The health and status of the feral honeybee (*Apis mellifera sp*) and *Apis mellifera mellifera* population of the UK

Catherine Eleanor Thompson

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

While declines in managed honeybee colonies are well documented, little is known about the health and status of feral honeybee populations. To date no studies have considered the wider pathogen burden in feral colonies, whether they represent a genetically distinct population, a remnant native population or a unique source of genetic resistance.

Chapter 2 investigates disease prevalence in managed and feral honeybee colonies. Deformed wing virus was shown to be 2.4 fold higher in feral than managed honeybees. Managed honeybee colonies not treated for *Varroa* showed similar levels of deformed wing virus to that of feral colonies. In the absence of managing the *Varroa* mite, feral populations are subject to potentially lethal levels of DWV. Such a finding provides evidence to explain the large decline in the feral population, and the importance of feral colonies as potential pathogen reservoirs is discussed.

Chapter 3 investigates the ecology, racial composition and survival of feral honeybee colonies. Over 47% of colonies were lost during the course of this study, confirming observations of large scale losses within the feral population. Only 12 colonies were seen to persist for 2.5 years, although the original queen swarmed or was replaced during this time. Feral colonies were shown to be genetically similar to local managed colonies, differing, albeit significantly, by only 2.3%. The implications for feral honeybee health are explored.

Feral colonies are highly introgressed and do not represent remnant populations of *A. m. mellifera*. Breeding efforts for the native bee are explored in Chapter 4 and 5. Honeybee colonies within breeding programs are shown to be of variable purity, but most successfully maintaining stocks at a higher level of *A. m. mellifera* than the background average from FERAs Random Apiary Survey samples. Methods to improve the success of breeding efforts and move the selection focus away from indices based wing morphometry are discussed.

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Chapter 1: Beekeeping in the UK, past and present

The honeybee

Apis mellifera, the honeybee belongs to the insect order Hymenoptera, which boasts over 100,000 species of sawflies, wasps, ants and bees (Weinstock et al. 2006). Most insects within the order Hymenoptera exhibit haplodiploid sex determination (males from unfertilized hapoid eggs and females from fertilized diploid eggs) which is thought to be a basis for the evolution and maintenance of eusociality (Weinstock et al. 2006). Hymenoptera diverged from Diptera and Lepidoptera over 300 million years ago to form the an ancient lineage of bees that evolved in tropical Eurasia and migrated north and west, reaching Europe at the end of the Pleistocene, 10,000 years ago (Weinstock et al. 2006).

The honeybee genus (*Apis L*.) is the most well recognised of all insects due to the component species services to agriculture, pollination and mankind (Kritsky 2010). This genus includes the giant honeybees (*Apis dorsata* and *Apis laboriosa)*, the dwarf honeybees (*Apis florae* and *Apis andreniformis), t*he eastern hive bees, (*Apis cerana*, *Apis nigrocinca*, *Apis koschevnikovi*, *Apis nuluensis)* and the western hive bees *Apis mellifera*, for which there are over 24 different races (Garnery & Solignac 1992).

A. mellifera can be grouped into four bio geographical branches: African (A), Oriental (O), Northern Mediterranean (C) and West European (M)(Jensen et al. 2005; Garnery et al. 1993). European honeybees (M-lineage) are thought to have survived the last glacial period in two refugia, one on the Iberian peninsula and one on the Balkan peninsula (C-lineage) (Jensen et al. 2005). After the glacial retraction 10,000 years ago the honeybees re-colonized Europe with the M-lineage (composed of *A. m. mellifera*) occupying north and west Europe and the C-lineage occupying central Europe (including *A. m. ligustica*, *A. m. carnica*, *A. m. cecropia* and others). Geographical barriers such as the Alps maintained the differentiation of subspecies (Jensen et al. 2005).

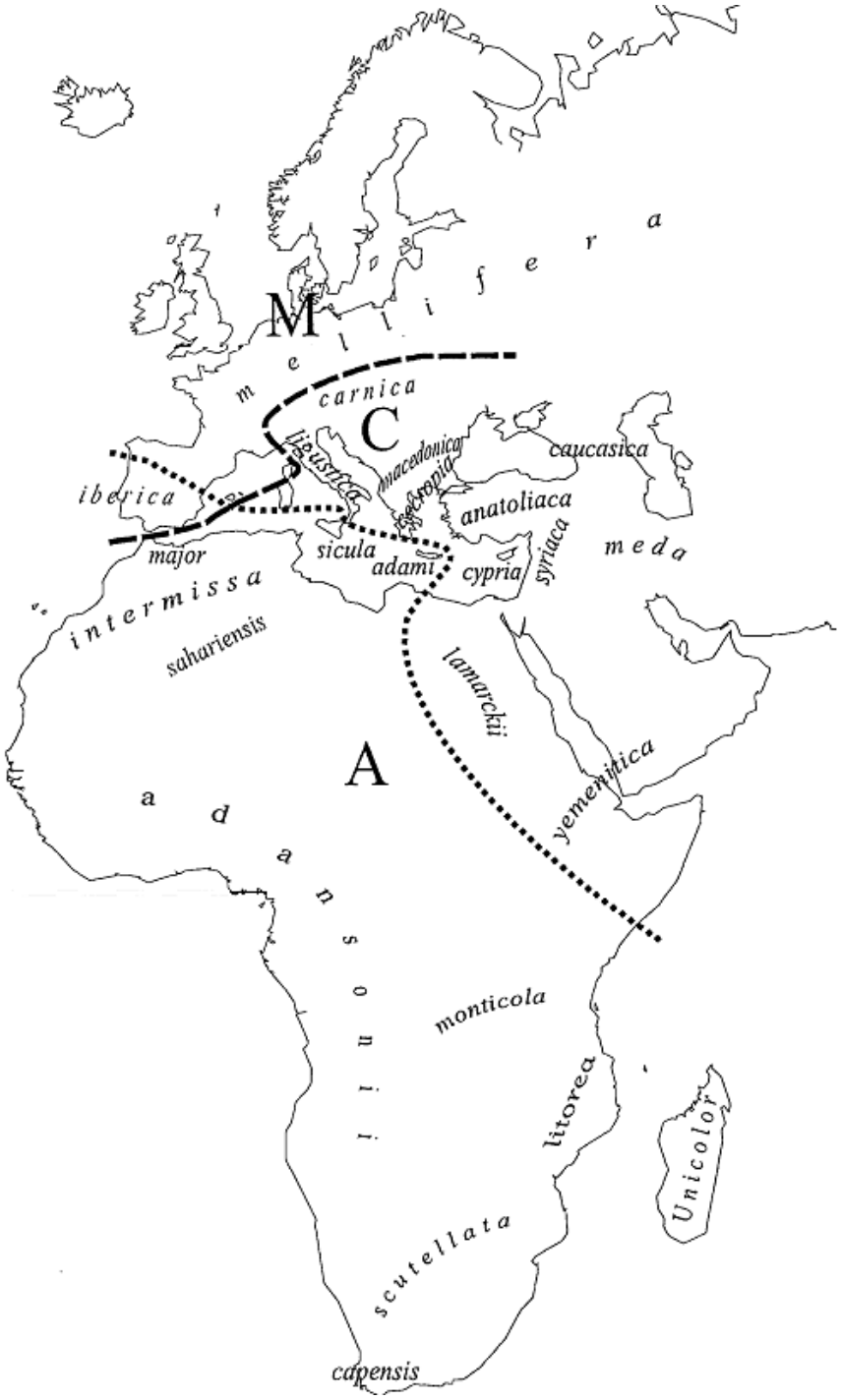


Figure 1: Honeybee subspecies adapted from Franck et al (1998)

Only *A. mellifera* is found in the UK, and there is evidence that the subspecies *A .m. mellifera* travelled into Britain across the European land bridge well before 8500BP (Prichard 2008; Carreck 2008). In fact it has been shown that the honeybee's range

was closely linked with hazel and lime distribution (Crane 1999). In 6500BP oak and hazel forests extended as far north as Skye in the west and Buchan in the east so as environmental conditions eased honeybees could have travelled with the advancing tree lines (Rackham 1998). Estimates by Prichard (2008) indicate wild honeybees could have reached Britain from remnant populations in France within 1100 years, if they were to swarm once every second year and travel a conservative 1.5km to their new colony site.

Once the land bridge was lost approximately 12,000 years ago (6500-6000 BC), the now 'British' honeybees would have continued to evolve independently. Analysis of British honeybee mitochondrial DNA reveals ancient queen lines that are similar to one another, but quite unique to Britain (Prichard 2008; Jensen et al. 2005).

Beekeeping

Beekeeping, annually contributes in the region of £120 billion to the world economy and £140 million in the UK (Gallai et al. 2009; POST 2010). Insect pollination, in which honeybees play a large part, benefits 75% of globally important crop species, and is a requirement for 35% of the world's crop production (Klein et al. 2007; Carreck & Williams 1998). Honeybees and their pollination services also benefit wild plants species and support wider ecosystems (Ollerton et al. 2011; Potts et al. 2010a; Biesmeijer et al. 2006). Unlike other European pollinators, honeybees also yield honey and other consumables such as wax and royal jelly (Van Engelsdorp & Meixner 2010).

Colony losses and the UK

There has been growing concern about the state of honeybee colony health, after large scale colony loss in the USA through Colony Collapse Disorder (CCD)(Van Engelsdorp et al. 2009; Van Engelsdorp et al. 2008). Although non *apis* pollinator species are seen to be declining, there is no current evidence for a global scale decline in the number of honeybee colonies or an immediate pollination crisis, although there has been a change in apicultural demography, with production becoming larger scale

and more business and cheap labour orientated (Potts et al. 2010; Aizen & Harder 2009; Aizen et al. 2008; Biesmeijer et al. 2006).

What is concerning globally, is that agriculture has become more pollinator dependent and in the future pollination demand could outstrip supply (Aizen et al. 2008). This may have serious consequences for feeding the growing human population, particularly in the developing world which has a 50% higher dependency on pollination than the developed world (Aizen et al. 2008; Brittain & Williams 2013; Calderone 2012). Native crop pollinators are also being lost at a faster rate in tropical regions, which may cause a detrimental cycle, of larger areas being converted to agricultural to compensate for reduced crop yield, thus placing increased pressure on remnant native pollinator habitats (Aizen & Harder 2009).

In the UK, rather than a marked reduction in honeybee colony numbers, media attention has caused resurgence in the hobby. However, new beekeepers face challenges for their hives from novel diseases, parasites, chemical contaminants in the hive, land use change, agricultural intensification and genetic homogenization (Potts et al. 2010a; Potts et al. 2010b; Abrol 2012).

Disease

Honeybees in the UK are have a range of diseases and parasites, some of which are novel like *Varroa*, and some of which act in combination with novel parasites to reduce colony health (Genersch 2010b).

Varroa

The *Varroa* mite (*Varroa destructor*) arrived in the UK in 1992 and is an ectoparasite which if left unchecked leads to colony death (Carreck et al. 2010). The *Varroa* mite causes direct negative effects by damaging developing honeybee larvae and pupae by sucking their hemolymph and reducing their hatching weight. Bees parasitized in this way usually begin foraging earlier and have a significantly reduced life span which may

be due to decreased learning abilities, impaired navigation ability and consequently a lower probability of returning to the colony (Rosenkranz et al., 2010).

Indirect effects of the *Varroa* mite are called Varroosis. This occurs when the *varroa* mite acts as a vector for viruses, most notably Kashmir bee virus (KBV), Slow paralysis virus (SPV), Acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV), and Deformed wing virus (DWV) (Chen et al. 2005). Indeed, honeybees can have multiple infections simultaneously, although it is not known what effect this has on the honeybees' physiology (Chen et al. 2005).

Doebler (2000) cites the arrival of *Varroa* mites as the 'biggest catastrophe to befall apiculture' (Doebler 2000). Indeed Varroosis is now considered to be the most destructive disease of honeybees worldwide (Boecking and Genersch, 2008, Rosenkranz et al., 2010) and the major cause of winter colony loss (de Miranda & Genersch 2010).

Other diseases

Nosema spp

After Varroosis, Nosemosis is one of the most prevalent adult honeybees diseases (Gisder & Genersch 2013). The microsporidian *Nosema apis* is correlated with reduced lifespan of individual bees, reduced performance of colonies, and increased winter mortality (Van Engelsdorp & Meixner 2010). In extreme cases it can even cause the death of colonies (Bailey 1981). In 2004 another Nosema species, *Nosema ceranae*, was found in the honeybee (Higes et al. 2006). *N. ceranae* doesn't exhibit the classic symptoms of *N. apis* such as crawling bees or dysentery but early research has found it to be more pathogenic (Higes et al. 2008).

Foul broods

There are two types of Foul Brood; American (AFB) and European (EFB). Both foul broods are a serious problem for beekeepers and are a notifiable disease in the UK, meaning beekeepers must report the infection to the local inspectorate at the National Bee Unit and treatment must be sought. AFB always leads to the destruction of the colony (Wilkins et al. 2007). Where European Foul brood (EFB) is detected, three potential avenues can be explored: 1) treatment of the colony with oxytetracycline (OTC; Terramycin), 2) a shook swarm method or 3) destruction of the colony (Wilkins et al., 2007).

EFB is caused by the bacterium *Melissococcus plutonius* (Forsgren 2010). EFB affects mainly unsealed brood, killing larvae when they are 4-5 days old, leaving a decomposing larva twisted around the wall of the cell (Forsgren 2010). If the comb is sealed affected larvae can be identified by sunken cell cappings (Forsgren 2010). Where a high proportion of cells are affected, the brood pattern appears patchy and gives off a foul odour giving the disease its name (Forsgren 2010). It is thought that outbreak of the disease may be due to colony stress (Forsgren 2010).

American foulbrood is caused by the spore forming bacterium *Paenibacillus larvae* (Genersch et al. 2006). AFB affects larvae in the early stages (between 12 and 36 hours after hatching) and bacteria colonise the midgut resulting in the breakdown of the larva into a 'brownish, semi-fluid, glue-like' state (Genersch 2010a). It is most easily identified by sunken cappings and a 'ropey mass' inside the cell. Spores are then distributed into the colony and are swallowed by the next host (Genersch 2010a). The spores are incredibly infectious and hardy, being able to retain infectiousness for up to 35 years (Genersch 2010a). It is for this reason that control demands the destruction of the colony.

Tracheal mites

Acarine is caused by the tracheal mite *Acarapis woodi* which infests the tracheal of adult honeybees, where it feeds on hemolymph and can act as a vector of viruses (Thompson & Wilkins 2013). It is thought to have been the cause of 'Isle of Wight disease' and the widespread colony losses in the early 1920s (Thompson & Wilkins 2013). Significant infestation by tracheal mites can lead to high levels of bee mortality, poor overwinter survival and individual bees may show symptoms of disorientation, dysentery and an inability to fly (Thompson & Wilkins 2013). In the UK, only low levels of acarine are seen, which may be due to the widespread use of miticides to control *Varroa* which also controls *Acarapsis woodi*.

Chemical contaminants in the hive

Honeybee colonies have been found to contain contaminants from two sources, the first being environmental and the second being apicultural. For this reason it is possible for honeybees to act as bio indicators of environmental health, however as yet it is unknown what effect the wide range of contaminants found in honey may have on the health of the colony.

Environmental

Industry and traffic produce toxic heavy metals, most significantly Lead (Pb) and Cadmium (Cd) which have been found in nectar and honey in colonies (Bogdanov 2006; Porrini et al. 2003). Little is known about the direct effect of heavy metals on the health of the honeybee colony, but assessing heavy metal concentrations in them is rapidly becoming an environmental assessment tool (Lazor et al. 2012). Recent research on solitary bees however, has shown that with increasing heavy metal concentration, there was a steady decrease in the number, diversity and abundance of solitary~~,~~ wild bees leading to a virtual absence of bees in the most polluted sites (Moroń et al. 2012).

Pesticides

There are a large number of pesticides in use in the environment and they have a myriad of effects on honeybees, both direct and indirect, depending on dose, and state of contact. Standard LD50 tests to assess safety of these products do not currently take into account potential sub-lethal effects (Desneux et al. 2007).

In honeybees pesticide application has been shown to; impair navigation, foraging and communication of the position of food resources within the hive, cause lack of co-ordination, bees to become preoccupied with self-cleaning, trembling and abdomen cleaning and foragers to fail to return to the colony (Desneux et al., 2007; Henry et al., 2012; Morandin & Winston, 2005; Thompson, 2003). Wu et al (2011) also noted reduced egg laying, early supersedure, increased queen cell rejection and reduced ovarian weight in queen bees (Wu et al. 2011). Decreased levels of house cleaning is also noted in honeybees and is of particular concern due to the high level of disease blighting some colonies (Thompson 2001).

Calls have been made to monitor sub-lethal effects of pesticides, but Thompson (2003) bemoans the lack of guidance available on the types of behavioural data which should be collected during laboratory, semi-field or field regulatory studies or how they should be interpreted as part of risk assessment (Thompson 2003). Morandin and Winston (2003) have gone some way to tackling this issue with the use of an 'artificial flower foraging array,' which provided encouraging results as a sensitive method for measuring sub lethal effects on bees treated with Imidacloprid. They argue that by altering flower design or tasks required to access a reward, artificial arrays could be modified to test for negative effects of pesticides on different aspects of foraging behaviour and on different types of bees' (Morandin & Winston 2005). Thompson (2007) argues however that it is important that ecological parameters measured from individuals as part of sub lethal effect trials, must be seen to have a detrimental effect at the colony level and it must be possible to replicate both in the field and in the laboratory (Thompson & Maus, 2007).

Herbicides, bactericides, fungicides

Herbicides, bactericides and fungicides have all been found in honey and pollen (Bogdanov 2006). Little recent literature exists on the effect of these compounds on honeybee health , but in Morton and Moffett's (1972) feeding trial certain herbicides were shown to vary widely in their toxicity and seriously reduce or eliminate brood production (Morton & Moffett 1972; Morton et al. 1972).

Individual studies of fungicides showed they had little effect on honeybees (Mayer & Lunded 1986), however when the combined effect of an azole fungicide and the insecticide deltamethrin was examined by Vandame and Belzunces (1998), a significant effect on honeybee thermoregulation was found (Vandame & Belzunces 1998). A major concern is how best to test the interplay between all the chemicals the honeybees are exposed to (Mullin et al. 2010; Chauzat et al. 2009). In a study of pollen samples in 2007 in the USA , 45 pesticides including toxic metabolites belonging to seven chemical classes of insecticides were found together with fungicides and herbicides (Mullin et al. 2010). In some cases fungicides have been shown to increase the already high toxicity of certain insecticides (Iwasa et al. 2004).

Apicultural contamination

Varroacides are an unsurprising source of contamination within honeybee hives as they are used long-term for *Varroa* control (Bogdanov 2006). Water soluble varroacides such as formic acid, oxalic acid and cymiazole can be found dissolved in honey but are not soluble in beeswax so do not accumulate (Wallner & Fries 2003) . Lipid soluble varroacides, however, such as bromopropylate, coumaphos and fluvalinate are stable and accumulate in colonies over time (Wallner & Fries 2003) . Wallner (1999) showed that bees from contaminated hives contain varroacides in the fat tissue of their bodies. The effect of these residues on honeybee health is not known (Wallner 1999). Wallner advocates the use of natural acaricides such as thymol and

organic acids such as oxalic and formic acid which do not leave significant residues if used properly (Bogdanov 2006; Wallner 1999).

Agricultural intensification

Agricultural intensification is most detrimental to solitary bee and *bombus* species as they rely on native vegetation for nesting habitat and local flower plants (Potts et al. 2010a; Gathmann & Tscharntke 2002). Honeybees are only effected by landscape context at a larger scale, as they are housed in hives and can forage at up to 10km away (Steffan-Dewenter et al. 2002) . The most important parameters for honeybees are insecticide use (see below) and agricultural intensification through monoculture , improved grassland, regular mowing and cutting and practices that result in fewer flowers (Batáry et al., 2010; Decourtye et al., 2010).

Vandame and Palacio (2010) studying the honeybee health in Latin America have not seen the colony collapse or catastrophic losses of adjacent USA. They suggest that a major cause of the apparent health of honeybees in Latin America is the low income agriculture that is practiced there (Vandame & Palacio 2010). It is characterised by a small heterogeneous field system on small farms in fragmented landscapes with low nitrogen and pesticide application (roughly half that of the US and Europe) (Vandame & Palacio 2010). One major factor is that honeybees found in Latin America are Africanized honeybees and have a naturally higher level of hygienic behaviour. Consequentially they have lower levels of *Varroa* and they never surpass the critical level (Vandame & Palacio 2010). However in Brazil where Africanized bees are also found, there has been expansion of crops for agrofuels and increased use of pesticides, and here large scale losses are becoming increasing common (Vandame & Palacio 2010). No colony collapse disorder style losses have been reported yet, but beekeepers do report an increase in the severity of *Nosema* and *Varroa*.

Monoculture and lack of pollen

Increasing intensity of agriculture can lead to a monoculture with a depauperate range of forage within flying distance of the honeybee colonies. Studies have shown that monocultures can lead to a deficiency in pollen nutrition for honeybee workers (Vandame & Palacio 2010). Bees normally select a mixed pollen diet, reducing the possibilities of vitamin, mineral or protein deficiencies, or of pollen toxin overload (Schmidt 1995). Mattila and Otis (2006) showed that colonies given a pollen supplement began producing workers earlier in spring, had a larger number of workers in April and May and consequently had an increased honey yield (Mattila & Otis 2006). Colonies with a reduced diversity of pollen do not get all the essential nutrients required for health (Brodschneider & Crailsheim 2010). Indeed poor nutrition can act as a stressor and consequently increase Nosema infection levels and possibly colony mortality (Fries 1993; Brodschneider & Crailsheim 2010). Wue et al (2011) found that honeybees fed on higher quality pollen were less susceptible to pesticide exposure than those on a poorer protein deficient pollen or pollen substitute diet (Wu et al. 2011). Alaux et al (2010) showed that increasing pollen diversity increased honeybee immunocompetence, showing a critical link between pollen diversity and honeybee health.

Genetic Homogenization

Honeybee queens practice polyandry and mate with multiple males when possible (Palmer & Oldroyd 2000). The larger the number of fathers the higher the fitness of the honeybee colony (Mattila & Seeley 2007; Tarpy & Seeley 2006; Tarpy 2003). For example recent research shows that *N. ceranae* infestation levels differ between paternal lineages, thus having multiple paternal lineages prevents possible catastrophic worker loss and colony mortality (Bourgeois et al. 2012). Matilla and Seeley (2007) also showed that colonies headed by queens mated by multiple drones had reduced disease intensity when inoculated with American Foul Brood. Eckholm et al (2011) showed that genetically diverse colonies collect more pollen than less diverse ones (Eckholm et al. 2010).

In the States, where large numbers of daughters were produced commercially from a small number of queen mothers, it has been proven that there is a marked reduction in genetic diversity (Delaney 2008). Delaney (2008) suggests that where the honeybee population relies on managed honeybee queens that have been bred in this way, the low genetic diversity may be having negative consequences for honeybee health. As well as reducing genetic diversity, inbreeding also alters the behaviour of the worker bees, producing symptoms such as inability to thermo regulate the nest, reduced recruitment activity to food sources, reduced hive cleaning and brood rearing and an increased sensitivity to parasites (Solignac 2005; Kraus 2005; Zayed 2009). Kraus (2005) found that inbred queens were also more aggressive.

Research by Dall'Olio et al (2007) in Italy has shown that intensive queen rearing from only a few mothers has eliminated genetic diversity there in native populations. Also, due to the ravages of *Varroa* there is a limited feral honeybee population to add genetic diversity (Delaney 2008). Solignac (2005) warns that unfortunately it is generally the rare alleles that are the first ones to disappear, compounding the significance of the loss.

The future for the honeybee

It appears that in modern beekeeping it is increasingly necessary to manage honeybees as farm animals rather than as a semi-domesticated species; i.e. treating them for pests and diseases, selectively breeding and importing specimens and enabling an increasing intensification of methods. However the honeybee has never really been considered wholly domesticated. This is due to its multiple mating breeding strategy at remote drone congregations, which is difficult to control and adapt. Artificial insemination and queen rearing are still not widespread in the smaller scale bee keeping of the UK. Indeed there is an increasing gulf between the more intensive methods of some honeybee farmers and the hobbyists, who instead of treading the path to total domesticity would prefer to see a return to 'old fashioned' beekeeping. This movement has been dubbed 'natural beekeeping' and has attracted a lot of support from new beekeepers prompted to take up the hobby as a result of media

reports of the decline of the honeybee and a future pollinator crisis (Doebler 2000; Chandler 2009).

The natural beekeeping movement

The natural beekeeping movement ranges from entirely let alone beekeepers (i.e those that neither open the hive, nor treat for *Varroa* or harvest honey), to those who want to have colonies from which to harvest honey, perhaps commercially, but who want to do this in an environmentally sensitive and sustainable manner (Chandler 2009).

Reduced or no Varroa treatment

Doebler (2000), states that we need a new beekeeper and not a new bee or mite. Indeed, Doebler (2000) insists that we must accept that *Varroa* is a continual problem that must be dealt with pragmatically and not simply endured. Some natural beekeepers use no or reduced *Varroa* treatment and where possible try to keep synthetic chemicals out of the hive and instead favour either formic, lactic or oxalic acid. Chandler (2009) states in his book 'The Barefoot Beekeeper' that he has no use for synthetic chemicals, relying instead upon creating the optimum conditions for the bees' survival and using natural medicine that causes least harm (Chandler 2009). This behaviour has been encouraged by a number of recent studies (see apicultural contamination above). Rosenkranz et al (2010) for example, warn that there is no *Varroa* treatment available which is both, safe, effective and easy to apply. However there is also no honeybee which is sustainably tolerant to Varroosis under temperate conditions (Peter Rosenkranz et al. 2010). Indeed, Le Conte et al (2007) report that untreated *Apis mellifera* colonies infested with *Varroa destructor* may survive for only one to two years in France, where queens could be expected to survive for up to 5 years in modern beekeeping (Le Conte et al. 2007).

