNA template. Singleplex reaction in red and multiplex in blue.

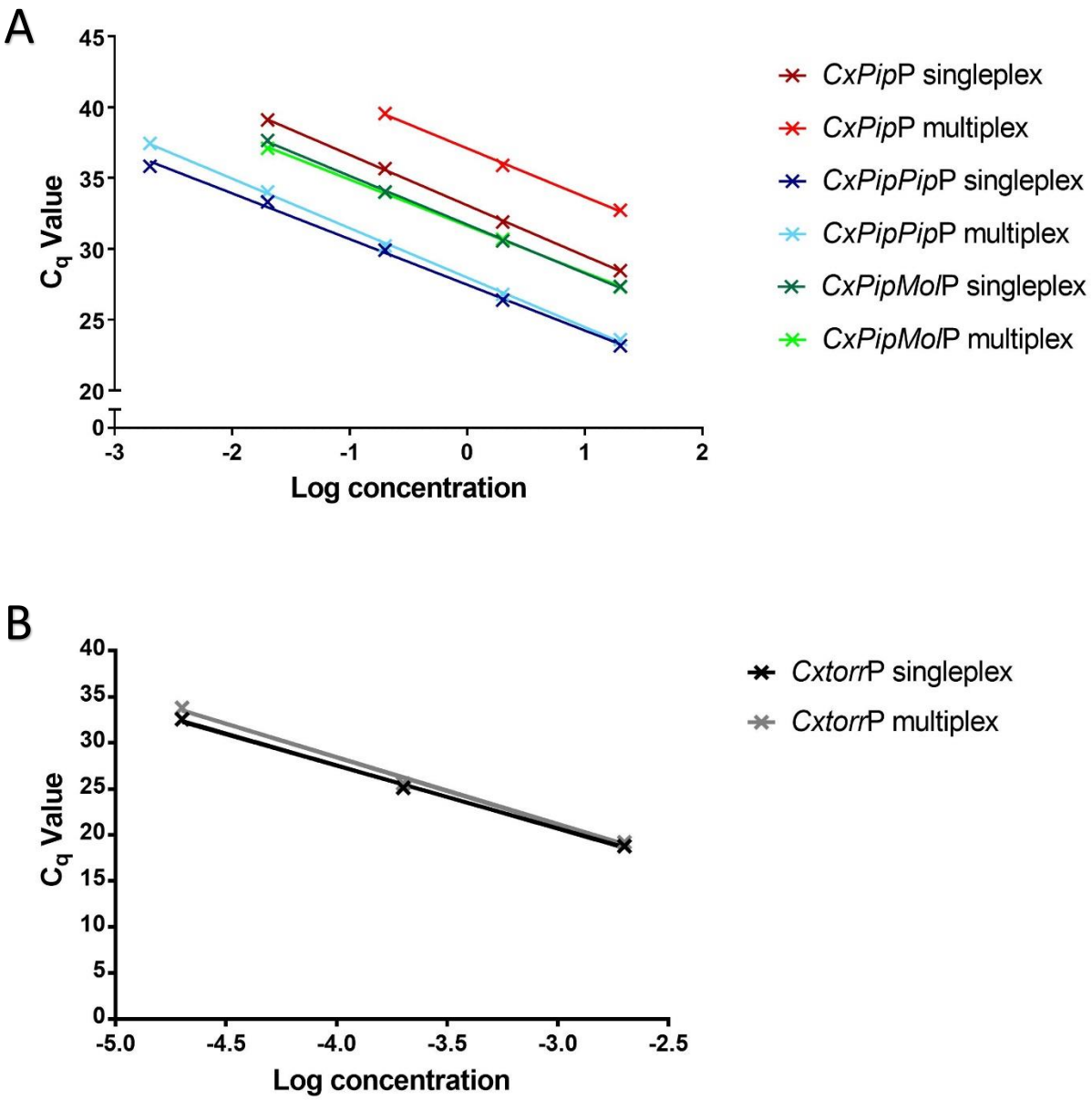
Standard curves were constructed and compared for both singleplex and multiplex reactions by performing 1 in 10 serial dilution of template DNA (starting concentration of 20 ng per reaction), for each primer/probe combination to determine the dynamic range (Appendix 1.7, amplification plot for *CxPipPip*P shown as an example) and efficiency of the assay (Appendix 1.8; Appendix 1.9). Dynamic range of the assay was shown to be 2 x 10^{-4} ng. Little or no variation between Cq values or ΔRN of singleplex and multiplex reactions were observed for probes *CxPipPip*P, *CxPipMol*P and *CxTorr*P however, Cq values for the *CxPip*P were found to increase and ΔRN values decreased when amplified in multiplex but were within acceptable limits. Furthermore, efficiency for all *Cx. pipiens* probes were acceptable for both singleplex and multiplex.