Maintaining the native honeybee

Another shift in beekeeping practice in the UK is a call for a return to the native honeybee, the dark bee *A. m. mellifera,* instead of other *Apis mellifera* races. *A. m. mellifera* is under threat from introgression of foreign genes due to mass importation of *A. m. ligustica, A. m. carnica* and the use of modified strains such as the Buckfast bee (Rúa et al. 2009).

It is thought that the native bee should be better adapted to the British climate and local conditions (Jensen et al. 2005). Although unique local ecotypes of *A. m. mellifera* have been identified in France, little is known about the extent of the remaining pure British population or if particular ecotypes exist (Garnery et al. 1998)

The earliest recorded importation of foreign (Italian) honeybees is AD 1859 (Pritchard, 2008). Following the Isle of Wight epidemic honeybees of several races were imported in quantity into Britain, including *A. m. mellifera* from Holland and France. Importation of foreign races is now widespread, particularly *A. m. ligustica* from Italy*, A. m. carnica* from Austria, or synthetic strains such as the Buckfast bee (Lodesani & Costa 2003). Indeed, queens could be brought into the UK from as far a-field as Hawaii (FERA 2010). As queens mate in mid air at drone congregation areas with drones from colonies as far away as 10km it is very difficult to control interbreeding between different honeybee strains (Lodesani 2005; Solignac 2005). Whilst this may sound the death knell for the UK's dark bee a recent paper by Jensen et al (2005) shows that little introgression of foreign genes have occurred in some colonies (Jensen, Palmer, Boomsma, et al. 2005). The honeybee colonies sampled in Jensen et al's, (2005) study were all managed by enthusiasts of the native British bee. To maintain the purity of their bees these beekeepers use wing morphometry techniques to assess race, a practice first devised by Ruttner (Ruttner 1988).

Wing venation has evolved slowly, and without any apparent environmental influence so for this reason can be used to determine race and history of subspecies of

honeybees (De La Rua & Serrano 2005). DrawWing software is a more recent addition to the armoury, and uses geometric morphometrics, i.e. it automatically creates a series of landmarks on a wing to measure shape (Tofilski 2008). DrawWing has the ability to create a consistent wing diagram regardless of the orientation of the wings of a sample which allows a number of samples to be scanned and processed simultaneously, while older systems such as Beemorph require manual wing positioning and cropping, are more time consuming and prone to human error (Prichard 2006; Tofilski 2004). A study by Tofilski et al. (2008) showed that geometric morphometrics were marginally better at discriminating honeybee species than standard morphometry (90.6% and 86.3% respectively) (Tofilski 2008). DrawWing is used exclusively in this thesis for assigning individual bees to subspecies.

To maintain the purity of their *A. m. mellifera* stocks beekeepers may also use practices such as the use of remote mating apiaries and artificial insemination to ensure the purity of their honeybees (Lodesani & Costa 2003). Remote mating apiaries use geographically isolated locations such as Spurn Point (a narrow land spit that extends out 3 miles into the North Sea)(personal comment John Dews) and central Wales (personal comment Albert Knight) to maintain purity of their bees by limiting the chance of mating drones other than the *A. m. mellifera* drones they transport.

Why the native bee?

Compelling scientific evidence for the superiority of *A. m. mellifera* for UK beekeeping is hard to find. A direct comparison between *A. m. mellifera* and the Buckfast bee was carried out by Hillard in 1968. The most pronounced difference seemed to be the conservative nature of the Irish *A. m. mellifera* strain. They maintained stores in the brood chamber as the summer came to an end and reduced the brood area earlier (Hillard 1968). Imported queens often belong to other subspecies and are adapted to a very different environment, although there is no proof that this affects their productivity (De la Rúa et al. 2009; Solignac 2005). It is also thought that native honeybees may be better adapted to native parasites due to co-evolution of parasite

and host, but again this hypothesis has yet to be tested (De la Rúa et al. 2009; Brown & Paxton 2009). Solignac (2005) notes that the observed proportion of foreign genetic markers in populations is often lower than the expected frequency given the rate of importation and this is possibly due to the low success rate of imported genes, although Solignac (2005) does counter that low levels of importation can benefit local populations by the introgression of adaptive genes (Solignac 2005).

It is where importation is widespread and continual that gene flow can destroy the genetic architecture of the local race (Solignac 2005; Dall'Olio et al. 2007; Garnery et al. 1998). Indeed, high polymorphism is not always synonymous with higher performance, which depends instead upon the race itself and the desirable characteristics favoured by the beekeeper (Solignac 2005; Zayed & Whitfield 2008) .

The native British bee has been found to be genetically distinct from other European populations and may as such be a worthy subject for conservation (Jensen, Palmer, Boomsma, et al. 2005; Prichard 2008). Jensen et al. (2005) believe the honeybee's high evolutionary potential for local adaption could provide a valuable gene pool for controlled breeding programs selecting for resistance against honeybee diseases. Thus it is in the beekeepers interest to maintain the widest possible genetic diversity for future adaptation.

An example of local adaptation is the Landes ecotype of *A. m. mellifera* in France for example, which has been shown to produce more brood to emerge at times of local flowering of heather. Louveaux (1966) crossed bees of the Landes ecotype with non ecotypic bees and found that intermediate brood cycles could be produced, indicating that the cycle was genetically determined (Louveaux et al. 1966; Louveaux 1973; Strange et al. 2007b). Louveaux (1973) identified three other ecotypes within France, each having a distinct brood cycle and adaptation to the local floral phenology (Strange et al. 2007a). However these ecotypes are now under threat from genetic introgression from imported honeybee strains (Strange et al. 2007b; Lodesani & Costa

2003). Strange et al (2007) laments the lack of research on local ecotypes and absence of provision for their conservation. In this respect the UK lags behind the rest of Europe (see table 1).

Table 1: Overseas *Apis m. mellifera* conservation projects

Country	Conservation
Sweden	Projekt NordBi 1990 http://www.nordbi.org/ Has two mating stations: Lurö in Lake Vänern and Hästliden close to Umeå. In Jämtland there are several safe mating apiaries in the north of the county. Some artificial insemination is used as well. Every strain used in the breeding program is controlled by cubital index, discoidal angle and mtDNA. Every year they attempt to measure the wings of all promising colonies.
Norway	In the area around Flekkefjord in southern Norway, bees other than *A. m. mellifera* are banned (Jensen et al. 2005).
Denmark	Whilst banning other strains of honeybee has worked in Norway, it has largely failed in Denmark. Although *A. m. mellifera* is officially protected on the island of Læsø there is widespread and increasing local opposition and hybridization with illegal bees. This is unfortunate as the Island of Læsø could be an ideal conservation site being too distant from the mainland for foreign drones to fly (De la Rúa et al., 2009).
Finland	Breeding program on remote islands.
La Palma, Canary Islands	Selection and protection of *Apis m. mellifera* began in 1996. Its first step was to characterize the insular honeybee populations through molecular data (De la Rúa et al., 2009). Regional laws established to control the conservation, recuperation and selection of the Canary black honeybee in 2001(De la Rúa et al., 2009). A natural mating area was established and saturated with

	black drones.
Germany	*A. m. mellifera* is considered to be extinct (Jensen, Palmer, Boomsma, et al. 2005).
Switzerland	There is the association of Swiss Mellifera Bee Buddies that was founded in 1993 to breed the native dark bee. In 2008 honeybees were protected by agricultural law. The association promotes the breeding, as well as the protection of the dark bee. To this end it maintains one of the most modern breeding programs in Europe coupled with the establishment of protected areas. There are six isolated mating yards, with one where all the bees are genetically tested for purity. There are also 23 local mating apiaries with anonymous testing. http://www.mellifera.ch/. Recent analyses of the genetic data shown a high proportion of hybrids in some areas however, suggesting that different management techniques should be more routinely checked using genetic methods (De la Rúa et al., 2009).

The Rio Biodiversity Convention for the conservation of biological diversity exists to protect genetic diversity and ecotypes yet *A. m. mellifera* is not included on the FAO's World Watch list for domesticated animals (Jensen et al. 2005). There is some hope within European legislation, however, as EU regulations have a directive for 'organic beekeeping', which is based on using indigenous honeybees rather than imported stock (Lodesani 2005). It states that a wide biological diversity should be encouraged and the choice of breeds should take account of their capacity to adopt to local conditions (Lodesani 2005). De la Rúa et al. (2009) advise that making this compulsory would be the best way to offer local ecotypes legal protection. Unfortunately attempts to formally protect current stocks of *A. m. mellifera* in Colonsay and Northern England have met with substantial opposition from UK conservation charities due to the perceived threat of competition with other species of native bees.

This thesis

The incidence of CCD in the USA has highlighted a global need to assess honeybee health, examine stressors and research a path to mitigate humanities negative impacts.

Chapter 1

As discussed in the Disease section above, there are a number of parasites, bacteria and fungi that can cause significant mortality to the honeybee. As very little research has been carried out on the feral honeybee population in the UK it will be important to examine:

A) How disease levels compare to the managed honeybee populations?
B) Whether feral honeybee colonies are a potential source of contamination for local managed hives, or a natural reservoir of evolving resistance?

This research is timely as it will be carried out in conjunction with the Food and Environment Research Agencies large scale project looking at race and disease levels within managed colonies.

Chapter 2

Chandler (2009) proposes setting up a network of 'conservation hives' that are totally untouched by man, to allow bees to evolve a natural resistance to disease and offer genetic diversity to managed hives. To some degree these honeybee colonies already exist in the UK population, in the form of feral or unmanaged hives. Despite reports of feral honeybee colonies being extinct, some colonies are still found (De la Rúa et al. 2009).

This thesis hopes to use feral honeybee colonies as a model to examine the potential success of natural beekeeping. Feral or unmanaged honeybees are free mating, not

treated for *Varroa* or exposed to beekeeper management. Where they are able to survive, they should be open to natural selection. Can a viable feral honeybee population, exposed to natural selection create a strain of bees resistant to viruses of managed bees? To this end, it is asked:

A) Are feral colonies simply an annual cast off from the beekeeping community or are they genetically distinct and evolved?

B) How long do feral colonies survive?

C) Are feral colonies more closely related to the native genotype *A. m. mellifera*?.

Chapter 3

As discussed in section 'Honeybee races and British honeybees', morphometry is the most accessible and widely used tool available for beekeepers. The initial morphometry work prescribed by Ruttner in 1988, involved laborious examination of 42 physiological and behavioural characters. Since then beekeepers have begun to focus primarily on wing morphometry as software developments have allowed a quick and automated system for measuring parameters from multiple scanned wing images (Tofilski 2004). The stripping back of the morphometry technique to a smaller number of characteristics has caused concern, especially where wing morphometry results are being used to select colonies for breeding programs. In this chapter the results of the most popular wing morphometry method (using scanned images in DrawWing software (Tofilski 2004)) are compared with the most recent microsatellite based assessment of race purity.

A) Do wing morphometry assessments of race compare with microsatellite assessments of purity?
B) Is morphometry an appropriate tool for use by beekeepers in breeding programs?

Chapter 4

Honeybee race is increasingly being explored, as it is thought that local ecotypes may be better adapted to local environmental and disease conditions(Costa et al. 2012). Unfortunately, local ecotypes and native honeybee races are being lost due to widespread importation of other races. In this chapter, the success of breeding programs focused on maintaining the purity of *A. m. mellifera* are examined.

A) How pure are our current stocks of *A. m. mellifera*?
B) Is there any significant effect of location on purity levels?
C) Do island populations of *A. m. mellifera* show evidence of inbreeding?

It is hoped that this research will contribute towards our understanding of the UK's honeybee populations health and status, but also allow beekeepers who would like to manage their colonies in a pollinator friendly way to make informed decisions based on scientific fact. The will to maintain genetic diversity, support the local bee and protect honeybee populations is very strong, and it is imperative that this positive attitude is carried forward as decisive action in years to come.

Chapter 2: Pathogen burdens on feral honeybees (*Apis mellifera sp*.)

Introduction

Feral colonies of honeybees are of particular interest to researchers as they are not subject to normal beekeeping husbandry practices, such as supplementary feeding, selective breeding or routine health screening (Doebler, 2000, Buchler et al., 2010). Most importantly feral colonies are not subject to any management to control the population of the ectoparasitic mite *Varroa destructor* (Rosenkranz et al., 2010, Genersch, 2010). As such, the UK population of feral colonies was thought to be decimated by the arrival of the *Varroa* mite in honeybee hives in 1992 and numerous anecdotal reports exist of the reduced feral honeybee population (e.g. Martin et al. 2012; Carreck et al. 2002).

Varroa has both direct and indirect impacts on honeybee health. The mite causes direct damage to the developing honeybee larvae and pupae by sucking their hemolymph and reducing their hatching weight (Rosenkranz et al. 2010). Bees parasitized in this way usually begin foraging earlier and have a significantly reduced life span which may be due to decreased learning abilities, impaired ability to navigate and consequently a lower probability of returning to the colony (Rosenkranz et al., 2010).

Indirect effects of *V. destructor* are termed Varroosis, whereby the *Varroa* mite acts as a vector for a variety of honeybee viruses, most notably Kashmir bee virus (KBV), slow paralysis virus (SPV), acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV), and deformed wing virus (DWV) (Boecking & Genersch 2008). Before the occurrence of *Varroa* mites, bee viruses were generally considered a minor problem to honeybee health (Rosenkranz et al., 2010). Recently, however, de Miranda and Genersch (2010) found DWV and ABPV to be significantly related to German winter colony loss, while Highfield et al (2009) attributed 67% of overwintering colony loss in Devonshire to DWV (Highfield et al. 2009). Indeed Varroosis is now considered to be the most destructive disease of honeybees worldwide (Boecking and Genersch, 2008,

Rosenkranz et al., 2010) and a major cause of winter colony loss (Genersch et al. 2010; Miranda & Genersch 2010).

The most impactful virus associated with *Varroa* is DWV, which is thought to have caused the loss of millions of honeybee colonies across the world (Martin et al. 2012). Colonies with high levels of DWV show evidence of a scattered brood nest, crippled bees, loss of coordinated social behaviour such as hygienic behaviour, queen attendance and rapid decline in the colony's bee population (de Miranda & Genersch 2010; Rosenkranz et al. 2010). Worker bees with crippled wings cannot fly and are ejected from the colony (Fries & Camazine 2001; Boecking & Genersch 2008). DWV is the most commonly found virus in *Varroa* infested managed honeybee colonies in Europe and the level of DWV transmitting mites is strongly negatively related to colony survival (de Miranda & Genersch 2010). Martin et al (2002) showed that the introduction of 15 DWV transmitting mites into a colony could cause a colony to collapse in only one year (Martin et al 2002). Unfortunately, it is impossible for the beekeeper to assess the level of DWV transmitting mites in a colony and a colony can appear healthy with a low level of *Varroa* mites but can have a high proportion of DWV transmitting mites that ultimately may lead to colony collapse (Martin et al 2002). Recent research by Schöning et al. (2012), suggests that even the honeybee cannot detect the presence of mites able to transmit a fatal DWV infection. Hygienic behaviour performed by the worker bees, where diseased brood is detected and removed, was found to be triggered by damage to the pupae and not the presence of the mite (Schöning et al. 2012).

It has been suggested that sufficient time has passed since the first exposure to *Varroa* mite infestation to allow selection pressure to act on bee populations, and that 'wild' honeybee populations are starting to rebound' (Doebler 2000). Indeed, shorter term selective breeding of managed colonies for '*Varroa* resistance' has been shown to lower *Varroa* numbers in some colonies (Le Conte et al. 2007). If found to be coping with Varroosis in the absence of active management, feral honeybee colonies could present important genetic stocks to improve managed honeybee breeding systems.

European honey bees living in the Arnot Forest in New York State were shown to be at the same density as in 1978, pre *Varroa*, and there have been reports of some populations of European honeybee races surviving for long periods without *Varroa* treatment (De Jong & Soares 1997; Kefuss et al. 2004; Fries et al. 2006; Thomas D Seeley 2007). Breeding programs have tried to take advantage of *Varroa* surviving populations by selecting mite tolerance, often called the *Varroa* sensitive hygiene trait (Tarpy et al. 2007; Danka et al. 2011; Büchler et al. 2010). The selection of this trait led to experiments using the Bond test ("Live and let Die!") in France and Sweden, where colonies were left untreated (Locke & Fries 2011; Büchler et al. 2010). The surviving colonies are then subsequently selected for honey production to attempt to create a race that is both *Varroa* tolerant and economically attractive (Büchler et al. 2010).

Some have suggested that feral honeybee colonies could present a risk to the managed population, harbouring disease agents and re-infecting managed stocks (Taylor & Goodwin 2001; Ratnieks & Nowakowski 1989; Taylor et al. 2007). For example, Ratnieks and Nowakowski (1989) showed that feral colonies occupy abandoned cavities infected with AFB, meaning that unmanaged colonies have the potential to spread spores to bees within the local environment (Goodwin et al. 1994). However, studies of comparative disease screening between feral and managed colonies are rare except where feral colonies have become the focus of eradication programs. In New Zealand for example, beekeepers were increasingly concerned about the arrival of the *Varroa* mite in South Island and proposed the removal of all feral colonies (Somerville et al. 2008; Taylor & Goodwin 2001; Goodwin 2004; Goodwin & Van Eaton 2001). The cull of feral colonies was later abandoned due to impracticality, and the fact that feral colonies exposed to novel *Varroa* infestation were thought to die within 18 months, thus reducing their potential impact on managed colonies (Doebler 2000; Le Conte et al. 2007).

Controlling communicable disease in managed honeybee populations is a challenge, given honeybees can move disease agents over great distances. Adult bees can be used to infer the infection state of a colony (Budge et al., 2010), theoretically allowing the disease state of a feral nest to be determined without the need for a destructive sample of brood. However, these methods have not been employed to estimate the pathogen burden of this important section of the pollinator community, and the pathogen burden on feral honeybee colonies is currently not known. This study presents the first evidence of pathogen burden in feral honeybees, compared to paired samples from local managed honeybee colonies.

Methodology

Site selection

Feral honey bee colonies were located by engaging the beekeeping community and the general public using several methods; (1) emails to beekeeping associations, both the main British Beekeeping secretary, but also secretaries of regional beekeeping associations; (2) notes on applicable internet forums such the natural beekeeping forum; and (3) an article in Beecraft, a popular monthly beekeeping magazine (Thompson, Budge & Biesmeijer 2010). Respondents were able to report their colony by email, letter, or using a bespoke website (www.honeybeeproject.co.uk).

Locations of feral colonies were selected based on a good history of activity at the nest site (1 year minimum) thus avoiding the inclusion of new swarms with no history of survival. Sites that were impossible to reach, being too high to be accessed safely were not selected. Colonies were limited to those within England due to financial and time constraints of visiting each site twice a year during the course of the study. From 100 reports, over 60 were visited and 34 feral colonies were selected. The managed apiary nearest the feral site was identified using a national beekeeping register called BeeBase (see www.nationalbeeunit.com) and the beekeeper was contacted by the

National Bee Unit to obtain permission for their inclusion in the study to create a pair. A paired managed colony was identified for each feral nest site and samples of adult honeybees collected from each pair of colonies on the same day in the Spring 2009 (feral sites = 34, managed sites = 34, total colonies screened = 68).

Each beekeeper that submitted a managed colony sample was asked to fill out a short questionnaire detailing *Varroa* management strategies. Varroa treatment was grouped into four categories: no treatment, standard treatment (i.e dosing with Varroacide one to two times per year), intensive (i.e. use of a Varroacide twice or more per year as well as other biotechnical controls such as comb trapping and drone brood removal), and unknown (with respect to *Varroa* treatment).

Colonies were screened for the presence of deformed wing virus (DWV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), slow paralysis virus (SPV), sacbrood virus (SBV), *Acarapis* spp., *Nosema apis*, *Nosema ceranae*, *Paenibacillus larvae* and *Melissococcus plutonius* using Real Time-PCR.

Other viruses thought to be transmitted by *V. destructor* include KBV, SPV, ABPV, and IAPV. Of these 4 only SPV was screened, due to the low prevalence across England and Wales (Budge, unpublished data).

Nucleic acid extraction from individual bees

Foraging *A. mellifera* adults were collected from each colony and stored for use in 100% ethanol at -70^{o}C. Twenty-four bees from each of the 34 paired colonies were selected. Whole bees were washed in molecular grade water, and individually disrupted with 2.3 mm silica beads in a Precellys lysis and homogenization bead beater at 5000 rpm for 30 seconds. Total DNA was extracted from each worker bee using a 10% Chelex solution with TE buffer. After disruption, 800 µl of 10% Chelex solution was

added to each crushed bee residue. The solution was heated to 95 ^{o}C for 5 minutes then centrifuged at 8000g for a further 5 minutes. We removed 200 µl of the upper aqueous DNA extract and centrifuged this again at 13,000 rpm for 5 minutes and removed 150 µl of the upper aqueous DNA. Finally, 20µl of extract from each individual bee was pooled with per colony (Highfield et al., 2009).

Purification of colony extracts

In total, 300 µl of the above DNA extract was added to 300 µl of 24:1 chloroform:IAA solution and the mixture spun at 8000g for 10 minutes. RNA was recovered by adding 100 µl of the upper aqueous layer to an equal volume of 4M LiCl. Samples were mixed well and left overnight. For DNA 100 µl of the upper aqueous layer was transferred into a fresh tube containing 50 µl of 5M NaCl and 100 µl isopropanol. For both DNA and RNA, each colony sample was vortexed and centrifuged for 10 minutes at 8000g. The aqueous layer was decanted and the nucleic acid pellet washed with 500 µl of 70% ethanol prior to a final spin for 4 mins at 8000 g. The ethanol was decanted and the pellet dried in a heated vacuum for 5 minutes at medium heat. Dried pellets were re-suspended in 150 µl of 1 x TE buffer and frozen at -20 ^{o}C until required.

Real time PCR analyses

PCRs were performed in 25 µl volumes, containing 7.25 µl of molecular grade water, 2.5 µl of buffer (Buffer A), 5.5 µl MgCl (25nM), 2 µl dNTP , 1 µl of forward and reverse primers, 0.5 µl of probe, 0.125 µl Taq polymerase, 0.125 µl MMLV and 5 µl DNA extract. All Taqman™ probes were covalently labelled with a reported dye (FAM) at the 5’ end and with a quencher dye (TAMRA) at the 3’ end (Table 2.1). Samples were run in triplicate reactions with positive and negative controls.

Reactions were run on an ABI Prism 7900HT (Applied Biosystems) with real-time data collection. Reverse transcription was performed at 48^{o}C for 30 minutes, followed by denaturing and enzyme activation at 95 ^{o}C for 10 minutes. This was followed by 40

cycles of denaturing at 95 ^{o}C for 15 seconds and a combined annealing and extension step for 60 seconds at 60 ^{o}C. Fluorescence values, amplification plots and threshold cycle (C_t) values were calculated using SDS 2.2 (Applied Biosystems).

Table 2.1. Primers used in this study. The 5′-terminal reporter dye for each *TaqMan®* probe was 6-carboxyfluorescin (FAM) and the 3′ quencher was tetra methylcarboxyrhodamine (TAMRA) or Minor groove binding (MGB) as indicated.

Target	Primer name	Sequence (5'-3')
Acarapis spp.*	*Acarapis* F1	GCCATAAGACATCACTCGACTATTCT
	Acarapis R1	TCATTTAAACTTCATGATACTCTCAATCA
	Acarapis T	TGCGCAATGCAACTAGTCCTCTAAAGAC
BQCV[1]	BQCV8195F	GGTGCGGGAGATGATATGGA
	BQCV8265R	GCCGTCTGAGATGCATGAATAC
	BQCV8217T	TTTCCATCTTTATCGGTACGCCGCC
CBPV[1]	CBPV304F	TCTGGCTCTGTCTTCGCAAA
	CBPV371R	GATACCGTCGTCACCCTCATG
	CBPV325T	TGCCCACCAATAGTTGGCAGTCTGC
DWV[1]	DWV958F	CCT GGACAAGGTCTCGGTAGA A
	DWV 9711R	ATTCAGGACCCCACCCAAAT
	DWV 9627T	CATGCTCGAGGATTGGGTCGTCGT
EF1[2]	EF 1 F	CTGGTACCTCTCAGGCTGATTGT
	EF 1 R	GCATGCTCACGAGTTTGTCCATTCT
	EF1 T	TGCTTCGAACTCTCTCCAGTACCAGCAG
*N. apis**	*N apis* F1	ATTTACACACCAGGTTGATTCTGC
	N apis R1	TGAGCAGTCCATCTTTCAGTACATAGT
	N apis MGB	TGACGTAGACGCTATTC
*N. ceranae**	*Nosema* c1 836F	TTG AGA GAA CGG TTT TTT GTT TGA G
	Nosema c1 974R	TTC CTA CAC TGA TTG TGT CTG TCT
	Nosema c1 865T	ATA ATA GTG GTG CAT GGC CGT TTT
M. plutonius[3]	EFB FOR	TGT TGT TAG AGA AGA ATA GGG GAA
	EFB rev2	CGT GGC TTT CTG GTT AGA
	EFB Probe	AGA GTA ACT GTT TTC CTC GTG ACG
*P. larvae**	Pl_R24_468F	TCCCCGAGCCTTACCTTTGT
	Pl_R24_538R	ACCTACGAACTTGACGCTGTCCT
	Pl_R24_489T	TGCTCATACCCGGTCAGGGATTCGA
SPV major[4]	SPV 8383F	TGATTGGACTCGGCTTGCTA
	SPV 8456R	CAAAATTTGCATAATCCCCAGTT
	SPV 8407T	CCTGCATGAGGTGGGAGACAACATTG

* Recently developed at FERA. In prep.

1 - Chantawannakul et al. 2006

2 – Martin et al 2012

3 – Budge et al 2010

4. - Miranda 2010

Quantification of PCR results

The relative amount of DWV, BQCV, *N. apis* and *N. ceranae* were analyzed using the comparative C_t method (Schmittgen and Livak, 2008) using the pathogen assay as the target and Elongation Factor 1 (EF1) as the reference assay. A known positive sample was diluted 1:10 through 6 levels and the PCR efficiencies between the target and reference were deemed to be equivalent (Table 2.2).

Table 2.2 PCR efficiencies by reaction for each disease or parasite

Reaction	**PCR efficiencies (%)**
DWV	92
BQCV	107
N. apis	104
N. ceranae	107
EF1 (reference)	96

Statistical analysis:

The data were analysed by Restricted Maximum Likelihood (REML) to account for the paired structure of the data. The pairs were therefore included in the model as a random effect whilst the treatment of interest (managed vs feral colonies) was included as a fixed effect. Further, the data were log-transformed prior to analysis to correct for right skew. All analyses were completed using GenStat 14.1 ((VSNInternational 2011).

Results

Paired samples

Over 100 responses were received from which 34 paired samples were selected across England, from as far north as the Scottish border and as far south as Cornwall (see Figure 2.1).

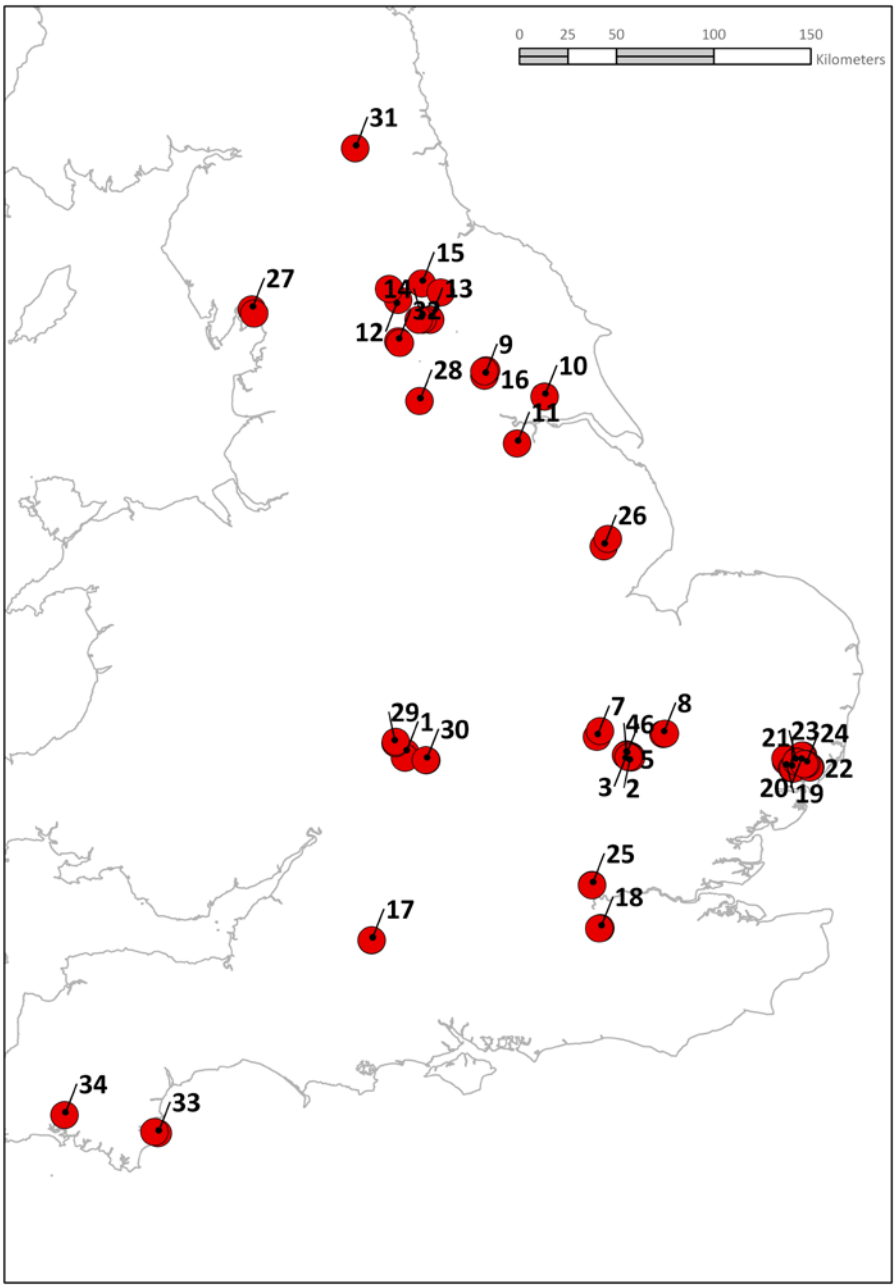


Figure 2.1: Location of the paired feral and managed honeybee locations across England.

Table 2.3: Number of feral and managed honeybee colonies with positive qPCR results for low incident diseases.

Disease	*Feral Positives*	*Managed Positives*
***Acarapis* spp.**	1	4
CBPV	3	4
M. plutonius	0	1
P. larvae	0	0
SBV	1	0
SPV	0	0

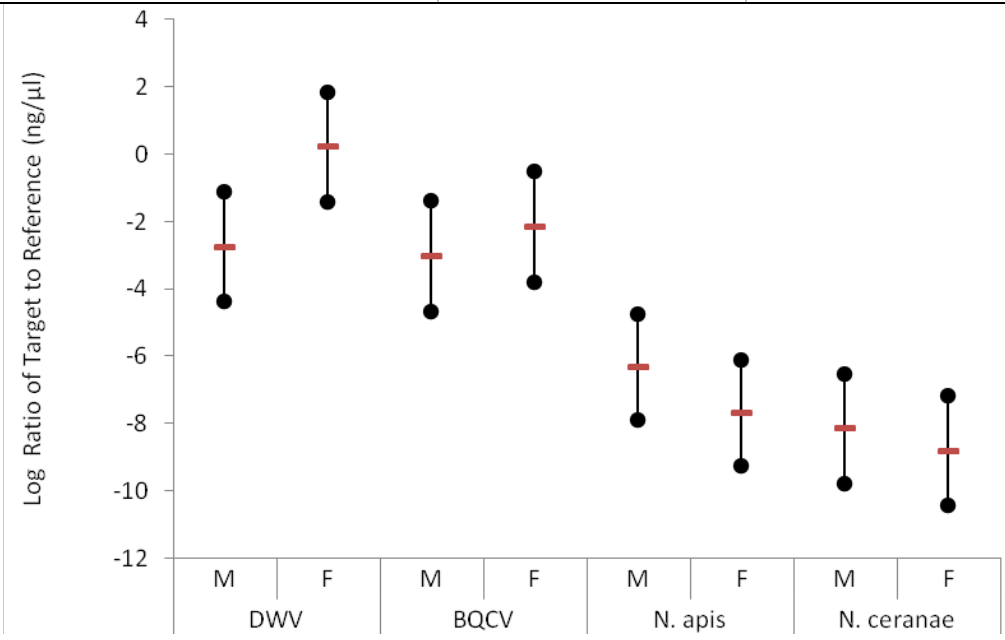


Figure 2.2: The Restricted Maximum Likelihood model estimates for the four most commonly found pathogens. Predictions are on the log scale with 95% confidence intervals. * denotes a significant different between managed (m) and feral (f) colonies.

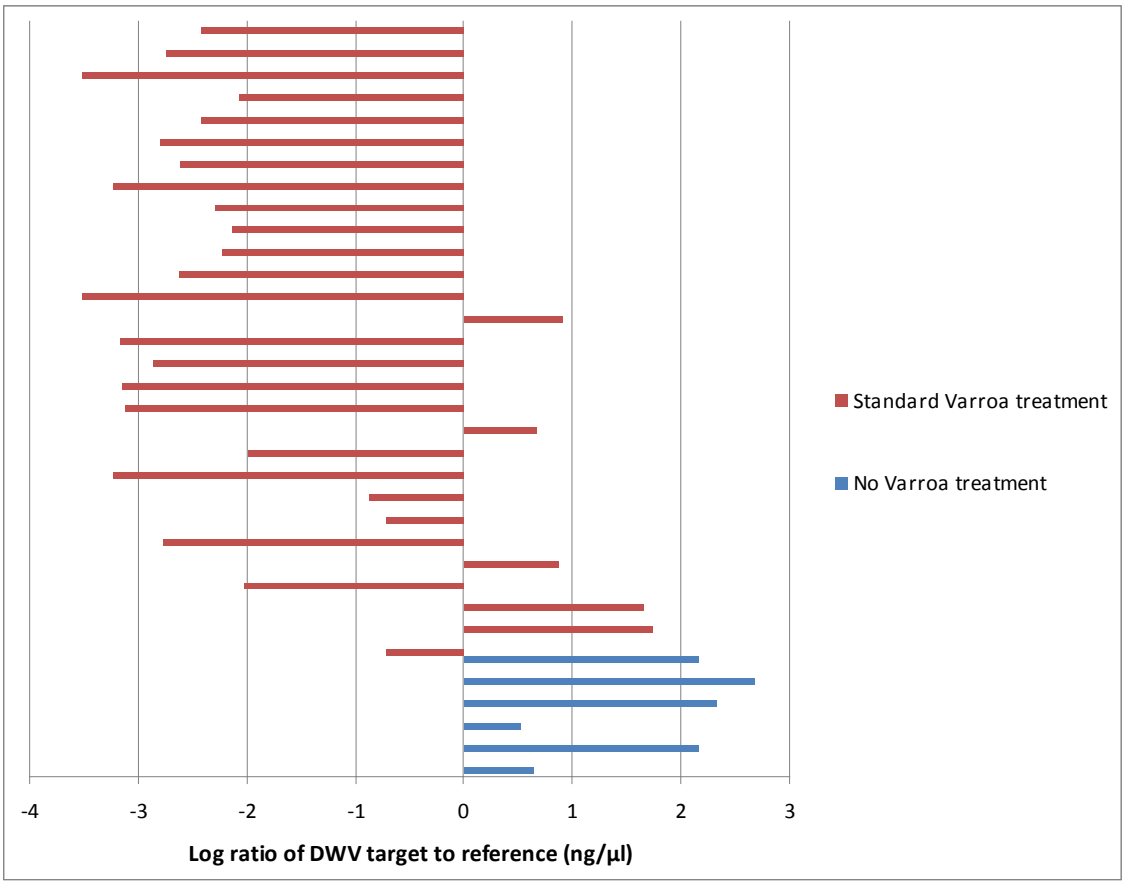


Figure 2.3: Showing the effect of *Varroa* treatment on managed colony log DWV levels. Blue indicates colonies where no *Varroa* treatment was used at all. Red indicates colonies where a standard application of *Varroa* treatment was used (i.e dosing with Varroacide one to two times per year).

EFB, AFB, SPV, SBV, *Acarapis* spp. and CBPV had such low prevalence that it was impossible to ascertain any trend between managed and feral colonies. There was only one colony that tested positive for *M. plutonius* and this was managed (Table 2.3).

All colonies were positive for DWV, BQCV, *N. apis* and *N. ceranae*. This reflects the most commonly found diseases of honeybee colonies in the UK (Bailey et al., 1981). There was no significant difference in the titre of *N. apis* or *N. ceranae* between managed and feral colonies ($F = 1.70$, d.f =1, 33, $p = 0.20$, $F = 0.52$, d.f. = 1,33, $p = 0.48$). Both these microsporidia were found in all colonies. There was also no significant

difference in the level of BQCV between feral and managed colonies (F= 1.11, d.f. = 1,33, p = 0.30). Only DWV was significantly different between managed and feral colonies and was highest in feral colonies (F = 6.41, d.f = 1,33, p = 0.02) (figure 2.2). A small subsection of the managed honeybee colonies that were not routinely treated for Varroa showed similar levels of deformed wing virus to that of feral colonies (figure 1.3). An independent samples t test revealed a significant difference in the log DWV levels between standard varroacide treated managed colonies (M=-1.83, n=28, SD= 1.61 (2.d.p)) and untreated managed colonies (M= 1.74, n=6, SD= 0.92 (2.d.p)), t (32) = 5.21, p=<.001 two tailed).

Discussion

Only DWV levels differed between feral and managed colonies. DWV was 2.4 fold higher in feral colonies; a figure that most likely reflects the absence of a *Varroa* control system. Indeed a significant difference in DWV levels could be seen between managed honeybee colonies treated and not treated for *Varroa* (Figure 2.3). As most managed colonies are treated to reduce the effects of the *Varroa* mite, this protects them from the possibility of pathogen spill over from local untreated colonies (Colla & Otterstatter 2006; Otterstatter & Thomson 2008; Power & Mitchell 2004) . What is uncertain, given the lack of knowledge about the density of the feral honeybee population, is whether feral colonies increase *Varroa* levels and associated disease levels in managed treated populations (Martin et al. 2012) .

Varroa treatment is strongly recommended for beekeepers as without it colonies are vulnerable to the effects of associated viruses such as DWV and ABPV that have a significant effect on mortality (Danka et al 2011, Harris et al 2011; Dainat et al. 2012; Martin et al. 2012). Dainat et al (2012) showed that *V. destructor* and DWV were associated with decreased honeybee life span, particularly in winter bees, leading to high winter losses. It is currently not known what threshold of DWV a colony can survive but assessment of feral honeybee survival and further dosing studies should illuminate this area further (Miranda & Genersch 2010; Kukielka et al. 2008). It is likely

that the threshold for colony mortality from DWV relies on a cumulative effect of other compromising stress factors such as a poor monofloral diet and exposure to pesticides (Alaux et al. 2010; Moritz et al. 2010).

It has been postulated that feral colonies surviving untreated with *Varroa* may offer a potentially useful source of resistance to local beekeepers (Kukielka et al. 2008; Y Le Conte et al. 2007). In experiments where *Varroa* surviving honeybee colonies have been mated with non *Varroa* surviving strains, Danka et al (2011) found a useful level of resistance. Indeed, Doebler (2000) argues that if widespread use of pesticides for *Varroa* treatment continues, beekeepers are effectively eliminating the pressure on bees and remove the development of tolerance. However, Spivak and Reuter (2001) warn that the apparent survival of feral colonies may in fact be due to concerted *Varroa* treatment of managed colonies that maintains *Varroa* at a tolerably low level for some feral colonies. If the current widespread treatment of Varroa came to an end in the UK it is likely that the lack of natural resistance in the population and the consequential boom in the *Varroa* population would cause massive feral and managed colony losses, particularly in areas of high beekeeping density (Fries & Bommarco 2007; Rosenkranz et al 2010). Another potential difficulty with advocating a no *Varroa* treatment program is that natural selection for a stable parasite-host relationship might create a bee not favoured by commercial beekeepers i.e. having a lower brood production, higher prevalence of brood diseases, overwintering with a smaller number of bees and a less gentle behaviour (Rosenkranz 2010).

Furthermore, although some *Varroa* tolerance breeding programs appear to have encouraging results, they are usually carried out in remote locations where researchers can draw on extensive feral populations that have survived without *Varroa* treatment for some time (Wallner & Fries 2003; Seeley 2007). Unfortunately in the UK, particularly England and Wales, we do not have large, remote and untreated honeybee populations to draw from (see Chapter 4). Indeed, the geographic proximity of commercial beekeepers and untreated colonies may prohibit meaningful breeding programs for *Varroa* resistance in England and Wales.

In Sweden for example, some colonies have been shown to survive for over 10 years without treatment, and this is attributed to a balanced host-parasite relationship, where reduced honeybee brood production and reduced *Varroa* fecundity co-evolve to reduce the negative effect of *Varroa* infestation (Locke & Fries 2011). Seeley (2007) also ascribed survival of untreated of feral colonies in Arnot forest to the evolution of a-virulence in the *Varroa* mites. Parasite a-virulence is seen to evolve where vertical transmission of the parasite (from parent to offspring) is favoured over horizontal transmission (infectious spread between colonies i.e. by drifting or robbing) (Bull 1994). This process requires the parent colonies to be healthy enough to procreate (Bull 1994; Seeley 2007). Seeley (2007) notes that in the Arnot forest, feral colonies are at a low density and widely separated so in this environment, vertical *Varroa* transmission would be strongly favoured.

V. destructor can also act as a vector for BQCV which may explain the slightly higher, though not significantly different level found in feral colonies (Bailey 1981). It had been documented that BQCV has a close association with *N. apis,* but in this study BQCV levels seemed to reflect DWV levels more closely than either *N. apis* or *N. ceranae* (Highfield et al. 2009).

Feral colonies had lower levels of both *N. apis* and *ceranae*, although not significantly so. It is possible that as feral honeybees are exposed to fewer stressors in the form of beekeeper manipulation e.g. direct damage to comb and propolis, death of bees during beekeeper activity, cross contamination between hives, honey removal, pollen harvesting etc that they have higher immunocompetence and show less signs of other stress factor diseases (Büchler et al. 2010). A larger sample of colonies would be required to further explore this relationship. Van Engelsdrop et al (2009), identified over 200 honeybee colony stress factors in an aim to elucidate a cause of colony collapse disorder. No single factor was identified despite *N. ceranae* coming under immense scientific scrutiny. Instead it has been postulated that colony loss is likely to be due to an accumulative effect of a wide range of stress factors of which high *Nosema* levels is indicative but not necessarily causal (Van Engelsdorp et al. 2009; Ratnieks & Carreck 2010; Neumann & Carrek 2010). Indeed, research by Aufauvre et al (2012) shows that the combined effect of *N. ceranae* and an environmental

concentration of the insecticide fipronil will cause a significantly higher bee mortality compared to the sum of the effects induced by each agent acting alone (Aufauvre et al. 2012). It seems likely that more significant colony stress factors and combinations will be identified as further wide ranging ecological examinations of honeybee health are carried out (Ratnieks & Carreck 2010; Alaux et al. 2010; Neumann & Carrek 2010; Moritz et al. 2010; Vanbergen et al (in review)).

Foulbroods cause most consternation for the beekeeper as they are notifiable diseases in the UK, and in the case of AFB, lead to the infected colony being destroyed (Selwyn Wilkins et al. 2007). EFB progresses more slowly and there are now options to treat the infected colony with oxytetracycline or perform a shook swarm (where worker bees and the queen are shaken into a new colony on fresh comb), although colony destruction is still favoured in some cases (Wilkins et al. 2007). In New Zealand, Perry (1994) found that only a low proportion of feral colonies tested positive for AFB and then with a lower number of spores than managed colonies. In this study only one colony was positive for EFB and this was a managed colony. EFB is found at a relatively low incident level in the UK (Budge et al. 2010), so the small sample size makes it impossible to draw any meaningful conclusions about the true level of foulbrood infection in the UK's feral colonies.

Conclusions

This study was the first to monitor disease incidence in the UK's feral honeybee population in any meaningful way. The absence of any *Varroa* treatment has a significant effect on DWV levels, both between feral and managed colonies and within the managed population. Further research must be carried out to assess how long feral colonies of the UK are able to survive with such high DWV levels, and whether there is any unique genetic, behavioural or environmental factor that is enabling them to survive, also how high *Varroa* levels in the feral honeybee population effect managed honeybee colony disease levels.

Chapter 3: UK feral honeybees: nest-sites, longevity and genetic make-up

Introduction

It was thought that the UK's feral honeybee population had been decimated by the arrival of the *Varroa destructor* mite in 1992 as numerous anecdotal reports exist of sudden declines (Carreck et al. 2002; Carreck 2008). *V. destructor* and the resulting *Varoosis* is now considered to be the most destructive disease of honeybees worldwide and the major cause of winter colony losses (Boecking & Genersch 2008; Rosenkranz et al. 2010; de Miranda & Genersch 2010; Carreck et al. 2010; Neumann & Carrek 2010). In the years immediately after the arrival of the *Varroa* mite, untreated managed colonies were reported to survive for as little as eight months before collapse and beekeepers suffered catastrophic losses of their colonies (Doebler, 2000).

In recent years however, anecdotal reports have suggested a resurgence of the feral honeybee population in the UK, and international studies have shown the persistence of some untreated colonies for a number of years (Yves et al. 2007, De la Rúa et al. 2009, Seeley 2006, Fries et al 2006, Le Conte et al 2007). In France, for example, Le Conte et al (2007) showed that their *Varroa* 'surviving bees' lived on average 9.5 years without *Varroa* treatment with five colonies out of the group surviving more than 11 years (Le Conte 2007). Another unrelated population in Sweden has also been shown to survive over 10 years without *Varroa* treatment (Locke et al. 2012).

It has been proffered that feral bees and unmanaged colonies that have survived may supply a solution to the *Varroosis* crisis by offering a natural source of *Varroa* tolerance or resistance. Some researchers argue that the continued use of *varroacide* among the beekeeping population prevents the evolution of *Varroa* tolerance behaviour by removing the selective pressure and high adaptive value of *Varroa* tolerance. Furthermore, there is concern that synthetic varroacides build up in the wax foundation of some colonies (Dietemann et al. 2012; Locke et al. 2012). Water soluble varroacides such as formic acid, oxalic acid and cymiazole can also be found dissolved in honey, and lipid soluble Varroacides such as bromopropylate, coumaphos and fluvalinate can be found in wax and fat tissue in honeybees (Wallner & Fries 2003; Wallner 1999; Dietemann et al. 2012). The effects of these residues on honeybee health are unknown, although it is feared that exposure to a myriad of pollutants,

agricultural sprays and apicultural products could be detrimentally affecting colony longevity. Even use of organic acids is not without its difficulties due to the variation in efficacy with ambient hive temperature and the relatively fine margin between a lethal dose for the mite and a lethal dose for the treated bees themselves (Genersch 2010). Dietemann et al (2012) conclude that there is no truly satisfactory Varroacide available at present.

Concerns over the safety and sustainability of Varroacide use, and the upsurge of new beekeepers who are motivated more by ecological principles than traditional apiculture has sparked a new debate on the validity of *Varroa* treatment. The apparent survival of feral colonies is often cited as evidence for overtreating managed colonies and thus as an argument to reduce treatment (Chandler 2009). This assertion is premature however, as to date no rigorous scientific study has been carried out on the feral honeybee population of the UK. It is not known for example whether the feral colonies are actually surviving or merely being replaced by regular swarms from nearby colonies. Also, little is known about the ecology and genetic make-up of feral honeybees. For example, to what degree are feral colonies related genetically to managed honeybee populations and do feral colonies truly represent a separate *Varroa* tolerant gene pool?

Accurate records of pre-*Varroa* populations were not kept in the UK and very little research was carried out on feral honeybees worldwide until their absence was noted by beekeepers and researchers. This means it is impossible to ascertain whether the anecdotal resurgence in the UK's feral honeybee populations is due to increased *Varroa* tolerance or just a greater number of novice beekeepers losing swarms.

This study set out to examine the status of surviving feral colonies. First, I will describe the conditions under which the feral colonies occur. Then, I will assess how long these feral colonies actually survive and, finally, I will assess whether feral colonies are different from nearby managed colonies. Conclusions on the state of the feral honeybee population in England and Wales are discussed with particular reference to their relevance to *Varroa* resistance breeding programs.

Methods

Finding and selecting feral colonies

See Chapter 2 for details.

Colonies where activity had been noted for a number of years were selected in favour of colonies without such history, to avoid sampling new swarms which may be less likely to survive due to unsuitable initial sites, for example an annual swarm was seen entering a metal statue, which had a relatively small cavity that would have been impossible to keep warm through the winter period. Sites that were impossible to reach or not safe to sample were also not selected.

Basic descriptive data were collected on all feral colonies sampled, including an estimate by the reporter of how long the colony had been in place; the location of the colony, e.g. tree, occupied house, other building; and the direction the colony faced. It was almost always impossible to get an estimate of colony volume.

Mean colony direction was calculated in R version 2.12.2 (Hornik 2012) using the packages Plotrix (Lemon 2006) and Cairo (Urbanek & Horner 2011), to create the polar plot.

Assessing feral colony longevity

Feral sites located in the spring of 2009 (n= 20) and some novel sites for autumn 2009 (n=16), were revisited biannually until autumn 2011 (36 sites in total, over two and half years). Samples of approximately 30 forager bees were collected from the entrance of the colony each time the colony was visited although only the first and final samples are used in this chapter. Where bees could not be encouraged to appear from the entrance, signs of cause of mortality were recorded. In most cases the cause of mortality was obvious: i.e poison or powder, vandalism, evidence of fire or colony removal for renovation and building work.

To explore whether the colony was headed by the same queen during the observation period, bees were collected during the first and final sampling period for genotyping (see below). Only 11 colonies were seen to have been continually occupied for the period of the study. Only the first and final sample from these 11 colonies were submitted for genotyping.