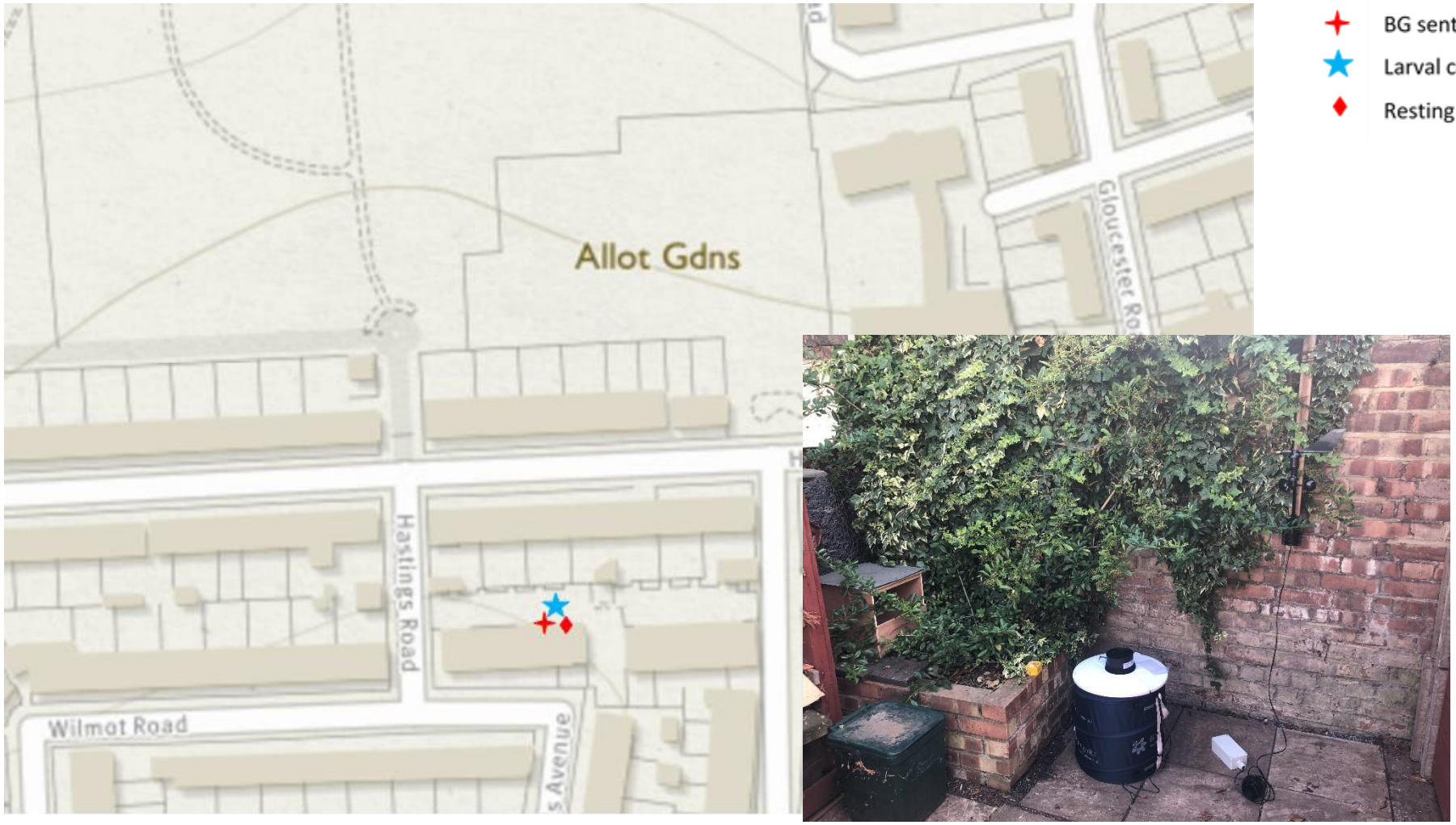
Appendix 1.7. Amplification plot showing the dynamic range for *CxPipPip*P probe for a 1 in 10 serial dilution of *Culex pipiens* f. *pipiens* DNA with a starting concentration of 20 ng.

Appendix 1.8. Slope gradient, R^2 values and PCR efficiency of all probes compared in singleplex and multiplex.