Comparing feral and managed colonies using DNA analysis

DNA extraction from samples

Forager *Apis mellifera* bees were collected from each colony and stored for use in 100% ethanol at -70^{o}C. Fifteen foragers from each of the 11 colonies were randomly selected. Whole bees were washed in molecular grade water, and crushed with 2.3 mm silica beads in a Precellys lysis and homogenization bead beater at 5000 rpm for 30 seconds. Total DNA was extracted from an entire worker bee using a 10% Chelex solution with 1 x TE buffer. Next, 800 µl of 10% Chelex solution was added to each crushed bee residue. This was heated to 95 ^{o}C for 5 minutes then centrifuged at 8000g for a further 5 minutes. 200µl of the upper aqueous DNA extract was removed and centrifuged again at 8000g for 5 minutes then 150 µl of the upper aqueous was removed and stored at -70^{o}C until required.

Sequencing of the samples

This microsatellite protocol was taken from FERA's 2009 to 2010 Random Apiary Survey project (RAS) and the 2010-2011 Defra seedcorn project assessing the diversity and provenance of managed and feral honeybees in the UK (Budge et al, in prep). The RAS project surveyed 361 colonies, genotyping one worker honeybee from each.

Extractions were diluted to a 1:500 concentration. Twelve microsatellites were selected for their variability and ability to discern between the common honeybee races in the UK (See *Bayesian analysis of populations* below and table 3.3). PCRs were performed individually in 10 µl volumes at two $MgCl_2$ concentrations as below in table 3.1:

Table 3.1: Primer master mix and conditions
1.2 µM $MgCl_2$ (840/814, 936/937):
4.475 µl of H_2O, 1.5 µl of 10x Buffer IV, 0.7 µl of $MgCl_2$ (25 µM), 0.045 µl of dNTPs (20 µM), 2 µl of BSA (1µg/ µl), 0.6 µl of Forward Primer (10 µM), 0.6 µl of Reverse Primer (10 µM), 0.08 µl of Taq.
1.5 µM $MgCl_2$ (828/829, 836/837, 876/877, 882/883, 938/939, 852/853, 864/865, 866/867, 950/951, 990/991)
4.275 µl of H_2O, 1.5 µl of 10x Buffer IV, 0.9 µl of $MgCl_2$ (25 µM), 0.045 µl of dNTPs (20 µM), 2 µl of BSA (1µg/ µl), 0.6 µl of Forward Primer (10 µM), 0.6 µl of Reverse Primer (10 µM), 0.08 µl of Taq.

Each colony PCR plate included a positive control and a blank. PCRs were run on a real-time PCR ABI Prism 7900HT (Applied Biosystems Inc., Foster City, CA). The first stage of the PCR was denaturing of the dsDNA at 94 ^{o}C for 5 minutes. This was followed by 35 cycles of denaturing at 94 ^{o}C for 30 seconds and annealing for 30 seconds at 72 ^{o}C. There was a 50 minute soak at 60 ^{o}C at the end. Polymerase chain reaction products were diluted in multiplex groups (table 3.2 below).

Table 3.2: PCR dilutions in multiplex pairs

Microsatellite pair	Volume of PCR contributed (µl)	H_2O for dilution (µl)
828/829	1	
836/837	1	146
840/841	1	
936/937	1	
950/951	2	147
990/991	1	
876/877	1	
882/883	1	147
938/939	1	
852/853	1	
864/865	1	147
866/867	1	

1 µl of the diluted multiplex mix was added to 10 µl of formamide and 0.3 µl of size standard ROX 500. Samples were sequenced on a 3130xl Genetic Analyzer. Peaks were scored using Genemapper software version 3.7.

Genetix software (Belkhir 2004) was used to calculate molecular statistics, and GST values. Arlequin software version 3.0 (Excoffier et al. 2005) was used to compare the populations using an AMOVA (Analyses of Molecular Variance to evaluate the amount of population genetic structure). A pairwise FST was calculated and bootstrapped with 1000 permutations. To check the validity of comparing multiple individuals from a colony (for the feral data) against the methodology for the RAS study which used a single queen, the analysis was repeated with a randomly selected individual from each feral colony (see table 3.3). Sampling individual feral bees and comparing them to the managed population (RAS data) did not produce a significantly different result from grouping all individuals from a colony into the feral sample, so data from multiple individuals was used in this study.

Table 3.3: Fst values and significance levels for feral verses managed population comparisons using randomly selected individuals from feral colonies.

Repeats of the FST calculations and bootstrapping on randomly selected individuals from each colony	GIS	Fst (%)	P Values (3.d.p)
1	0.0263	1.49 *	0.029
2	0.0237	1.89 *	0.013
3	0.0259	2.07 *	0.007
4	0.0294	2.96*	0.001

Bayesian analysis of populations (BAPs) to identify race and genetic separation

39 highly variable microsatellites or SSRs were tested to cluster and discriminate between the races of queens (Corander & Marttinen 2006; Budge et al, in prep). This project used 12 of the most variable and discriminatory microsatellites to assess the

component races of feral honeybee colonies (highest Gst) (See table 3.4). The genetic makeup of the feral worker honeybees was compared alongside that of the reference queens using BAPS software version 5 (Corander & Marttinen 2006). The BAPS data is presented as a proportion of the 11 races or clusters found within each worker bee (Hawaian *Carnica*, Hawaian *Ligustica*, Spanish *Iberica*, Malta *Rutneri*, New Zealand *Ligustica*, Australian *Ligustica,* Slovenian *Carnica*, Greek *Macedonica*, UK *Mellifera*, French *Mellifera* and German *Carnica*) (see figure 3.9).

Table 3.4: The 12 microsatellite primer sequences used for assessing racial proportion.

Microsatellite Loci	**Sequence**
828/829	UN012-F [HEX]CGACCTAACAGTTGCTTCGTG
	UN012-R ATTGTTCCTTGCCACGATACA
836/837	K0190-F [6FAM]ACGAATCGAGCCTCGACC
	K0190-R ATGCAAGTTTCAAAGGCACG
840/841	AP271-F [HEX]CATGATGCGAGTCTCCTGGC
	AP271-R GCATTAATTGCGCTGCGTC
852/853	UN151-F [HEX]AAATTAGCGTACGTGAGCGG
	UN151-R CGTCATAGTCCACCTACCAACG
864/865	K0264-F [HEX]TCTCGTGGAATGGCCTAAGAG
	K0264-R ATACACGCGCACATTCGC
866/867	6230-F [6FAM]CGATCAGCAAAGGCGTATCC
	6230-R GGATGTTCGCGTTAACGTAACTG
876/877	K0352B-F [HEX]ATCAATCTCCTCGCGATCG
	K0352B-R CAAGTTACACAATCCTCGCACC
882/883	UN375-F [HEX]TTCGGACAGGATGCTGCA
	UN375-R GGCCGACTTTCGTCACTGA
938/939	K0911-F [6FAM]GGAACAAAGTGGAGCGAATTACT
	K0911-R CTCGAGGATCCTTAATCGTGG
936/937	AT064-F [HEX]TATGCAGAGCGCGATACATC
	AT064-R TCTGCTTGTTTCGTCGCAG
950/951	AT129-F [6FAM]GCTAACGGGGTAACGGGATC
	AT129-R CCATCCAGACCACGCCTACA
990/991	AT192-F [HEX]GCCTGCTTCTCCTCACTTTC
	AT192-R AGATTTTGAAAGAGGGTCGC

Colony survival

Queen genotypes were inferred from worker offspring microsatellite data using COLONY software 2.0.3.0 (Wang & Santure 2009). Suggested queen genotypes were compared between 2009 and 2011 samples.

Change in race composition

The percentage UK *mellifera* in each colony, as calculated through the BAP analysis was compared between spring 2009 and autumn 2011. Statistics were performed in IBM SPSS version 20 (IBM 2012).

Results

Colony locality of UK feral honey bees

Feral colonies were found in a wide variety of locations (figure 3.1). A chi-squared test shows there was no significant difference in the proportion of colonies in trees, houses or non-housing sites (n=37) $\chi 2 = .054$, p=.973). Although no significant preference was found between nest cavity sites, it is possible that house walls convey some advantage due to passive warmth. The disadvantage for honey bee colonies nesting in houses or close to human inhabitation is that they are at greater risk of removal or destruction. No significant difference was found between natural colony mortality and destruction by man in the different nesting locations (n=15, $\chi 2 = 1.667$, p=.197; figure 3.2).

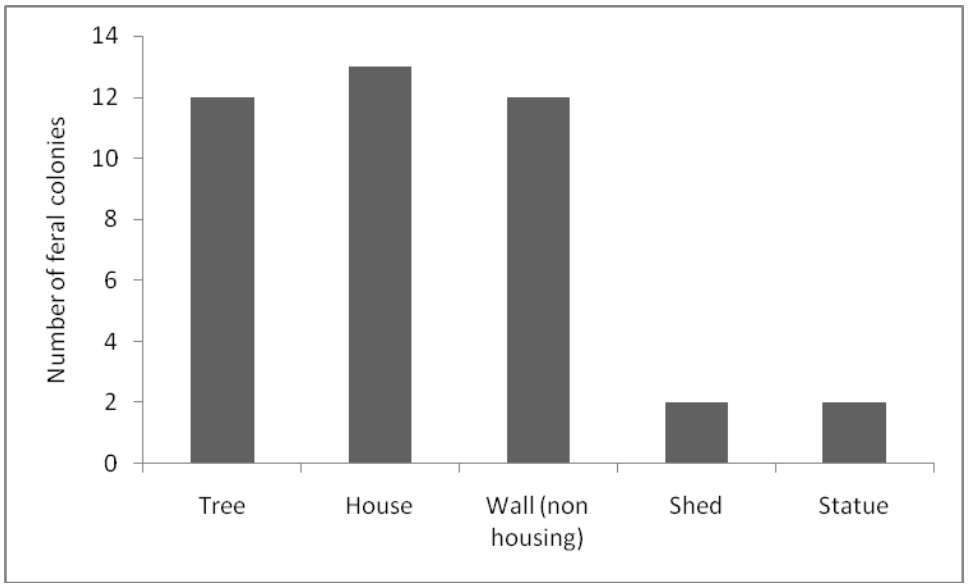


Figure 3.1: Location where feral colonies were found.

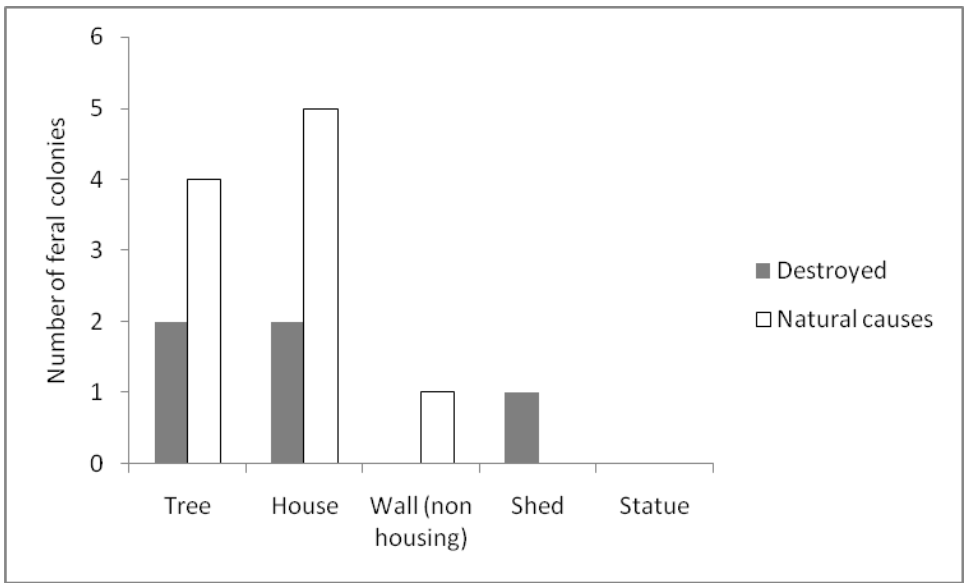


Figure 3.2: Whether colony mortality was due to natural causes or human destruction

Estimates of colony longevity varied widely between individual cases with some estimates of survival seeming unlikely.

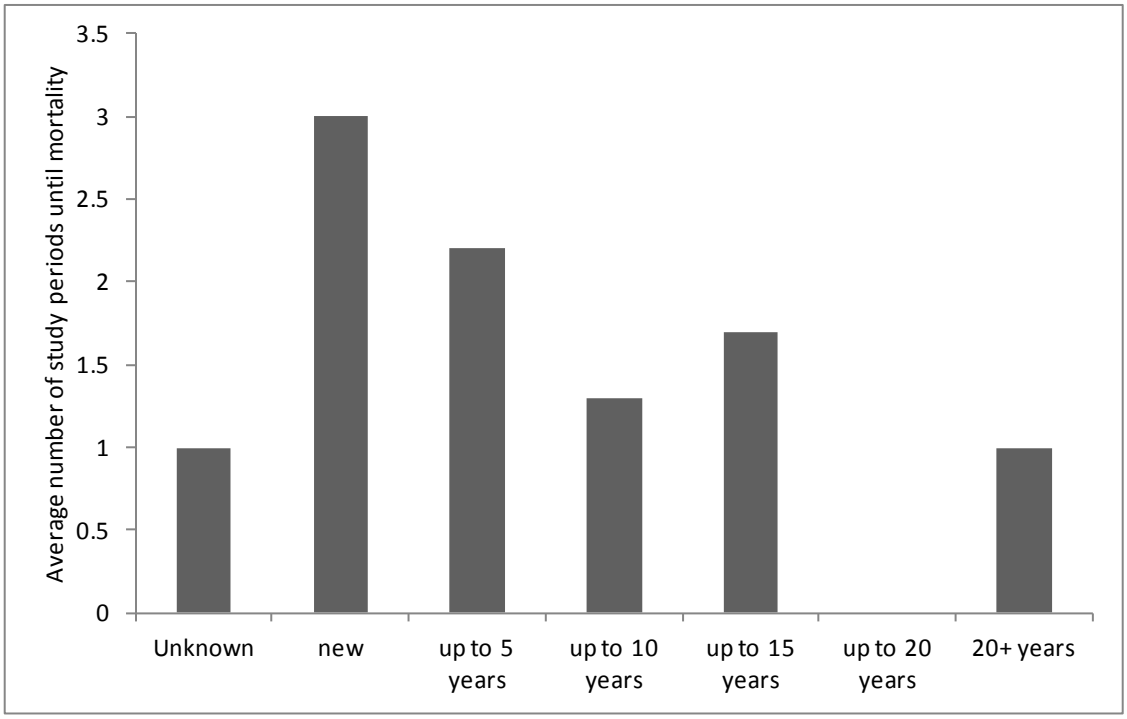


Figure 3.3: Average number of study periods (1 period = six months) until colony mortality for sites with different assumed longevity.

The most significant trend in feral colony ecology is that colonies tend to face predominantly South East. Of 40 sites where aspect was noticed the mean direction was 143.87^{o} (figure 3.4). The samples that appear to be facing due north are perhaps misleading as they were located on the roof of a house, so although the entrance of the colony was facing North it is likely that they were warmed by the sun for much of the day.

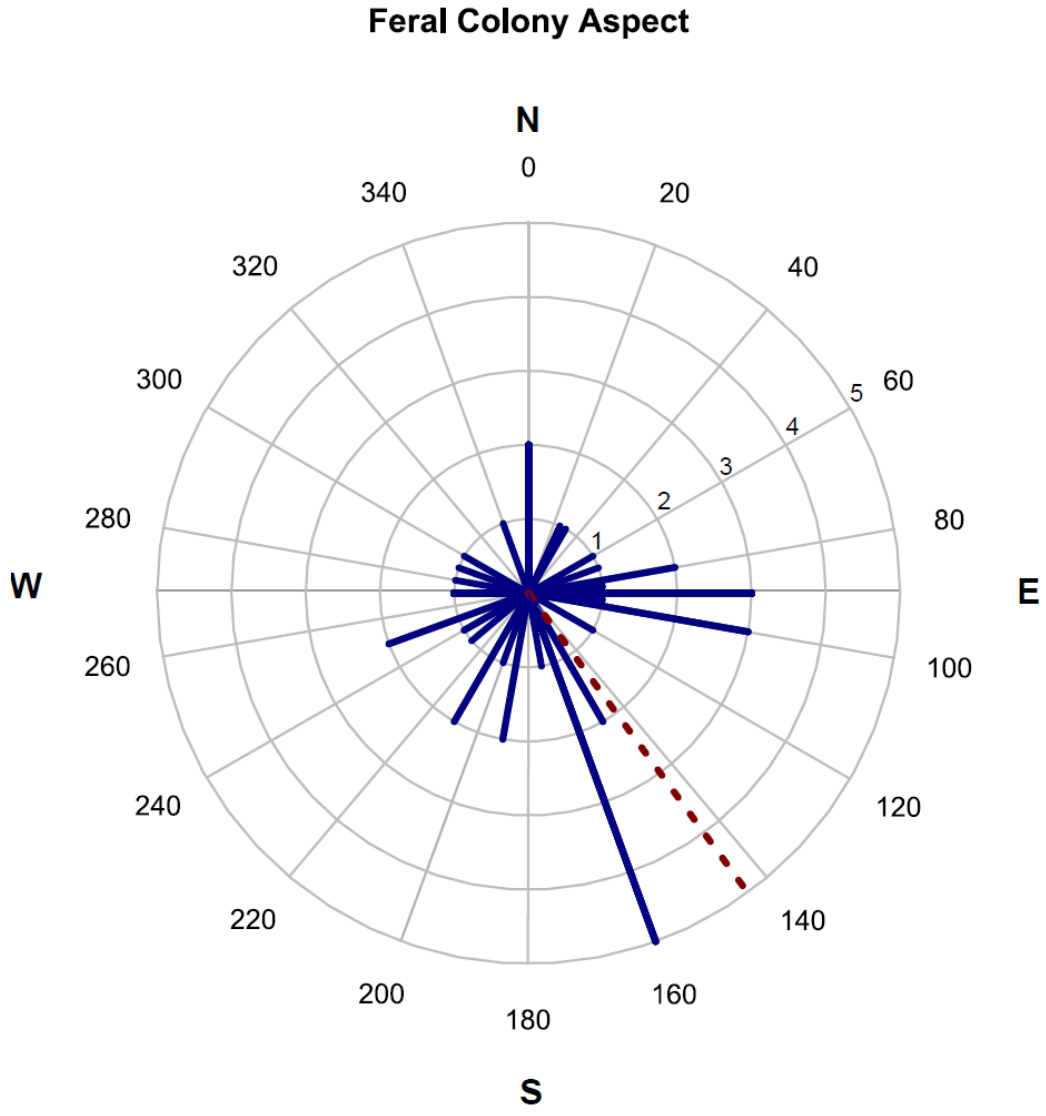


Figure 3.4: Feral colony aspect

Longevity of feral colonies

Out of the 36 feral colonies monitored, 47.22 % (n=17/36) died out or were destroyed within the 2.5 year period (45 % of the colonies studied for 2.5 years n=9/20 and 50 % of the colonies studied for 2 years n=8/16). This yields total losses of 30% in 2010 (6/20) and 21.43% in 2011 (3/16), for colonies sampled for the full 2.5 years. Seasonal colony loss is shown in figure 3.5. These figures for colony loss are likely to be an under representation of the true loss, as survival was determined by honeybee presence. The highest colony loss was in Autumn 2010 where 19% of colonies were lost. Usually beekeepers see largest losses over winter but these figures were bolstered by the large number of colonies destroyed during the summer, i.e. near parks, near children's playgrounds etc.

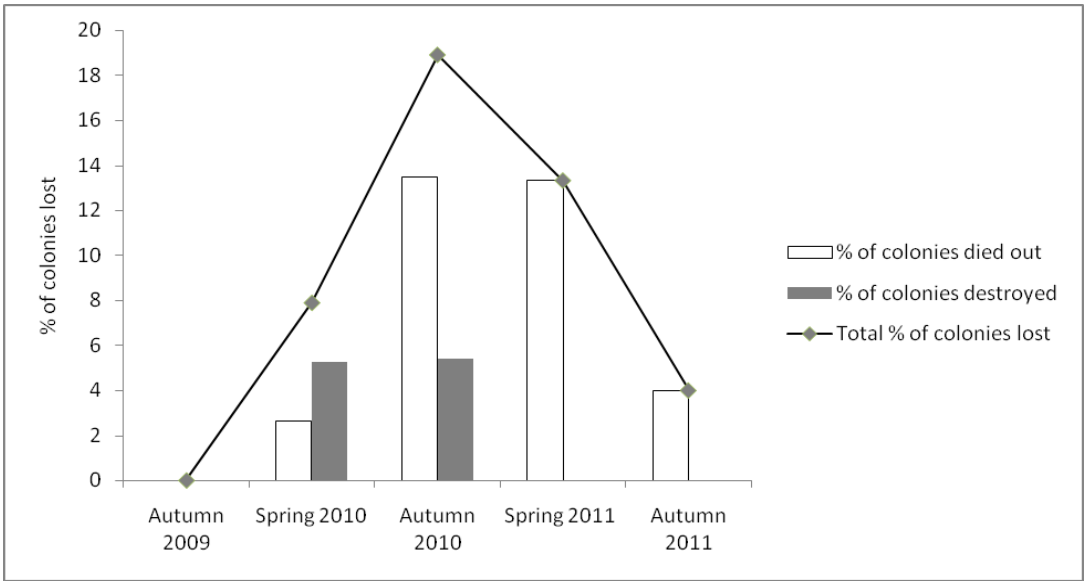


Figure 3.5: Colony loss through natural mortality or human destruction

Eleven colonies appeared to survive for the 2.5 year period. The first and last sample (Autumn 2009 and Autumn 2011) of these colonies was genotyped to assess queen survival. None of these colonies had the same queen for the duration of this period (see appendix for inferred queen genotypes). Therefore, it can be assumed that the original queen was either replaced or swarmed during the course of this study.

Genetic comparison of feral colonies compared to the Random Apiary survey managed populations

Of a total of 471 individuals (248 RAS plus 223 feral samples), Gst, a measure of total genetic differentiation attributable to sub-population differentiation was 0.0168 or 1.7%. Fst showed a small but significant difference between the feral and managed population of 2.33% $p<0.05$. Fst is directly related to the variance in allele frequency among populations (Holsinger & Weir 2009).

Table 3.5: Gst values for a comparison between the UK's feral and managed honeybee population

Feral colony heterozygosity (Hs)	Managed colony heterozygosity (Ht)	Gst
0.6954	0.7073	0.0168

The RAS data was characterised for 8 UK regions (Eastern, North Eastern, Northern, South East, South West, South, Wales, Western). The relationship between race and latitude was explored but no genetic difference was found between the RAS regions: Fst = 0.076%, P= 0.44. Therefore, there was no analysis of feral colonies per region due to the small regional sample size and the lack of a geographic structure to compare against.

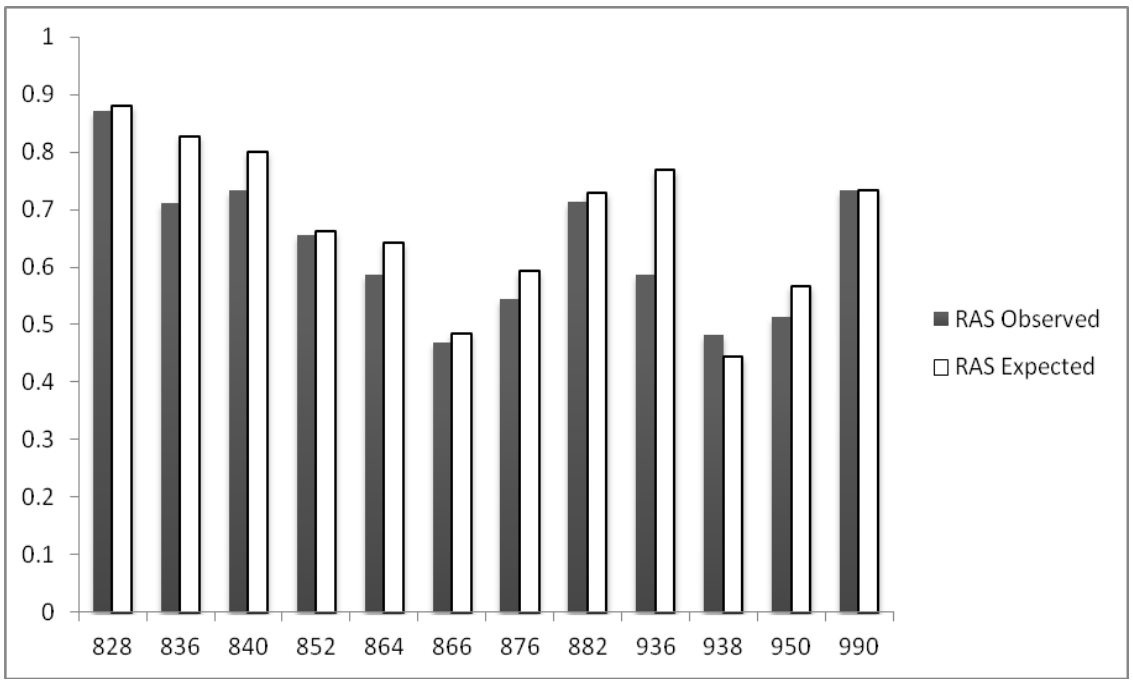


Figure 3.6: Random apiary survey / managed population expected and observed allele heterozygosity

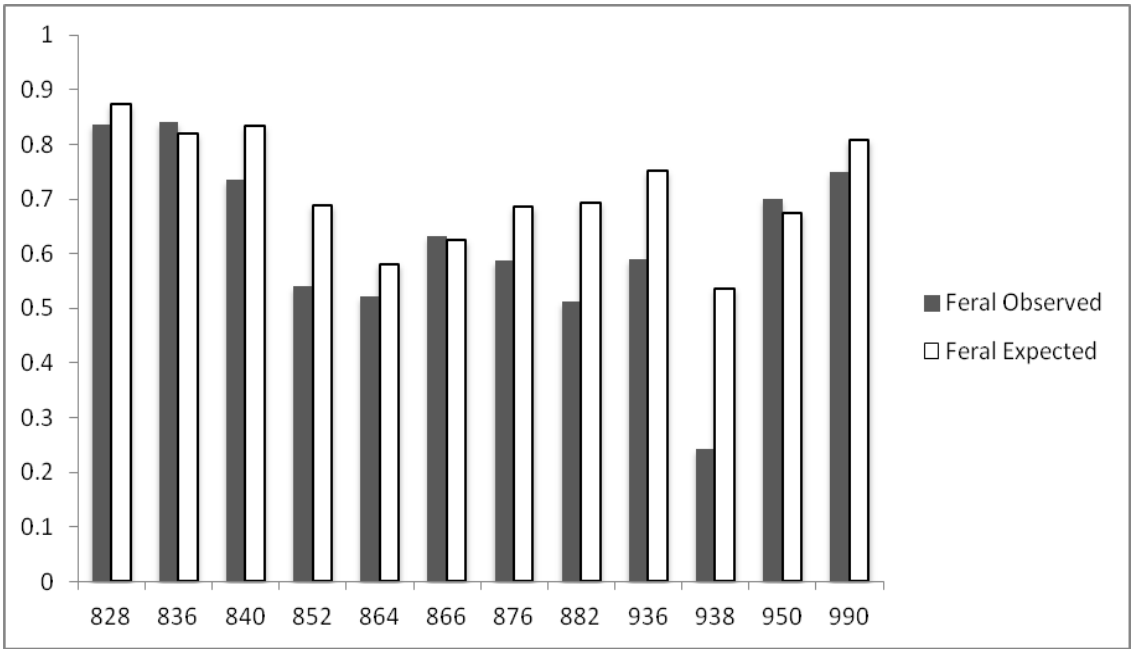


Figure 3.7: Feral population expected and observed allele heterozygosity

Table 3.6: Gst values by locus for feral (sub population) and the total population

Locus	Subpopulation heterozygosity (Hs)	Total population heterozygosity (Ht)	Gst
828	0.8761	0.8804	0.0049
836	0.8228	0.8257	0.0035

840	0.8166	0.8193	0.0033
852	0.6750	0.6822	0.0106
864	0.6110	0.6122	0.0021
866	0.5547	0.5596	0.0087
876	0.6386	0.7289	0.1239
882	0.7108	0.7124	0.0022
936	0.7594	0.7633	0.0050
938	0.4900	0.4986	0.0173
950	0.6203	0.6290	0.0138
990	0.7700	0.7764	0.0082

Heterozygosity values were calculated by locus in Genetix software (Belkhir 2004). There are no alleles which are obviously linked to the feral population as they all have relatively low Gst values (table 3.6). Only locus 938 was lower in feral populations than expected (figure 3.6 and 3.7).

The reason for the lack of significant different in total population or locus heterozygosis is the almost total genetic overlap found between managed (RAS) and feral populations. This is best illustrated by figure 3.8, the principle component analysis of feral and managed populations.

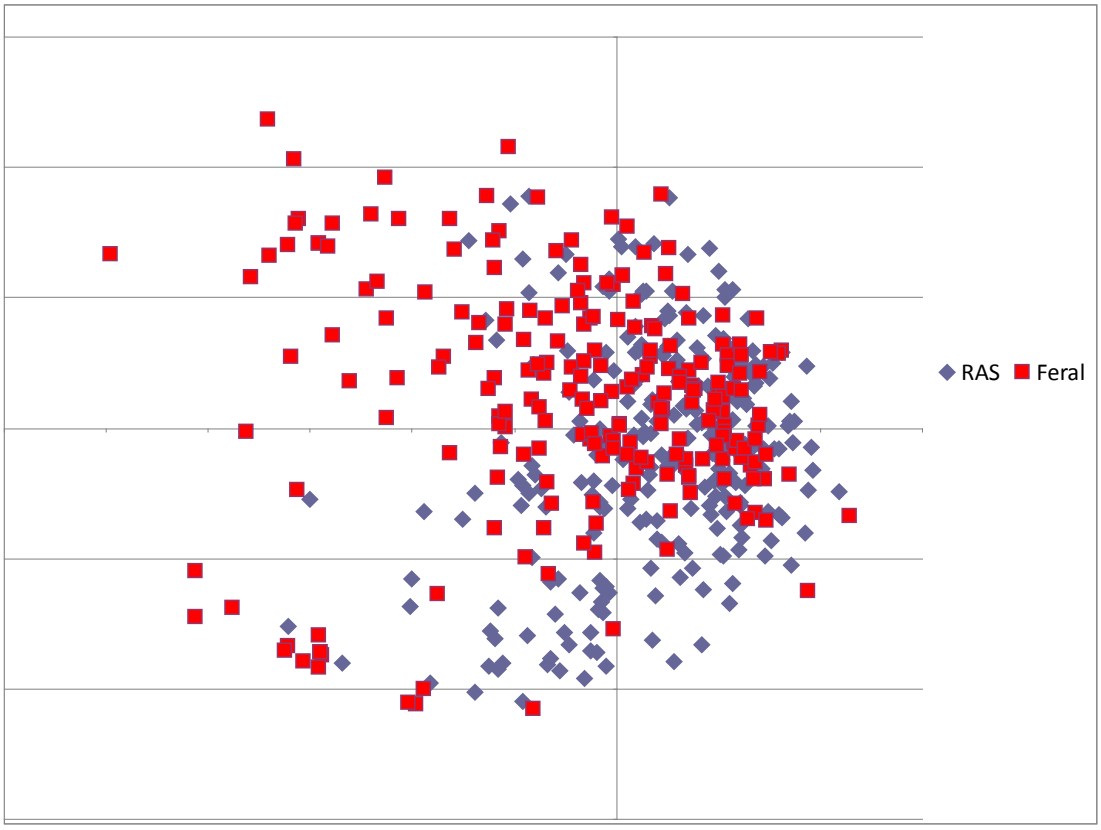


Figure 3.8: Bayesian analysis of population principle component analysis comparing feral and managed populations.

Feral colony race composition

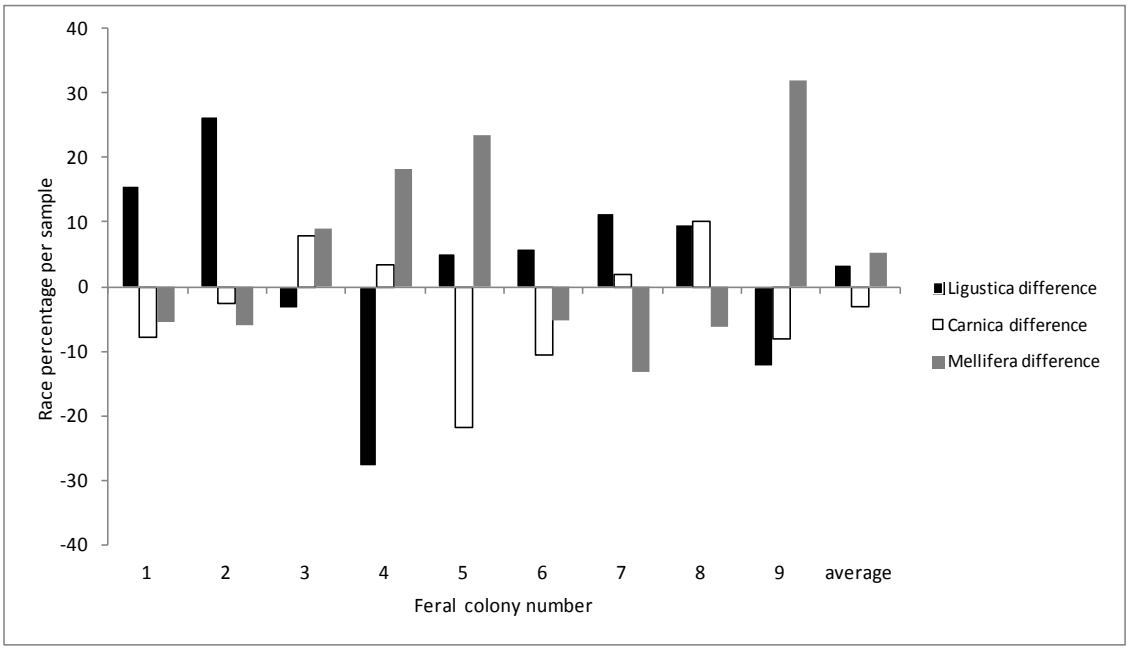


Figure 3.9: The difference in percentage race composition by colony between 2009 and 2011. There is no general decline of *mellifera* in feral samples but actually a small increase.

Bayesian mixture models attempt to identify a hidden population structure by clustering individuals into genetically divergent groups. FERA's project assessing the diversity and provenance of managed and feral honeybees in the UK, examined 259 reference queens from Australia, France, Germany, Greece, Hawaii, Malta, New Zealand, Slovenia, Spain and the UK.

Percentage common race composition was calculated for each feral colony and managed RAS individuals through the BAP protocol/method. A Wilcoxon Signed Rank test was used to assess the difference between percentage *A. m. mellifera* in feral colonies in 2009 and 2011 and between feral *A. m. mellifera* levels and RAS *A. m. mellifera* levels. There was no significant difference between percentage *A. m. mellifera* in feral colonies between 2009 and 2011 (z=-1.056, p=.291) (figure 3.9) or between feral and RAS *A. m. mellifera* levels for either 2009 (z= 1.194, p= .847) or 2011 (z=-.098, p=.922).

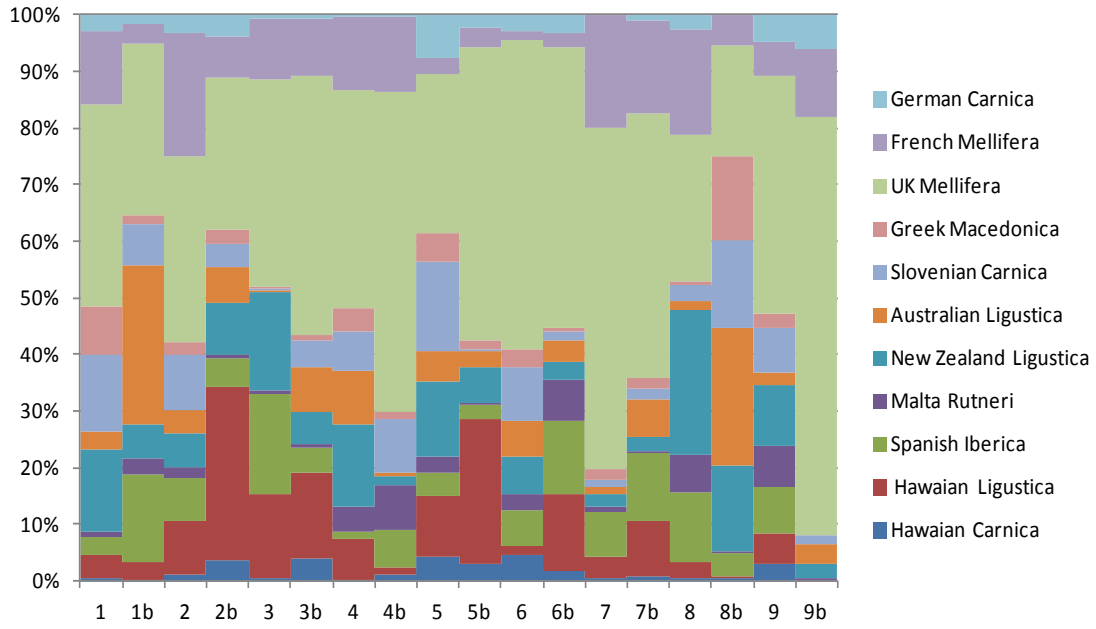


Figure 3.10: Average colony race composition of the 9 feral colonies samples in 2009 and 2011 (b) compared to FERA reference genotypes. Two colony samples were not included in this figure due to the failure of some microsatellites.

Feral colonies were highly introgressed (figure 3.10), with almost all colonies but 9b representing a hybrid of a number of races. *A. m. mellifera,* historically the native race, was still the predominant component but *A. m. ligustica* and *A. m. carnica* accounted for up to 30% of the genetic makeup. Figure 3.10 also shows the marked difference in genetic make up between study periods and supports the earlier conclusion that the queen was different between samples.

Discussion

Feral colonies were found in a surprising array of locations; with little preference between man-made and natural cavities, high above ground or below the soil in a tree stump (see figures 3.11 and 3.12). Some colonies were in situations where they came into close contact with people such as above a door ledge, whilst others lay further away in garden walls and copses. Previous research suggests that honeybee swarms favour deciduous tree nesting sites however this was not shown in this study (Ruttner 1988; Seeley & Morse 1978).

It is possible that cavity type data could have been skewed by reporter bias, as colonies are more likely to be noticed nearer to human settlements. Alternatively it is likely that feral honeybee populations are limited by the availability of suitable cavities (Ruttner 1988). Seeley's (2007) study showed that there was a rapid uptake of bait hives in Arnot Forest and a number of swarms were reported that had begun to build their comb in trees, exposed to the elements. Trees with large and thus suitable cavities are in danger of being felled, and access to houses is usually limited. Unattended honey stores from deceased colonies are highly attractive to worker bees and likely to be removed by robbing from surrounding honeybee colonies or attract a new swarm. The premium on suitable cavities may therefore contribute to the appearance of perpetual feral colonies with regular colonisation from local swarms.

Figure 3.11: Image of a feral colony in a metal statue.

The red arrow indicates the entrance.

Figure 3.12: Image of a feral colony in the wall cavity of a Tudor house.

Photo courtesy of Peter Edwards.

Unfortunately, due to the nature of the feral colonies it was impossible to estimate cavity volume, wax residue levels or level of propolis; all factors which could directly affect the health of the feral colonies (Wallner 1999; Seeley & Morse 1978). Seeley and Morse (1976) noted that feral honeybees line the entirety of the interior of a tree cavity with a propolis envelope which has been shown to enhance immunity in honeybees. Simone et al (2009) showed that honeybees from hives with extracts of two sources of honeybee propolis had a significantly lowered expression of two honeybee immune-related genes. Propolis also allows improved nest defence by restricting the colony entrance, and the maintenance of nest homeostatis (Seeley & Morse 1976). Propolis use is heavily linked to race however, with *Carniolan* bees being favoured by beekeepers for their minimal use of propolis. As the feral colonies found in this study did not represent a separate population you would not expect to see a difference in propolis use compared with managed honeybee colonies, or an effect of propolis use on colony health. It would be interesting to explore propolis levels in remote feral populations (Silici & Kutluca 2005).

Colony volume could be of particular importance as some colonies appeared extensive (figure 3.13). Where large expanses of comb are available it is possible that the colony may be able to regulate their disease burden by moving about the comb. This behaviour is seen in other natural systems such as bats, which avoid ectoparasites build up within roost sites by varying roosting location (Bartonička & Růžičková 2012; Lewis 1995).

One significant aspect of feral colonies is that the entrance tends to face South East (Seeley & Morse 1978). The only colonies found with North facing entrances were those in a roof, so the colony cluster within the roof might still receive warmth from the sun for most of the day. Rosenkranz et al (2010) suggested that environmental factors may act subtly and indirectly on honeybee parasites via the host, such as in the quantity of brood produced and the extent of hygienic behaviour. Warmth from the sun may also allow thermoregulatory savings and allow worker bees to begin flying earlier in the day. Temperature and radiation were shown to be the major factors influencing honeybee flight initiation by Burrill & Dietz (1981). This is of particular significance in the winter, where warmer temperatures allow worker bees to take cleansing flights, reducing the spread of *Nosema* within the colony (Moeller 1978). Indeed Alber and Campagna (1970) suggest that apiaries should be exposed to maximum sunshine during winter to facilitate the cleansing flights (Marino & Campagna 1970).

Figure 3.13: Removing a feral colony from an old house Courtesy of : http://www.makingbeehives.com/blog/removing-a-honeybee-colony-from-an-old-house

The true density of feral colonies is not known in the UK but knowledge of the local feral population could be useful for increased understanding of local honeybee population demography, particularly for the control of disease or for racial breeding programs. Unfortunately such data are difficult and time consuming to collect, and will alter between the seasons.

Feral colony longevity

Out of the thirty six feral colonies monitored, nearly half (47.22 %) were lost during the course of the study period. This yields annual losses of 30% in 2010 and 21% in 2011. This is marginally higher than some reports of managed honeybee colony losses although losses fluctuate between years and regions (Budge et al. 2010). Carreck & Aston (2011) report average losses of 17.7% in the 2009-2010 period and 13.6% in the 2010-2011 period. Although this study represents a small subsample of the overall feral honeybee population, and also includes destructive loss, it seems likely that the absence of beekeeper interference and honey removal cannot compensate for the lack of *Varroa* treatment. As seen in Chapter 1, feral colonies had a significantly higher level of deformed wing virus (DWV) than treated managed counterparts. DWV is transmitted by the *Varroa* mite, so high DWV levels were indicative of high *Varroa* levels within the colony (Martin et al. 2012; de Miranda & Genersch 2010; Rosenkranz et al. 2010). High *Varroa* levels and the resulting Varroosis are a significant cause of mortality for managed honeybee colonies and thus feral colonies as well (Boecking & Genersch 2008; Rosenkranz et al. 2010; de Miranda & Genersch 2010).

Of the 11 colonies that seemed to survive no queen was shown to survive the entire 2.5 year period. Often a number of possible queen genotypes were presented for a colony. This is probably an artefact of the sampling protocol, where workers were collected at the entrance of the colony. When determining queen genotypes from workers in managed colonies, young recently emerged bees are generally sampled from the centre of the hive to ensure they have descended from the queen in the colony and are not drifter or robbing bees. As the presence of brood could not be established there is also a possibility that bees present at the entrance were simply robbing an abandoned cavity. As all initial and subsequent queen genotypes (even where multiple) were different, this was seen as sufficient proof that no single queen survived the period.

Most colonies swarm annually after a year old and Seeley (1978) showed that feral honeybee colonies of central New York State produced on average 0.92-0.96 swarms a

year, whilst Winston (1980) showed a higher value of 3.6 daughter colonies a year in the population of Kansas honeybees. During a swarm the old queen leaves the colony after the production of sealed queen cells, with about 50-60% of the colonies worker bees (Fries et al. 2003). Unfortunately in this study it was impossible to assess the relatedness of the remaining queen to the former queen i.e. whether she was a daughter or unrelated as no data were collected regarding paternal allele frequency and there was a relatively small number of workers sampled.

Seeley (1978) calculated that feral honeybee colonies have an average life span of 2.1 years, although it could be as long as 5.6 years when colonies that die during the most difficult first year are discounted. Therefore it seems unlikely that colonies are surviving as long as they are estimated to. This study found that newer colonies were surviving for longer periods, whilst older and supposedly more established colonies succumbed more quickly (figure 3.3). It is possible that older colonies are at higher risks of mortality due to disease accumulation within the colony cavity. However, it is also highly probable that reports of colony longevity from beekeepers and members of the public are inaccurate. Colonies can swarm and nest sites can be reoccupied un-noticed and no colonies were monitored constantly.

From anecdotal reports it was expected that older established colonies would survive longer, i.e. there was something favourable about the site such as extensive remnant honey stores, good local forage, advantageous position etc. However it was the colonies only recently established that appeared to be continually occupied across sampling periods. This may reflect simple demographics, i.e. younger colonies and queens have longer to live. Alternatively it is possible that recently developed colonies on newly produced comb have less of a disease burden (Seeley 1978).

The fact that feral colonies appear to have a relatively high mortality rate, and that colonies which appear to have survived for the longest, tended to succumb more quickly (figure 3.3), supports the observation that feral colonies may be transient with a relatively high turnover.

The genetic difference between feral and managed bees

Fst is a measure of genetic differentiation that is directly related to the variance in allele frequency among populations, and to the degree of resemblance among individuals within populations (Holsinger & Weir 2009). There was a very small but significant genetic difference between the feral and managed honeybee populations of the UK (Gst =0.017, Fst = 2.33% $p<0.05$). The reason for this significant difference is hard to determine. It is possible that the high levels of importation within the managed community create higher allelic diversity, especially where these imports fail without beekeeper management (Costa et al. 2012).

The small difference between the two populations made the assessment of feral loci that may hint at positive attributes such as disease resistance difficult. Given the high disease levels and the proportion of feral colonies lost during the course of this study it seems likely that feral colonies were at best seriously reduced, at worst eliminated with the arrival of *Varroa,* and that colonies sampled here were not remnant survivors, but predominantly a by-product of beekeeping. The lower levels of Varroa due to widespread treatment of managed colonies may enable the survival of feral colonies (Spivak & Reuter 2001).

Feral colonies are unlikely to be able to evolve natural resistance to *Varroa* as they interbreed with local treated colonies, which have a lower selection pressure for *Varroa* tolerance. To create a *Varroa* tolerance breeding program it has been suggested that experimental colonies must be genetically remote from other honeybee stocks, although this defeats the aims of allowing the majority of beekeepers access to Varroa tolerant bees (Wallner & Fries 2003; Seeley 2007).

The knowledge that there is unlikely to be a remote honeybee population that may evolve resistance or tolerance to *Varroa* means that the future of the honeybee population of the UK is in beekeeper hands (Meixner et al. 2010; Dietemann et al. 2012). This puts a large responsibility on the shoulders of beekeepers, for both

maintaining health stocks of bees and preserving genetic diversity for the future and may require a large cultural shift from the entire beekeeping community.

Feral populations are not significantly more native type, and instead represent a highly introgressed admixture of populations. It seems likely therefore that the feral honeybee population of England and Wales is a consequence of managed beekeeping and does not constitute a separate population. As drones can attend a congregation to mate with gathering virgin queens up to 15 km away and there are only a handful of sites with no registered beekeepers within 10 km, there are few areas in England and Wales where an isolated honeybee population could exist (Jensen et al. 2005).

It was proposed that feral honeybees may be more native type (*A. m. mellifera*) as the native race may be better adapted to the UK's climate and landscape. No difference in *mellifera* levels was found between feral and RAS (managed) colonies. This re-iterates the assertion that feral populations are cast-offs from managed populations. Honeybee importation has been routine in the UK for many years so most managed colonies represent hybrids of a number of races such as the popular Italian *ligustica* or even the briefly imported Hawaian *ligustica* and *carnica*, (De la Rúa et al. 2009; DEFRA 2010; Lodesani & Costa 2003).

Another suggestion by beekeeper is that feral colonies may convert to native type over time due to loss of un-advantageous foreign genes (Solignac 2005). Further introgression of feral colonies was not seen in this study. The small differences observed could be purely due to the natural variation in the managed population, and only a larger feral sample could illuminate this further.

Seeley (1978) noted that the honeybees in the Ithaca region of New York were hybrids of several European races imported from American apiculture. It is possible that the highly introgressed levels of both feral and managed colonies proffers some advantage in the changing environmental and disease landscape through hybrid vigour although preliminary research by Costa et al. (2012) suggests that local races are more adapted to local environmental conditions. Moreover, it is not clear which honeybee is best

adapted to the UK climate, because living conditions have been changed, e.g land use change, and are likely to change even more in the future due to global warming.

Chapter 4: Assessing the effectiveness of wing morphometry for assigning *A. m. mellifera* race to UK honeybees

Introduction

The accurate identification of honeybee race and introgression levels is critical for race specific breeding programs where stocks are at risk of hybridisation. In the UK for example, the native race is *A. m. mellifera* but widespread importation of *A. m. ligustica*, *A .m. carnica* and the Buckfast bee, itself a hybrid of many honeybee races, has resulted in most stocks being considered hybrids (Ruttner 1988). Honeybee race can be determined by either morphometric or molecular means.

Morphometry

Morphometric analysis of honeybee race was pioneered by Ruttner (1988). Precise measurements of 42 body, wing and behavioural characteristics were taken from pure, non-introgressed colonies to determine distinct parameters of race. These parameters have been used extensively for the identification of further subspecies as well as for the successful assessment of the *Apis mellifera* L evolutionary pathway (Meixner et al. 2007; Sheppard & Meixner 2003).

Wing morphometry is often favoured over broad morphometric analysis in population discrimination studies, as the data can be readily accessed from scanned images of wings. Measures of behavioural parameters for example, can be subjective and some taxonomic measurements can be difficult to record consistently. Furthermore, the honeybee wing has been cited as a reliable measure as it is thought to evolve slowly, and without any apparent environmental influence (De La Rua & Jerrano 2005; Tofilski 2008).

Wing measurements are generally summarised into three commonly used indices: the cubital index, the hantel index and the discoidal shift angle (see figure 4.1) (Bouga et al. 2011). In 1994 Adam Tofilski devised new software that used geometric morphometry to assess honeybee wings. This software automatically creates a series of landmarks on a wing, measures wing shape, and creates a consistent wing diagram

regardless of the orientation of the wings of a sample (Tofilski 2004). This significant improvement allowed a number of samples to be scanned and processed simultaneously, while older systems such as Beemorph required manual wing positioning and cropping of individual wings. Not only was Tofilski's new software less time consuming and less human error prone, it was also shown to be better at discriminating honeybee species than standard morphometry (90.6% correct assessments compared to 86.3% in beemorph) (Tofilski 2008). Tofilski's high through put system requires only a scanner and laptop, and has been widely embraced by UK beekeepers as a tool for assessing hybridisation in their stocks (Patterson 2012).