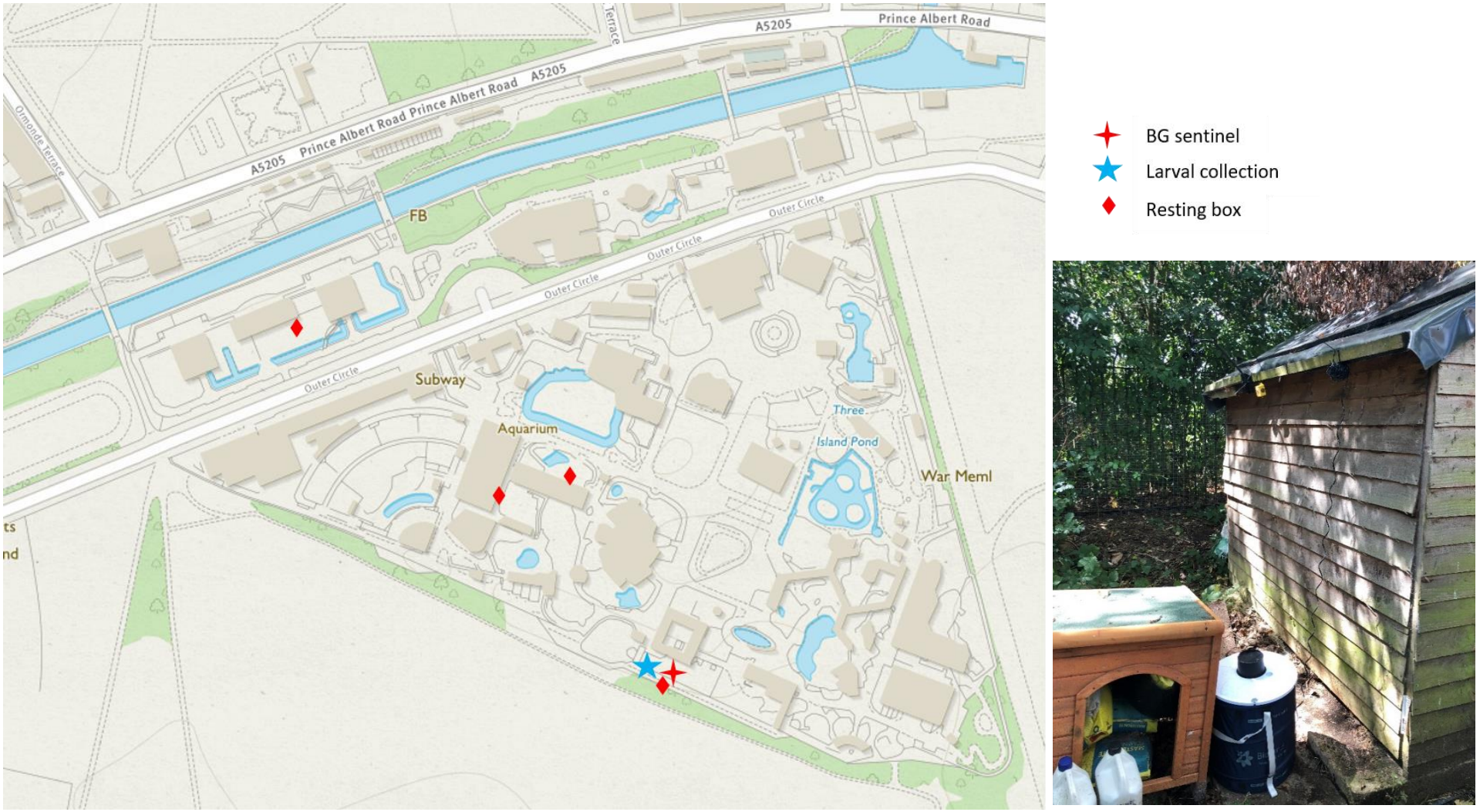
Probe	Single/multi	Gradient ± SE	R^2	Efficiency
***CxPip*P**	Singleplex	-3.571 ± 0.0455	0.9997	90.56
	Multiplex	-3.421 ± 0.1416	0.9983	96.03
***CxPipPip*P**	Singleplex	-3.230 ± 0.1022	0.9970	103.98
	Multiplex	-3.496 ± 0.0563	0.9992	93.22
***CxPipMol*P**	Singleplex	-3.439 ± 0.0667	0.9992	95.34
	Multiplex	-3.278 ± 0.0443	0.9996	101.87
***CxTorr*P**	Singleplex	-6.902 ± 0.3257	0.9978	39.60
	Multiplex	-7.309 ± 0.5303	0.9948	37.03