Molecular analysis

Much of the work on honeybee evolution and race to date has, however, not used wing morphology, but instead examines the mitochondrial DNA (mtDNA) region between the cytochrome oxidase subunits I and II genes (CoxI–CoxII intergenic region) (ÖzdÏL et al. 2009; Garnery et al. 1993; Garnery & Solignac 1992; Garnery et al. 1998). *Dra*I restriction of this area has revealed more than 50 restriction fragment length polymorphisms (RFLPs) (De La Rua & Serrano 2005; Arias & Sheppard 1996). Recently, the sequencing of the honeybee genome has provided new possibilities for genetic studies of race and evolution through nuclear DNA (Weinstock et al. 2006). Microsatellites are abundant within the *Apis mellifera* genome and support the evolutionary path way proposed by morphometric studies, for example African races are seen to have a higher number of alleles and heterozygosity than the more recent European races (Jensen et al, 2005, Estoup et al., 1995, Solignac et al., 2003). Microsatellites are a powerful tool for honeybee characterization, and thus for conservation as they are highly efficient at differentiating populations, subspecies, levels of introgression and determining relatedness (Jensen et al. 2005; Dall'Olio et al. 2007; Queller et al. 1993; Brookfield & Parkin 1993; Solignac et al. 2007; Baudry et al. 1998). Microsatellites also have distinct advantages over morphometric and mitochondrial analysis as the determination of race is faster and requires a smaller sample size (Estoup et al. 1995). Microsatellite markers can also be more variable than mitochondrial markers and are thus superior at detecting population differentiation and population structure (Jensen et al. 2005).

Comparative studies of both methodologies

Although many researchers use morphometry, molecular tools or a mixture of both, there is a large variation in the exact methodologies used (Bouga et al. 2011). For example, the assessment of wing morphometry can differ in the venation junctions used, the morphometry analytical software used and in the statistical analysis (Bouga et al. 2011). Mitochondrial and microsatellite studies differ in the precise markers used (Bouga et al. 2011). This discrepancy between researchers prevents solid comparisons and frustrates larger scale research.

In 2007, the project for 'prevention of honeybee COLony LOSSes (COLOSS)' cited having a common method for determining race as one of the main goals of the working Group 4: Diversity and Vitality (Bienkowska et al. 2009) . In 2010 the National Bee Unit based at the Food and Environment agency (FERA) in the UK, began work on establishing a universal set of microsatellites that could distinguish between the most common races in the UK. Over 100 microsatellites were chosen from each major gene block across the newly sequenced honeybee genome, for maximum discriminatory ability (Weinstock et al. 2006).

This novel assessment of the UK's honey bee racial components has allowed, for the first time, a comparison between microsatellite and morphometric analysis of hybridisation. In this study, we set out to assess how wing morphometry performs on UK honey bees, and compare wing morphometry results to purity assessments using the newest microsatellites both at the colony and individual worker level.

Methods

Samples

Morphometric data were collected from over 30 worker wings per colony, for all feral and managed *A. mellifera* colonies sampled during the course of this thesis (280 colonies, over 8500 wings).Colony level microsatellite and morphometric data was assessed from 20 feral and 32 managed colonies (n=52).

Individual morphometric and microsatellite results were assessed for 10 feral colonies collected in 2009 (n= 86), where corresponding wings and DNA extracts were labelled so a direct comparison could be made.

Morphometry

Wings were removed from the bee specimens, labelled (for subsequent microsatellite comparison), and stored in 100% ethanol until processed. Wings were allowed to dry until free of alcohol residue then placed under glass slides to ensure an image of a flat wing. Wings were scanned using an Epson Perfection V300 Photo scanner, at 4800dpi resolution using positive film strip mode. DrawWing software version 0.45 was used exclusively in this study as the best example of modern wing morphometry, to record the cubital, hantel and discoidal shift index (see figure 4.1) (Tofilski 2004; Tofilski 2008). These indicies were determined by Ruttner (1988) to be the most reliable for race identification. The DrawWing software struggled to correctly identify venation junctions in wings that had damage to the tip of the wing. In these cases landmarks were placed by eye. The output data for individual wings produced by DrawWing were entered into the Excel macro Morphplot version 2.2 to yield results by colony for *A. m. mellifera* parameters (i.e. an assessment of *A. m. mellifera* purity) (P. Edwards 2007). A morphometric purity percentage is obtained by plotting two indices against one another to record how many worker honey bees from the sample fall within the indices parameters (see figure 4.2). All images have been labelled and retained.

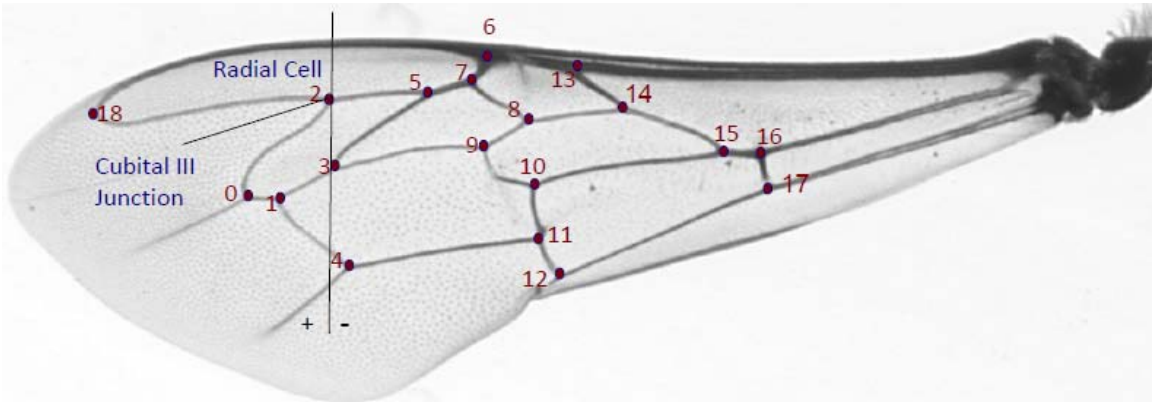


Figure 4.1: Wing diagram produced by DrawWing version 0.45 (Tofilski 2004).

Cubital Index is calculated by dividing distance 1 to 0 by distance 3 to1. Discoidal Shift Angle is determined by the offset of point 4 in relation to the Radial Cell and the Cubital III Junction. Generally when point 4 is shifted towards the body of the bee the value is negative, but if towards the wing tip the value is positive. The Hantel Index is the distance between points 0 and 3 divided by the distance between 8 and 2 (http://www.cybis.se/cbeewing/pertxt/index.htm).

Calculations of purity of *Apis mellifera mellifera* are based on the following values (Ruttner et al., 1990): Cubital Index: 1-2.1, Discoidal Shift Angle: -10 to 0 and Hantel index: 0.7 to 0.923

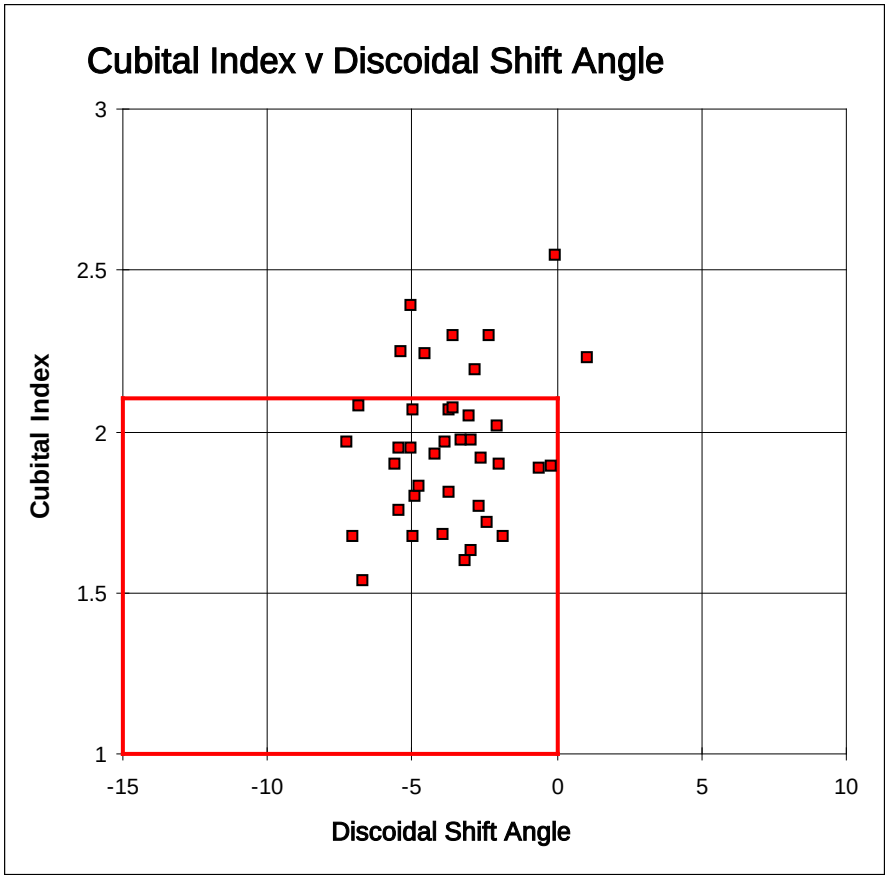


Figure 4.2: An example of the assessment of colony purity through morphometry for cubital index verses discoidal shift angle in MorphPlot version 2.2 (Edwards 2007). The red box indicates the parameters for *A. m. mellifera* for the two indices. 31 out of 39 worker honeybee wings fell within the parameters for *A. m. mellifera* so colony *A. m. mellifera* purity was cited as 79%.

DNA extraction from samples

Worker bees were collected from each colony and stored for use in 100% ethanol at -70^{o}C. Fifteen workers from each colony were randomly selected. Whole bees were washed in molecular grade water, and crushed with 2.3 mm silica beads in a Precellys lysis and homogenization bead beater at 5000 rpm for 30 seconds. Total DNA was extracted from an entire worker bee using a 10% Chelex solution with 1 x TE buffer. Next, 800 µl of 10% Chelex solution was added to each crushed bee residue. This was heated to 95 ^{o}C for 5 minutes then centrifuged at 8000 g for a further 5 minutes. 200 µl of the upper aqueous DNA extract was removed and centrifuged again at 8000 g for 5

minutes then 150 µl of the upper aqueous was removed and stored at -70^{o}C until required.

Microsatellite analysis

This microsatellite protocol was taken from FERA's 2009 to 2010 Random Apiary Survey project (RAS) and the 2010-2011 Defra seedcorn project assessing the diversity and provenance of managed and feral honeybees in the UK (Budge et al., in prep). Extractions were diluted to a 1:500 concentration. In total, 12 microsatellites were selected for their variability and ability to discern between the common honeybee races in the UK (See *Bayesian analysis of populations* below and table 4.3). PCRs were performed individually in 10 µl volumes at two different $MgCl_2$ concentrations as below in table 4.1:

Table 4.1: Primer master mix and conditions
1.2 µM $MgCl_2$ (840/814, 936/937):
4.475 µl of H_2O, 1.5 µl of 10x Buffer IV, 0.7 µl of $MgCl_2$ (25 µM), 0.045 µl of dNTPs (20 µM), 2 µl of BSA (1µg/ µl), 0.6 µl of Forward Primer (10 µM), 0.6 µl of Reverse Primer (10 µM), 0.08 µl of Taq.
1.5 µM $MgCl_2$ (828/829, 836/837, 876/877, 882/883, 938/939, 852/853, 864/865, 866/867, 950/951, 990/991)
4.275 µl of H_2O, 1.5 µl of 10x Buffer IV, 0.9 µl of $MgCl_2$ (25 µM), 0.045 µl of dNTPs (20 µM), 2 µl of BSA (1µg/ µl), 0.6 µl of Forward Primer (10 µM), 0.6 µl of Reverse Primer (10 µM), 0.08 µl of Taq.

Each colony sample included a positive control and a blank. PCRs were run on a real-time PCR ABI Prism 7900HT (Applied Biosystems Inc., Foster City, CA). The first stage of the PCR was denaturing of the dsDNA at 94 ^{o}C for 5 minutes. This was followed by 35 cycles of denaturing at 94 ^{o}C for 30 seconds and annealing for 30 seconds at 72 ^{o}C. There was a 50 minute soak at 60 ^{o}C at the end to ensure amplification. Polymerase chain reaction products were diluted in multiplex groups (table 4.2 below). 1 µl of the diluted multiplex mix was added to 10 µl of formamide and 0.3 µl of size standard ROX

500. Samples were sequenced on a 3130xl Genetic Analyzer. Peaks were scored using Genemapper software version 3.7.

Table 4.2: PCR dilutions in multiplex pairs

Microsatellite pair	Volume of PCR contributed (µl)	H_2O for dilution (µl)
828/829	1	
836/837	1	146
840/841	1	
936/937	1	
950/951	2	147
990/991	1	
876/877	1	
882/883	1	147
938/939	1	
852/853	1	
864/865	1	147
866/867	1	

Genotyping

Bayesian analysis of populations (BAPs)

Bayesian mixture models attempt to identify a hidden population structure by clustering individuals into genetically divergent groups. FERA's project assessing the diversity and provenance of managed and feral honeybees in the UK, examined 259 reference queens from Australia, France, Germany, Greece, Hawaii, Malta, New Zealand, Slovenia, Spain and the UK. in total, 39 microsatellites or SSRs were tested to cluster and discriminate between the races of these queens (Corander & Marttinen 2006). This project used 12 of the most variable and discriminatory microsatellites to assess the component races of feral honeybee colonies (highest Gst) (See table 4.3). The genetic makeup of the feral worker honeybees was compared to that of the reference queens using BAPS software version 5 (Corander & Marttinen 2006). The BAPS data is presented as a proportion of the 11 races or clusters found within each worker bee (Hawaian *Carnica*, Hawaiian *Ligustica*, Spanish *Iberica*, Maltese *Rutneri*, New Zealand *Ligustica*, Australian *Ligustica,* Slovenian *Carnica*, Greek *Macedonica*, UK *Mellifera*, French *Mellifera* and German *Carnica*) (see figure 2.9 in Chapter 2). This

proportion was compared with percentage colony purity figures from morphometric software and individual worker morphometric indices.

Table 4.3: The 12 microsatellite sequences used for assessing racial proportion.

Microsatellite Loci	Sequence
828/829	UN012-F [HEX]CGACCTAACAGTTGCTTCGTG
	UN012-R ATTGTTCCTTGCCACGATACA
836/837	K0190-F [6FAM]ACGAATCGAGCCTCGACC
	K0190-R ATGCAAGTTTCAAAGGCACG
840/841	AP271-F [HEX]CATGATGCGAGTCTCCTGGC
	AP271-R GCATTAATTGCGCTGCGTC
852/853	UN151-F [HEX]AAATTAGCGTACGTGAGCGG
	UN151-R CGTCATAGTCCACCTACCAACG
864/865	K0264-F [HEX]TCTCGTGGAATGGCCTAAGAG
	K0264-R ATACACGCGCACATTCGC
866/867	6230-F [6FAM]CGATCAGCAAAGGCGTATCC
	6230-R GGATGTTCGCGTTAACGTAACTG
876/877	K0352B-F [HEX]ATCAATCTCCTCGCGATCG
	K0352B-R CAAGTTACACAATCCTCGCACC
882/883	UN375-F [HEX]TTCGGACAGGATGCTGCA
	UN375-R GGCCGACTTTCGTCACTGA
938/939	K0911-F [6FAM]GGAACAAAGTGGAGCGAATTACT
	K0911-R CTCGAGGATCCTTAATCGTGG
936/937	AT064-F [HEX]TATGCAGAGCGCGATACATC
	AT064-R TCTGCTTGTTTCGTCGCAG
950/951	AT129-F [6FAM]GCTAACGGGGTAACGGGATC
	AT129-R CCATCCAGACCACGCCTACA
990/991	AT192-F [HEX]GCCTGCTTCTCCTCACTTTC
	AT192-R AGATTTTGAAAGAGGGTCGC

Statistical analysis

Correlations were calculated in SPSS statistics software version 20 (IBM 2012). Indices values for individual worker honeybees were converted to binomial format using the thresholds described by Ruttner (1988) (Cubital index: 1-2.1, Discoidal Shift Index: -15 – 0, and Hantel Index: 0.7 – 0.923). A Mixed effects model and generalized linear model with binomial errors were performed in R (Hornik 2012), to compare the ability of microsatellite data to predict whether morphometric data would fall within *A. m.*

mellifera thresholds. A lower AIC was obtained for the model with random intercepts verses random slopes for all indices. A comparison between the LME with colony as a random effect, and a GLM was conducted to show no random effect of colony.

Results

Assessment of colony-level purity

Figure 4.3 shows a map produced with wing morphometry data from 280 colonies across the UK. Over 30 individual worker wings were sampled per colony, and the number of wings that fell within pre-defined parameters for the cubital and discoidal shift index were used to give a percentage purity of *A.m. mellifera* for each colony. The results indicate that areas of high beekeeping density and thus high bee importation like the centre of London have low percentage purity, while Scottish islands, Anglesey and Cornwall have high percentage purity. However, when data for colony-level purity is compared both by wing morphometry and microsatellites a clear lack of relationship can be noted (figures 4.4 to 4.7).

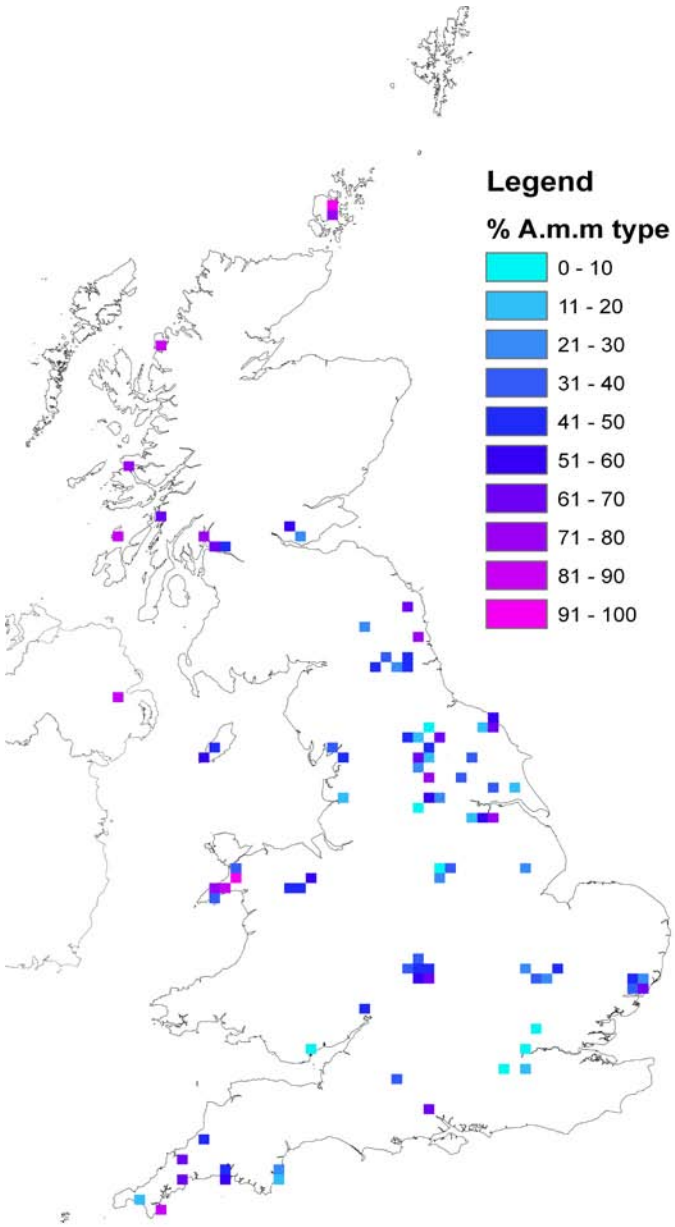


Figure 4.3: *Mellifera* purity according to wing morphometry.

The average percentage of *A. m. mellifera* purity per 10km square is given based on the percentage of workers per colony falling within the *A. m. mellifera* parameters for cubital Index and discoidal shift angle (see figure 4.2; Ruttner 1988).

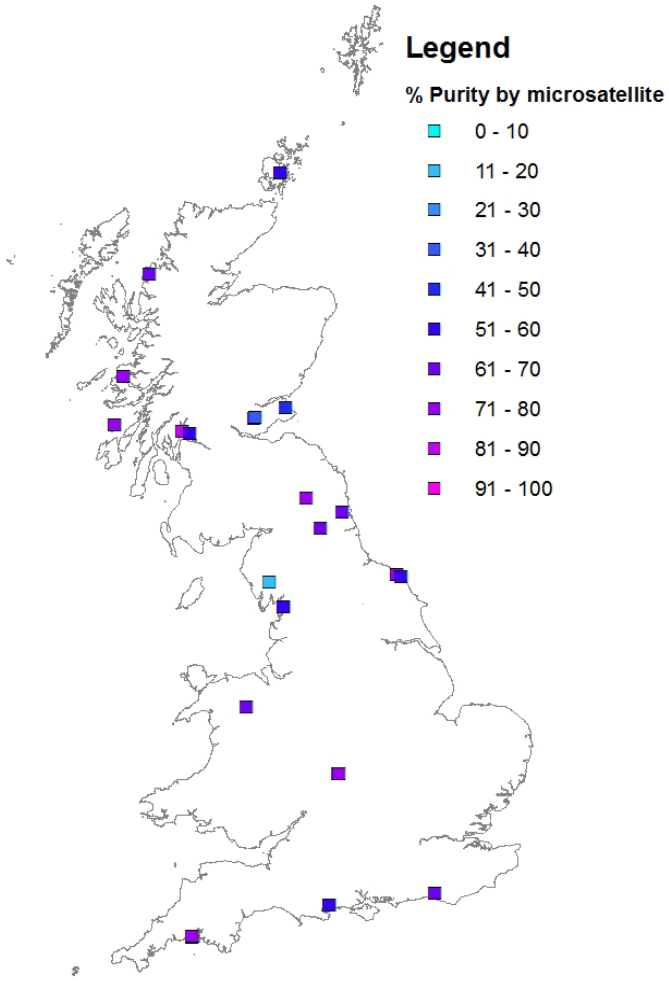


Figure 4.3b: *Mellifera* purity according to microsatellite data for individual colonies. This does not give such an intuitive picture of *mellifera* purity because in modern beekeeping, a remote beekeeper is as able to import foreign queens through the post as one in a more densely populated location.

Figures 4.4 to 4.7 represent a comparison of a colony level assessment of morphometric and microsatellite purity. The convention is to plot two indices against one another so the number of worker honey bees falling within the pre-defined parameters can be expressed as a percentage. There is a very poor correlation between the two methods (see table 4.4). Even though assessment may be limited by

the relatively small number of samples at the very high (>80%) or very low levels of *A. m. mellifera* purity (<20%), the correlations are so weak it is safe to conclude that the two methods provide widely differing assessments of purity levels of *A. m. mellifera*.

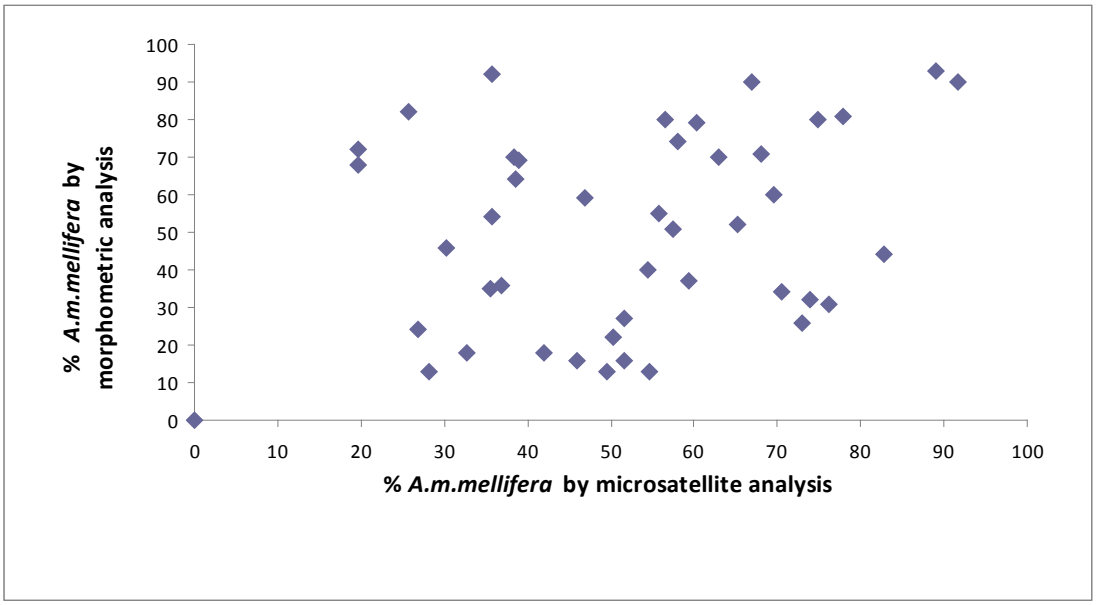


Figure 4.4: The morphometric analysis is based on the percentage of workers with Cubital Index and Discoidal Shift Angle values that fall within pre-defined parameters (Ruttner 1988).

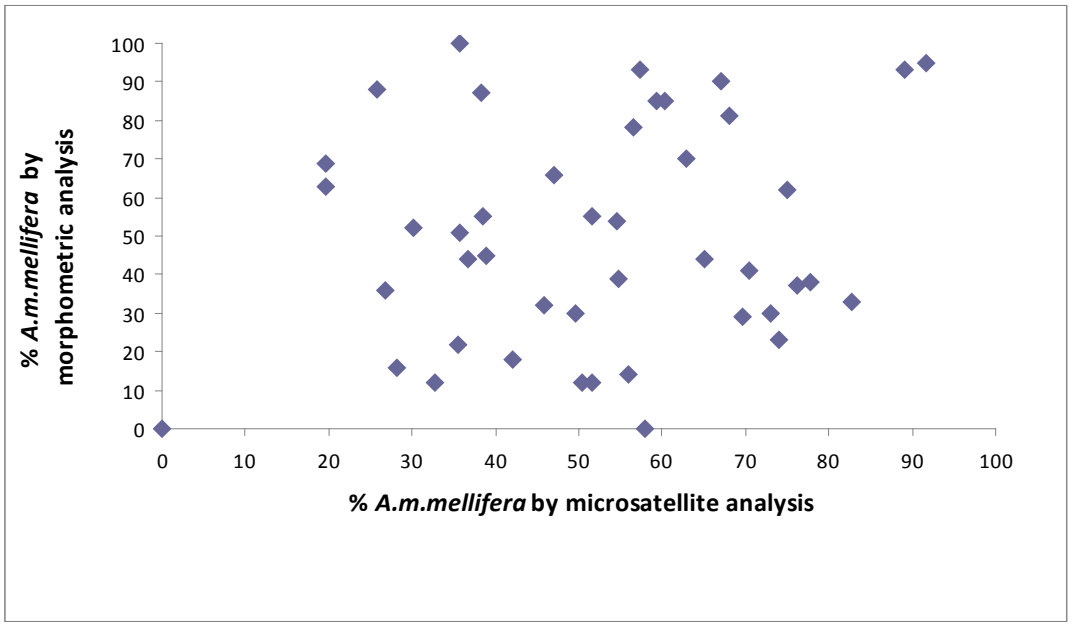


Figure 4.5: The morphometric analysis is based on the percentage of workers with Discoidal Shift Angle and Hantel index values that fall within pre-defined parameters (Ruttner 1988).

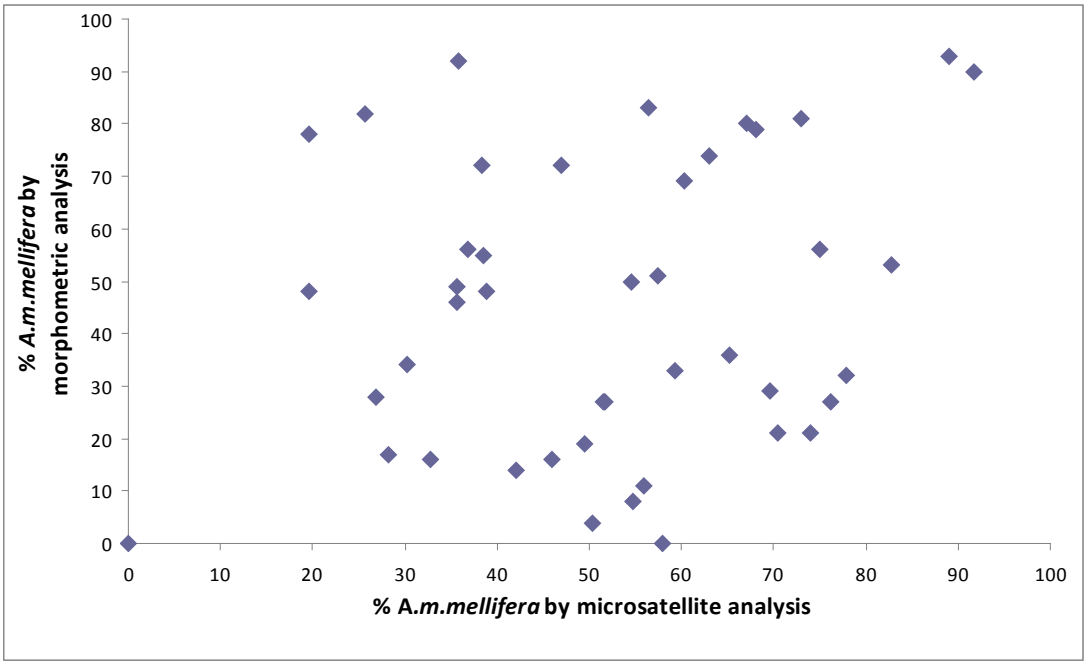


Figure 4.6: The morphometric analysis is based on the percentage of workers with Cubital Index and Hantel index values that fall within pre-defined parameters (Ruttner 1988).

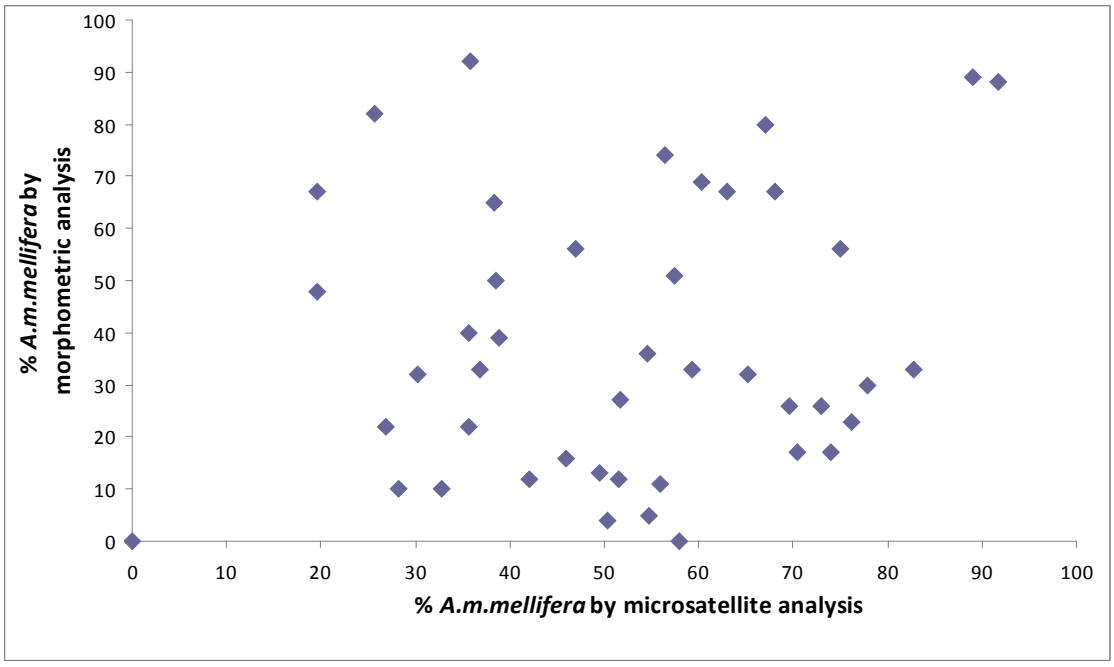


Figure 4.7: The morphometric analysis is based on the percentage of workers with Cubital Index, Discoidal Shift angle and Hantel index values that fall within pre-defined parameters (Ruttner 1988).

Table 4.4: Pearson's correlation values for colony level morphometric and microsatellite analysis of *A. m. mellifera* purity	
Relationship	**Pearson's correlation value**
% *A. m. mellifera* by microsatellite and by morphometry (Cubital index/Discoidal Shift Angle)	.298
% *A. m. mellifera* by microsatellite and by morphometry (Discoidal Shift Angle/Hantel index)	.191
% *A. m. mellifera* by microsatellite and by morphometry (Cubital index/Hantel index)	.205
% *A. m. mellifera* by microsatellite and by morphometry (Cubital index/Discoidal Shift Angle/Hantel index)	.183

As there appears to be an almost random relationship between morphometric data and microsatellite data at the colony level, a mixed effects model with binomial errors was carried out to compare whether individual honey bee microsatellite *A. m. mellifera* percentage values could predict whether individual morphometric indices were within *A. m. mellifera* thresholds i.e. below 2.1 for cubital index, below 0 for discoidal shift angle and below 0.7 for hantel index (see figure 4.8). Microsatellite *A. m. mellifera* percentage values had an ability to predict whether morphometric data would be within morphometric thresholds for discoidal shift angle data (p=.041) and hantel index data (p=.034) but not cubital index (p=.056) (see table 4.5).

Values of over 44% for *A. m. mellifera* microsatellite data predicted that the individual wing would be found within the *A. m. mellifera* threshold for morphometry data. Values below 24% for *A. m. mellifera* microsatellite data predicted that the individual

wing would be found outside the *A. m. mellifera* threshold for morphometry data (figure 4.8). In figures 4.9 to 11 show that samples with very high or very low levels of *A. m. mellifera* indicative molecular markers are usually separated by the morphometric division. Values at an intermediate level of molecular purity (20-70%) appear to be almost randomly distributed inside or outside the *A. m. mellifera* morphometric thresholds. It is only in figure 4.9 for cubital index where values of high and low microsatellite *A. m. mellifera* values are not well separated by the morphometric *A. m. mellifera* divide.

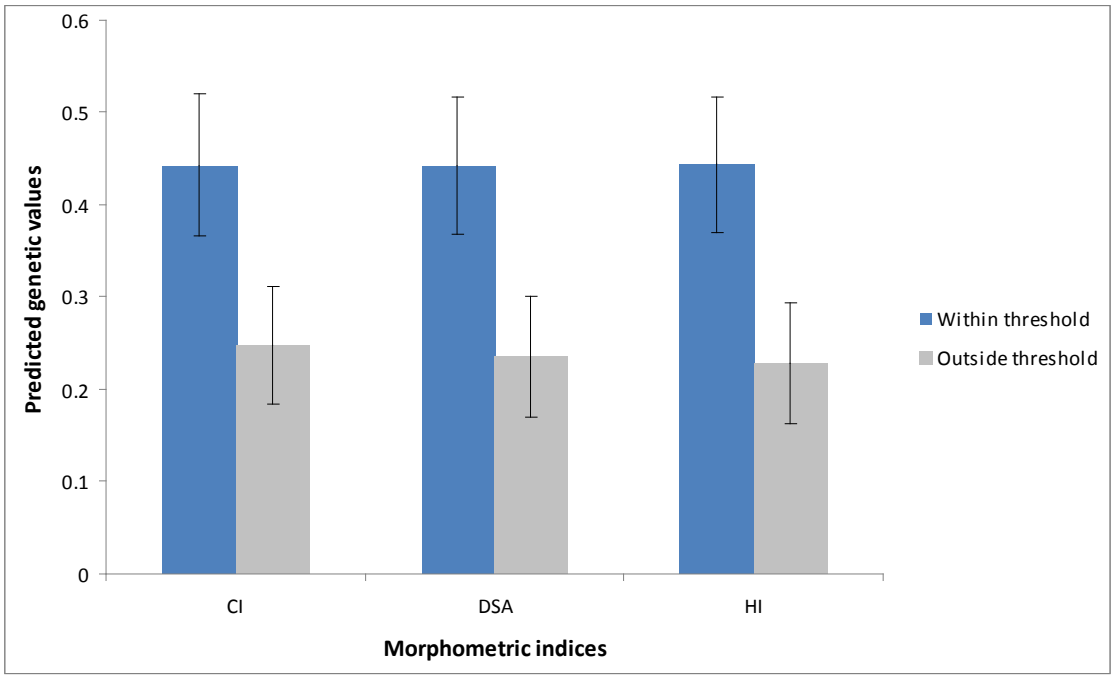


Figure 4.8: Predicted values for microsatellite *A. m. mellifera* percentage, within or outside morphometry thresholds (+/- 1 standard error).

Table 4.5: Predicted values within and outside *A. m. mellifera* thresholds for the microsatellite data. * denotes significant at the 0.05 level.			
Index	**Predicted value within *A. m. mellifera* thresholds (3.d.p)**	**Predicted Value outside *A. m. mellifera* thresholds (3.d.p)**	**P value (Degrees of freedom =1) (3.d.p)**
Cubital Index	0.443 (SE +/- 0.078)	0.248 (SE+/- 0.064)	0.056
Discoidal Shift Angle	0.442 (SE +/- 0.075)	0.235 (SE+/- 0.065)	0.041 *
Hantel Index	0.444 (SE+/- 0.074)	0.229 (SE+/- 0.066)	0.034 *

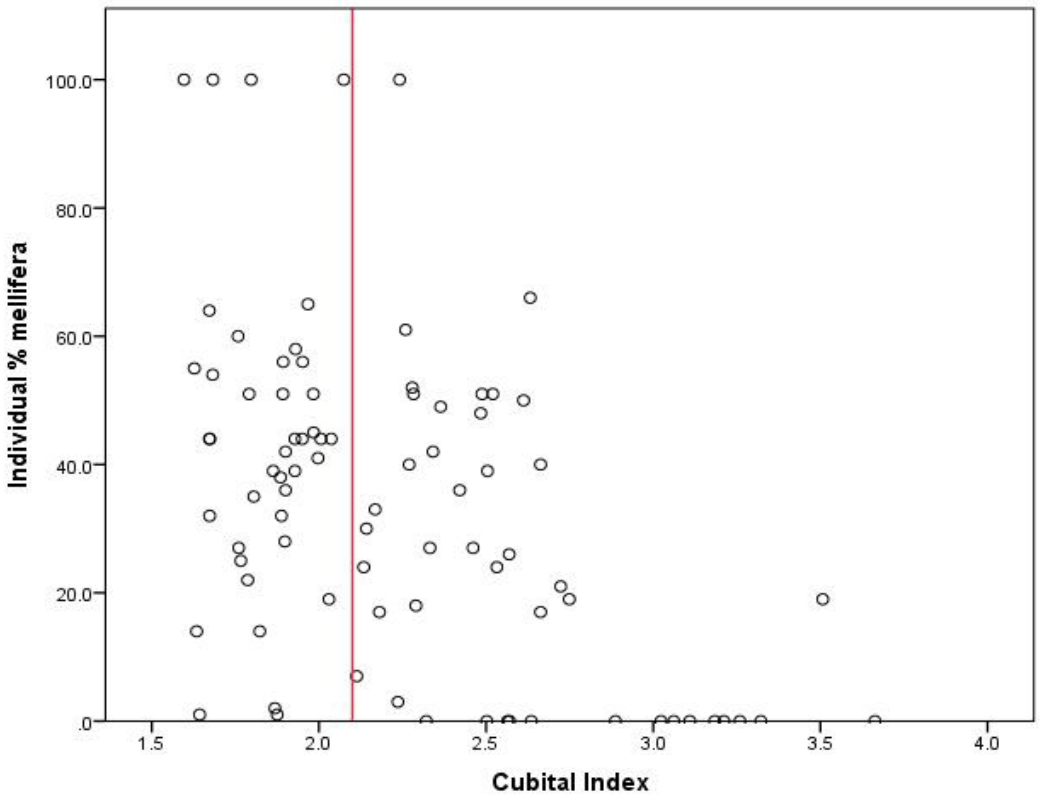


Figure 4.9: Individual microsatellite percentage values against cubital index values. The red line denotes the Cubital index upper threshold of 2.1.

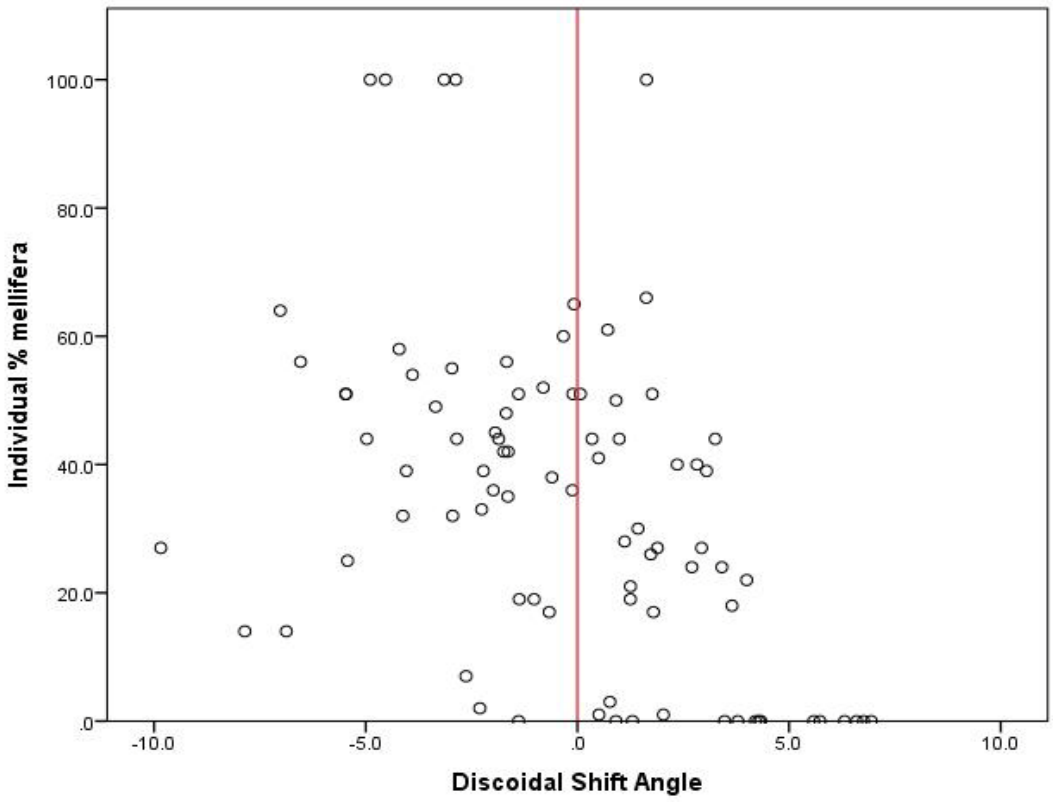


Figure 4.10: Individual microsatellite percentage values against discoidal shift angle values. The red line denotes the discoidal shift angle upper threshold of 0.

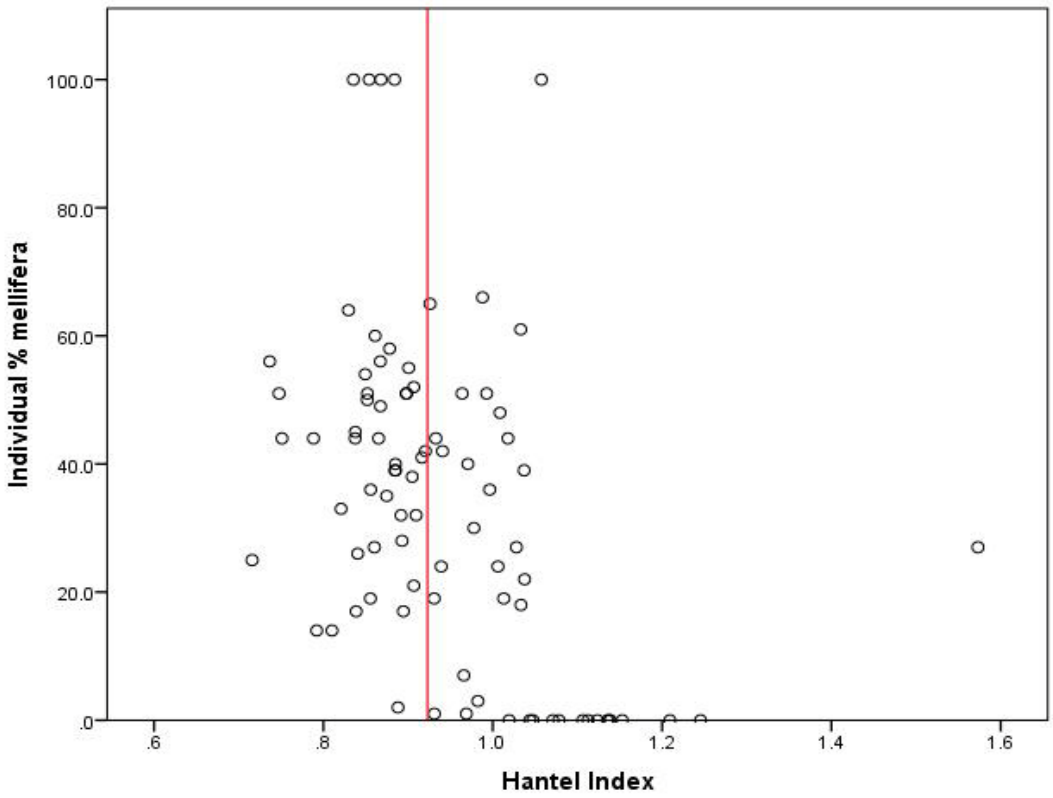


Figure 4.11: Individual microsatellite percentage values against hantel index values. The red line denotes the hantel index upper threshold of 0.923.

Discussion

While wing morphometry appears to give intuitive values of purity (i.e. the purest colonies are in the most remote location such as North Wales, the West coast of Scotland and the tip of Cornwall), the values of colony percentage *A. m. mellifera* from microsatellite data does not correlate strongly with morphometry data.

It is perhaps unsurprising that morphology of a single trait such as forewing venation is not a suitable substitute for the 42 morphometric measurements originally suggested

by Ruttner. In Tofilski's (2008) study, forewing venation was described by coordinates of 18 vein junctions and centroid size or by four distances and eleven angles (Tofilski 2008). It seems likely that reducing an assessment of race to three indices which focus on a small area of the forewing is an oversimplification, even though it has been advocated widely to be reliable and sufficient (Bouga et al. 2011). In his original work, Ruttner (1988) used other morphometric features in addition to the wing indices to draw conclusion on the race of an individual honeybee. He cites that bees with long abdominal cover hair and a larger body size with a broader abdomen should be screened for a CI lower than 1.85, and only then regards the taxonomic diagnosis to be completed. Other features he considers include the length of the 3rd and 4th tergite, proboscis length, fore wing length, hind leg length, distance between the wax plates and the colour of the third tergite. Cooper (1986) also details nineteen behavioural characteristics indicative of *A. m. mellifera* , which include low temperature flight, reluctance to fly when snow is lying, longevity, a conservative nature with honey stores, a small peak brood size, tight winter clustering near the hive entrance, convex white honey capping, compact brood pattern and compact honey storage pattern. In short, it is unwise to rely on a single character to guide breeding programs as repeated selection can result in honey bees with the particular morphological trait selected, i.e. a particular arrangement of venation on the forewing, but not necessarily the other favourable qualities of the race selected for (Soland-Reckeweg 2006).

The classic morphometry designed by Ruttner (1988) was primarily used for race discrimination to invoke an evolutionary pathway. Samples were collected from relatively pure and geographically isolated colonies. The recent use of morphometry as

a tool to assess introgression is a corruption of the original tool (Soland-Reckeweg 2006; Estoup et al. 1995). In essence, as described in figure 4.12, where once morphometry was used to examine whether colony A was different from colony B, it is now being used largely to examine the race components of C. Figures 4.9 to 4.11 show that this region is the most unreliable in terms of predicting whether an individual will fall into *A. m. mellifera* thresholds.

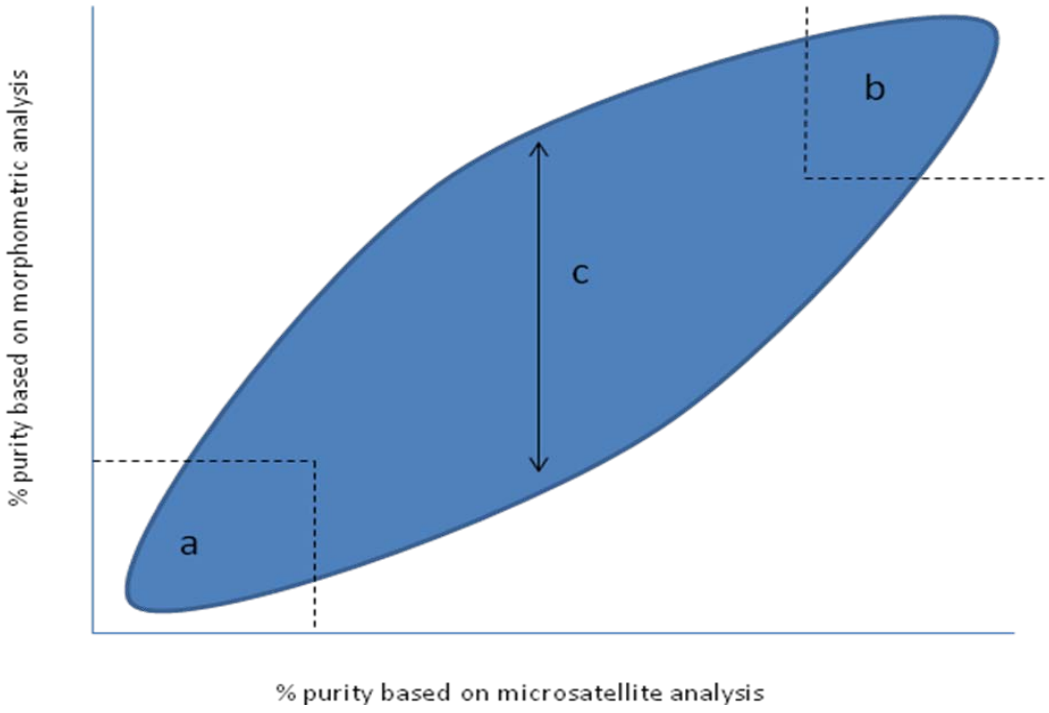


Figure 4.12: Visualisation of the validity of morphometry
A and B are where the results of morphometry tend to resemble results of genetic analysis, i.e. distinguishing one race from another and being used as they were intended when proposed by Ruttner, C is where the results of morphometry tend to deviate wildly from microsatellite results. These colonies tend to represent hybrids of two races and are difficult to discern with morphometry. Situation C represents the colonies normally assessed by morphometry in the UK.