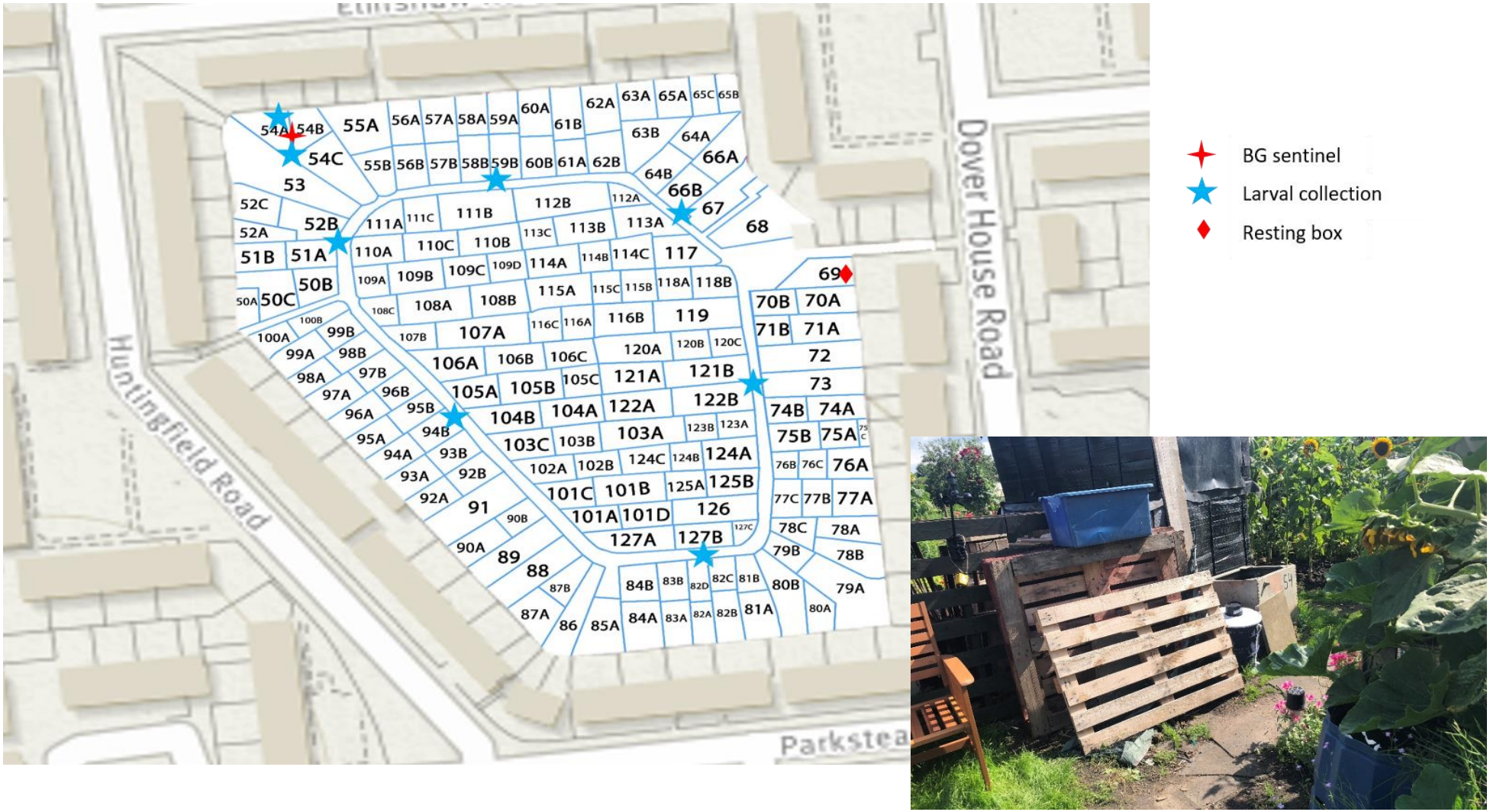
Appendix 1.9. Standard curve of 1 in 10 dilution series of each of the *Culex pipiens* probes (A) and *Culex torrentium* probes (B).



Appendix 2. Map of the garden site ("UR1") which was used for the survey of adult and larval *Culex pipiens* biotypes (Section 4.2.1) and assessment how environmental factors influence life history traits of *Culex pipiens* (Sections 4.2.2 and 4.2.3). Shapes indicate locations of BG sentinel traps, larval habitats and resting boxes.



Appendix 3. Map of the zoo site ("UR3") which was used for the survey of adult and larval *Culex pipiens* biotypes (Section 4.2.1). Shapes indicate locations of BG sentinel traps, larval habitats and resting boxes.



Appendix 4. Map of the allotment site ("UR4") which was used for the survey of adult and larval *Culex pipiens* biotypes (Section 4.2.1). Shapes indicate locations of BG sentinel traps, larval habitats and resting boxes.