The misuse of wing morphometry is a significant problem. It is widely promoted as a tool for assessment of race purity e.g. Patterson 2012, and is also one of the few

accessible scientific tools available to beekeepers trying to maintain the purity of *A. m. mellifera* in their colonies and assess introgression levels (Tofilski 2008). It is cheap and easy to use, with supported software and lots of existing data for comparisons. Other behavioural characteristics that could be recorded, such as colour of the queen and worker bees, temperature of flight, position of stores in the brood comb, wax capping colour and thriftiness, are often subjective and assessments would differ between beekeepers (Tofilski 2008).

In 1991, Moritz showed that German breeding programs relying on wing morphometry had failed to maintain purity and had high levels of introgression (Moritz 1991). Mortiz (1991) emphasised that identification of hybrids with this technique is unreliable. The Cubital index was unimodal across the *A. m. mellifera* and *A. m. carnica* hybrids, which meant that when beekeepers thought they had selected for relatively pure *carnica* bees they in fact had predominantly hybrids (Moritz 1991). Moritz (1991) warned that if beekeepers were to re-identify other biometry by multivariate statistics they would run the risk of repeating the failure of their current project by placing intense selection pressure on a few characters that do not reflect the race as a whole (Moritz 1991). Tofilski (2008) suggests that use of the DrawWing software could be improved by using all the landmark data collected: 18 wing venation junctions and associated angles. Distance of these 18 landmarks can then be superimposed on reference wings, and the differences can be calculated (Tofilski 2008). However, for success this process requires a convincing pure reference sample as well as a level of statistics that may be prohibitive to beekeepers. Moritz (1991) suggests abandoning biometry to return to a state where colonies are selected purely

on positive attributes such as honey production and non-aggressive behaviour. Fortunately since this research, molecular techniques for assessing hybridisation have improved in accuracy and accessibility (Jensen et al. 2005; Soland-Reckeweg 2006; Tofilski 2008; Solignac et al. 2003; Estoup et al. 1995).

The future of black bee breeding in the UK: genetic screening

The markers used as part of the Defra Seedcorn project assessing the diversity and provenance of managed and feral honeybees in the UK were chosen for maximum variability and were able to definitively separate race. This microsatellite tool kit was selected from across the honeybee genome to avoid the risk of selecting single attributes or characters, unlike wing morphometry. In Sweden, genetic testing is used routinely to assess hybridisation in breeding populations and has enabled a marked reduction in hybrid queens, after generations of reoccurring hybridisation using morphometric methods (Bouga et al. 2011). Breeders are said to now be focusing more on the productivity of their colonies than their cubital index (Bouga et al. 2011).

In the UK, our honeybee breeding programs lag behind the rest of Europe. Routine genetic testing is still rare, even when colonies are considered to be part of a breeding program and there is currently no scientific institution offering purity assessment. Having now established a robust protocol for race assessment in the UK it seems likely that high throughput and low cost hybridisation assessment will be made available to beekeepers. This should be used in conjunction with other bee breeding approaches such as the use of remote breeding apiaries, protection areas and the selection of positive behavioural attributes (Soland-Reckeweg 2006).

Chapter 5: To what extent are current breeding programs protecting *Apis mellifera mellifera* in the UK?

Introduction

Apis mellifera mellifera is considered to be the native honeybee race of the United Kingdom, yet little is known about the extent of its population or if particular ecotypes exist. The purity of *A. m. mellifera* is under threat from introgression of foreign genes due to mass importation of *A. m. ligustica, A. m. carnica* and the use of hybrid strains such as the Buckfast bee.

A. m. mellifera ranges from Northern Spain to 60°north in Scandinavia, and extends from the edge of the Atlantic ocean as far east as the Ural Mountains. It was the predominant race found in the United Kingdom until the "Isle of Wight disease" struck in the early 1900s (Meixner et al. 2007; Moritz et al. 2010; Carreck 2008). Then beekeepers started to repopulate their stocks with bees from Europe, and more recently from around the world (Mutinelli 2011). British bee keepers tend to favour *A. m. ligustica* for their large productive colonies size which can render high honey yields in good years, and *A. m. carnica* for their gentle nature and minimal propolis use (Ruttner 1988). Consequently, *A. m. mellifera* has suffered a strong reduction in its original distribution (Soland-Reckeweg 2006).

Recent high profile losses of honeybee colonies across many parts of the world have highlighted the need to protect honeybee genetic diversity, and have returned the focus to the virtues of the native honeybee (De la Rúa et al. 2009). First results from the COLOSS (Prevention of Colony Losses) project shows a significant effect of honeybee genotype on the ability to cope with local environmental conditions (Meixner et al. 2010; Costa et al. 2012). Costa et al. (2012) state that genetic adaptation to the local conditions influences a colony's population dynamics, health status and productivity. They conclude that local breeding activities should be encouraged and European honeybee diversity should be maintained to retain an important resource for breeding disease and stress resistance (Costa et al. 2012).

A. m .mellifera enjoyed a large native range, suggesting an inherent ability adapt to changing habitats. Indeed, it has been seen that *A. m. mellifera* can exist as different

ecotypes (Soland-Reckeweg 2006). The Landes ecotype of *A. m. mellifera* in France for example is specialized in that it increases its brood production so that the maximum colony worker numbers coincide with the flowering times of the local heather (Strange et al. 2007b). Louveaux (1973) was able to determine that this characteristic had a genetic basis as when they crossed bees of the Landes ecotype with non ecotypic bees they found that the cross produced intermediate brood cycles between the two parental races (Strange et al. 2007a). Louveaux (1973) went on to identify three other ecotypes within France that each show a distinct brood cycle and adaptation to the local floral phenology (Strange et al., 2007b). The extinction or hybridisation of *A. m. mellifera* could see the loss of many potentially beneficial genetic traits such as adaptation to high altitudes, cold climates and even some diseases (Soland-Reckeweg 2006). Moritz et al (2007) warn that population sizes of native honeybee races are already small, thus the task of conserving these stocks is becoming increasing difficult and urgent (Kraus 2005; Moritz et al. 2007).

Earlier research

In 1986, a study based on wing morphometry concluded that there were still 'near-native' dark bees in many regions of the UK (Bouga et al. 2011; Cooper 1986). In 2005, the EU funded the BABE project "Beekeeping and *Apis* Biodiversity in Europe". As part of this research Jensen et al (2005) assessed the level of *A. m. ligustica* introgression in populations of *A. m. mellifera* using four colonies from the UK (Colonsay, Whitby, Sheffield and the East Midlands). All populations showed some evidence of introgression, although unsurprisingly the colony on the remote Scottish island of Colonsay was the purest *A. m. mellifera* line. Jensen et al (2005) concluded that the *A. m. mellifera* population of the UK was genetically distinct from the other European populations examined and that conservation should be advised (Jensen et al. 2005). Colonsay, one of the sites sampled by Jensen et al (2005), is a small island situated on the West coast of Scotland, 8 miles or 12.9k from the nearest land, and is relatively low lying with little tree cover. It can experience severe gales in late autumn and early spring and has a relatively high rainfall throughout the year. It was noted that the bees on Colonsay were larger, browner and had specific behavioral qualities that suggested

ecotypic adaptation, such as flying close to the ground even on still and sunny days as an adaptation to strong wind (Jensen et al. 2005).

Conservation efforts

In the UK, whilst some beekeepers are trying to maintain pure *A. m. mellifera* stocks, there is currently no formal conservation project in place. Jensen et al (2005) conclude that to effectively conserve the remaining *A. m. mellifera* population authorities would have to grant them the same status as endangered races of other domesticated animals like cattle or sheep. Finding an appropriate measure has proved difficult. The Countryside and Wildlife Act 1981 (used previously to create reserves for Red deer on other Scottish Islands) was rejected as a measure of protection for the Colonsay honeybee population as the honeybee is not considered a domesticated creature.

The Rio Biodiversity Convention for the Conservation of Biological Diversity exists to protect genetic diversity and ecotypes, however *A. m. mellifera* is not included on the FAO's World Watch list for domesticated animals due to the same contention over whether they are a domesticated species (Jensen et al, 2005).

European legislation prevents restrictions on honeybee imports due to free and open trade laws. However it does include a directive to support organic beekeeping, which is based on using indigenous honeybees rather than imported stock (Lodesani, 2005). It states that 'a wide biological diversity should be encouraged and the choice of breeds should take account of their capacity to adopt to local conditions'(Lodesani, 2005). De la Rúa et al. (2009) advise that making this directive compulsory across Europe would be the best way to offer local ecotypes legal protection. However, in regions where legislation has been used to try and restrict beekeepers choice of honeybee race, such as on Læsø island, Denmark (EU regulation 1804/99), success has been limited and hard fought (Bouga et al. 2011; Jensen & Pedersen 2005; Meixner et al. 2010).

Attempts to formally recognize Colonsay as an *A. m mellifera* reserve has also suffered due to concerns about the effect of the relatively high density of honeybee colonies (brought to Colonsay some 20 years previously as a protective measure) on local

bumblebee and native bee species. The locally rare field cuckoo bumblebee *Bombus campestris* was found on Colonsay in 2009.

Most studies of honeybee competition showing a detrimental effect of honeybees, tend to detail sites where honeybees are a relatively recent arrival (Thomson 2004). In the UK there are few long term studies of bee dynamics (>4 years), so a detrimental effect of honeybees is difficult to prove (Steffan-Dewenter & Tscharntke 2000; Aebi et al. 2012; Goulson & Sparrow 2008). *B. campestris* can still be found on Colonsay 20 years after the introduction of honeybees and studies from other areas of the UK suggest that bumblebee declines are usually attributable to causes independent of honeybee density (Forup & Memmott 2005).

The UK lags behind the rest of Europe in native honeybee breeding and conservation. In Sweden for example, beekeepers have access to isolated mating apiaries, a colony grading system, genetic testing, formal government protection, funding and extensive literature on the subject (Thomas & Gallmann 2012; Soland-Reckeweg 2006; Soland-Reckeweg et al. 2009). In the UK conservation efforts are maintained solely by beekeepers with limited funds. There is an over reliance on wing morphometry to assess race purity, and more sophisticated methods such as artificial insemination and remote breeding apiaries are rarely used (Bouga et al. 2011). In 1964 Beowulf Cooper, a government entomologist, created what is now called the Bee Improvers and Bee Breeders Association (BIBBA) to bring about “the conservation, restoration, study, selection and improvement of strains of honeybees of native or near-native type suitable for Britain and Ireland” (Cooper 1986). In 2009, after some false starts, BIBBA, with the assistance of funding from the Cooperatives Plan BEE, launched ‘Project Discovery’ to tackle the lack of rigorous scientific assessment of purity of honeybee colonies and to assess a starting point for more formalised conservation efforts (http://www.co-operative.coop/Plan-Bee/).

In cooperation with BIBBA's Project Discovery and FERA's National Bee Unit this chapter examines the purity of current *A. m. mellifera* stocks in the UK and evaluates the current conservation measures through two main questions:

1) Are there any wild populations of native honeybees in the UK that could act as a source for conservation driven breeding programs?

2) Are current breeding efforts successful at maintaining *A. m. mellifera* at higher levels than managed background colonies and which programs work best?

Methodology

The first step in assessing the levels of *A. m. mellifera* in the UK was to examine whether any remnant native populations remained outside the managed population. Unfortunately, during the first year of this study (2009), comprehensive beekeeping density data was only available for England and Wales.

Search for remote Apis mellifera sp. colonies in England and Wales

If honeybee colonies are surviving remotely from beekeepers it is possible that they are remnant populations of the native UK honeybee, and relatively free from hybridisation with imported strains. Using data on beekeeper locations and colony number from FERA' (Food and Environment Research Agency's) BeeBase database, areas up to 10 km from the nearest known beekeeper were identified using the creation of buffer zones in ArcGIS version 10 (ESRI 2011). Ten kilometers is generally considered to be the maximum distance queens and drones will fly to mate (Lodesani, 2005, Solignac, 2005). As England and Wales are relatively densely populated by beekeepers only a few sites satisfied these conditions. Once these sites were further restricted on suitability of honeybee colonies i.e. forested landscaped but not open moorland, only a handful of areas remained. These were Kielder Forest, Ennerdale Forest and Tywi Forest and these are all Forestry Commission owned plantations.

Two out of three of these locations were checked during the summer of 2009 using pollination sampling techniques including: walked transects, pan trapping and luring with hot wax and honey (Visscher, 1982). The third site Kielder Forest was sampled in

the summer of 2010. Pan traps followed the protocol of Sutherland et al. (2012), with white, yellow and blue ultraviolet reflecting paint. Beekeeper density per 10 km square was calculated using Hawth's Analysis Tools for ArcGIS (Beyer 2004).

Collecting breeding program samples

BIBBA had called on members to submit samples of 30 worker honeybees from colonies they were trying to keep native type using morphometric analyses. Using this information, samples were requested from beekeepers who were actively involved in managing the purity of their colonies. Management varied from simply culling queens that didn't display colour or behavioural characteristics of *A. m. mellifera*, to more intensive stud book selection (Edwards 2010). The level of activity varied widely between beekeepers and colour of the queen or worker bees was the most commonly mentioned attribute used. Remote breeding locations had been used by older beekeepers, such as Spurn Point and Tywi forest but had fallen out of favour due to the protracted effort involved (personal comment Albert Knight, John Dews). Artificial insemination was not used by any participants, and genetic assessment had only been carried out on the Colonsay, Fylingthorpe, Iburndale (Whitby) and Sussex (moved from Sheffield) samples.

Beekeepers known to be selecting stocks to maintain *A. m. mellifera* purity submitted samples from across the UK (figure 5.6). Samples were split into four groups graded by local beekeeper density taken from FERAs BeeBase: (islands, very remote (<5 known beekeepers per 10 km^2), remote (<25 known beekeepers per 10 km^2) and areas of high beekeeping density (>100 known beekeepers per 10 km^2) (table 5.5 and table 5.6). Beekeeping density was estimated for Scottish samples from the Scottish Beebase database in 2012, although these figures are likely to be an underestimation due to the recent opening of their voluntary beekeeper registration scheme (King et al. 2010).

Comparing breeding program stocks to background managed data

Racial composition of the managed honeybee population in England and Wales was taken from FERA's 2009 to 2010 Random Apiary Survey project (RAS). In this project 361 randomly selected honeybee colonies were sampled, with one worker from each being genotyped following the protocol below.

Genetic analysis

DNA extraction

Worker *Apis mellifera* bees were collected from each colony and stored for use in 100% ethanol at -70^{o}C. Fifteen workers from each of the breeding program colonies were randomly selected. Whole bees were washed in molecular grade water, and crushed with 2.3 mm silica beads in a Precellys lysis and homogenization bead beater at 8000 g for 30 seconds. Total DNA was extracted from an entire worker bee using a 10% Chelex solution with 1 x TE buffer. Next, 800 µl of 10% Chelex solution was added to each crushed bee residue. This was heated to 95 ^{o}C for 5 minutes then centrifuged at 8000g for a further 5 minutes. 200µl of the upper aqueous DNA extract was removed and centrifuged again at 8000g for 5 minutes then 150 µl of the upper aqueous was removed and stored at -70^{o}C until required.

Sequencing of the samples

This microsatellite protocol was taken from FERA's 2009 to 2010 Random Apiary Survey project (RAS) and the 2010-2011 Defra Seedcorn project assessing the diversity and provenance of managed and feral honeybees in the UK (Budge et al., in prep). 12 microsatellites were selected for their variability and ability to discern between the common honeybee races in the UK (See *Bayesian analysis of populations* below and table 5.1).

Table 5.1: The 12 primer sequences used for assessing racial proportion.

Microsatellite Loci	**Sequence**
828/829	UN012-F [HEX]CGACCTAACAGTTGCTTCGTG
	UN012-R ATTGTTCCTTGCCACGATACA
836/837	K0190-F [6FAM]ACGAATCGAGCCTCGACC
	K0190-R ATGCAAGTTTCAAAGGCACG
840/841	AP271-F [HEX]CATGATGCGAGTCTCCTGGC
	AP271-R GCATTAATTGCGCTGCGTC
852/853	UN151-F [HEX]AAATTAGCGTACGTGAGCGG
	UN151-R CGTCATAGTCCACCTACCAACG
864/865	K0264-F [HEX]TCTCGTGGAATGGCCTAAGAG
	K0264-R ATACACGCGCACATTCGC
866/867	6230-F [6FAM]CGATCAGCAAAGGCGTATCC

	6230-R GGATGTTCGCGTTAACGTAACTG
876/877	K0352B-F [HEX]ATCAATCTCCTCGCGATCG
	K0352B-R CAAGTTACACAATCCTCGCACC
882/883	UN375-F [HEX]TTCGGACAGGATGCTGCA
	UN375-R GGCCGACTTTCGTCACTGA
938/939	K0911-F [6FAM]GGAACAAAGTGGAGCGAATTACT
	K0911-R CTCGAGGATCCTTAATCGTGG
936/937	AT064-F [HEX]TATGCAGAGCGCGATACATC
	AT064-R TCTGCTTGTTTCGTCGCAG
950/951	AT129-F [6FAM]GCTAACGGGGTAACGGGATC
	AT129-R CCATCCAGACCACGCCTACA
990/991	AT192-F [HEX]GCCTGCTTCTCCTCACTTTC
	AT192-R AGATTTTGAAAGAGGGTCGC

Extractions were diluted to a 1:500 concentration. PCRs were performed individually in 10 µl volumes at two $MgCl_2$ concentrations as below in table 5.2:

Table 5.2: Primer master mix and conditions
1.2 µM $MgCl_2$ (840/814, 936/937):
4.475 µl of H_2O, 1.5 µl of 10x Buffer IV, 0.7 µl of $MgCl_2$ (25 µM), 0.045 µl of dNTPs (20 µM), 2 µl of BSA (1µg/ µl), 0.6 µl of Forward Primer (10 µM), 0.6 µl of Reverse Primer (10 µM), 0.08 µl of Taq.
1.5 µM $MgCl_2$ (828/829, 836/837, 876/877, 882/883, 938/939, 852/853, 864/865, 866/867, 950/951, 990/991)
4.275 µl of H_2O, 1.5 µl of 10x Buffer IV, 0.9 µl of $MgCl_2$ (25 µM), 0.045 µl of dNTPs (20 µM), 2 µl of BSA (1µg/ µl), 0.6 µl of Forward Primer (10 µM), 0.6 µl of Reverse Primer (10 µM), 0.08 µl of Taq.

Each colony PCR plate included a positive control and a blank. PCRs were run on a real time PCR ABI Prism 7900HT (Applied Biosystems Inc., Foster City, CA). The first stage of the PCR was denaturing of the dsDNA at 94 ^{o}C for 5 minutes. This was followed by 35 cycles of denaturing at 94 ^{o}C for 30 seconds and annealing for 30 seconds at 72 ^{o}C. There was a 50 minute soak at 60 ^{o}C at the end. Polymerase chain reaction products were diluted in multiplex groups (table 5.3 below).

Table 5.3: PCR dilutions in multiplex pairs		
Microsatellite pair	Volume of PCR contributed (µl)	H_2O for dilution (µl)
828/829	1	
836/837	1	146
840/841	1	
936/937	1	
950/951	2	147
990/991	1	
876/877	1	
882/883	1	147
938/939	1	
852/853	1	
864/865	1	147
866/867	1	

1 µl of the diluted multiplex mix was added to 10 µl of formamide and 0.3 µl of size standard ROX 500. Samples were sequenced on a 3130xl Genetic Analyzer. Peaks were scored using Genemapper software version 3.7.

Bayesian analysis of populations (BAPs) to identify race and genetic separation

Bayesian mixture models attempt to identify a hidden population structure by clustering individuals into genetically divergent groups. FERA's project assessing the diversity and provenance of managed and feral honeybees in the UK, examined 359 reference queens from Australia, France, Germany, Greece, Hawaii, Malta, New Zealand, Slovenia, Spain and the UK. Over 40 highly variable microsatellites or SSRs were tested to cluster and discriminate between the races of these queens (Corander & Marttinen 2006). This project used 12 of the most variable and discriminatory microsatellites to assess the component races of feral honeybee colonies (highest Gst) (See table 4.1). The genetic makeup of the breeding program honeybees was compared alongside that of the reference queens using BAPS software version 5 (Corander & Marttinen 2006). The BAPS data is presented as a proportion of the 11 races or clusters found within each worker bee (Hawaian *Carnica*, Hawaian *Ligustica*,

Spanish *Iberica*, Malta *Rutneri*, New Zealand *Ligustica*, Australian *Ligustica,* Slovenian *Carnica*, Greek *Macedonica*, UK *Mellifera*, French *Mellifera* and German *Carnica*).

Statistical analysis

Statistics were performed in SPSS version 20 (IBM 2012) and GenStat 14.1 ((VSNInternational 2011).

Results

Search for remote Apis mellifera sp colonies in England and Wales

Figure 5.1 is likely to be an under representation of actual honeybee colony density, as at the time of assessment (2009), only approximately two thirds of practising beekeepers are voluntarily subscribed to the National Bee Units Bee base in England and Wales (King et al. 2010). Beekeepers in Scotland were only able to register from the 24th of June 2010 so density data were unavailable for this stage of the study in 2009. Honeybee colony density does tend to mirror human population density, as beekeepers tend to keep their colonies close to their home.

Figures 5.3, 5.4 and 5.5 detail the remote regions searched for honeybees in 2009, compiled from figure 5.2. Upland areas without forest were discounted in this search. No honeybees were found in areas remote from managed beekeeping in England and Wales (Table 5.4). It seems likely therefore, given that feral honeybees have a low survival, and closely reflect managed colony genotypes (see Chapter 2), that there are no remaining wild populations of *Apis mellifera mellifera* in England and Wales.

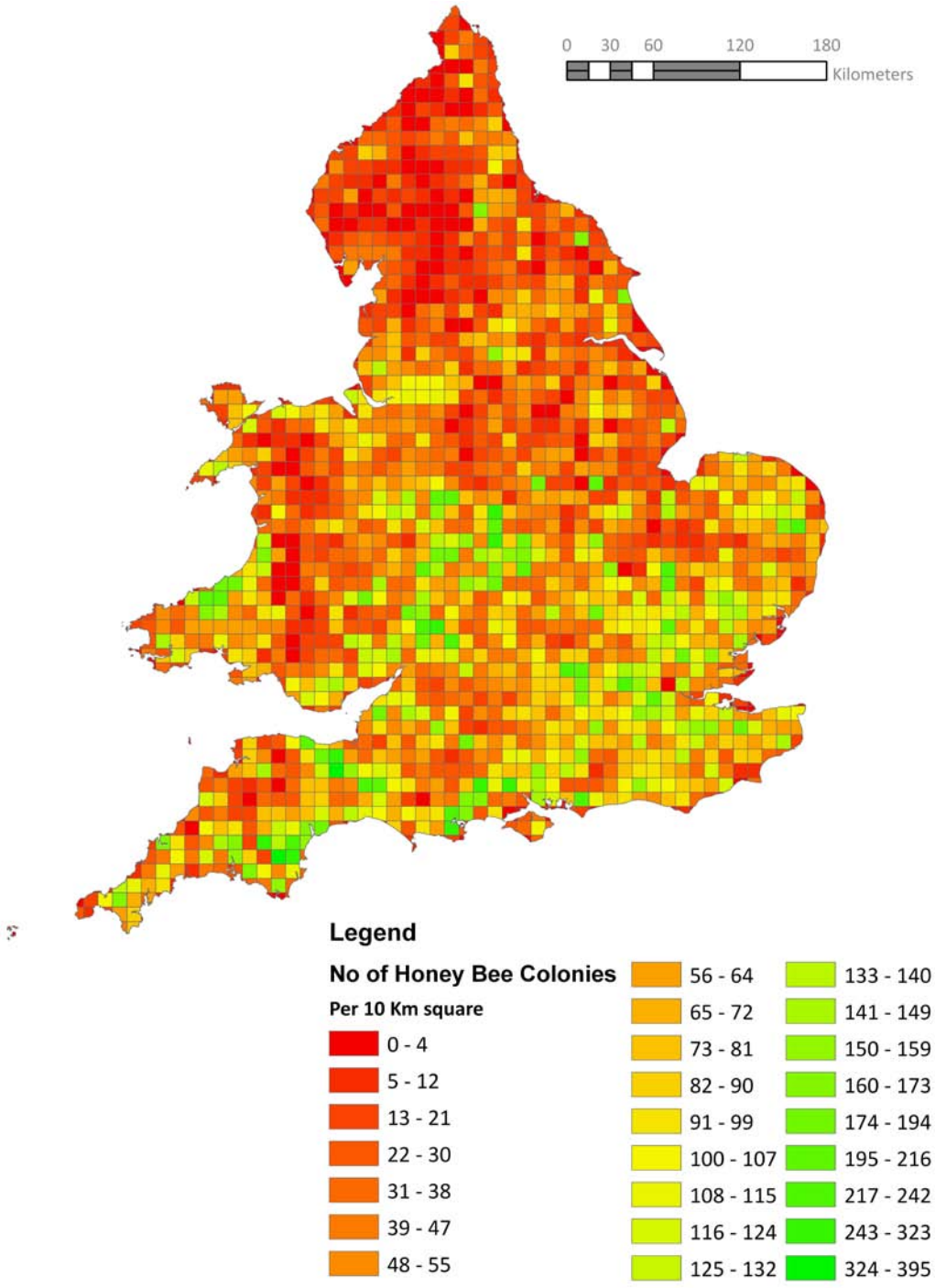


Figure 5.2: Honeybee colony density per 10 km^2 compiled from FERA's BeeBase (voluntarily reported beekeepers).

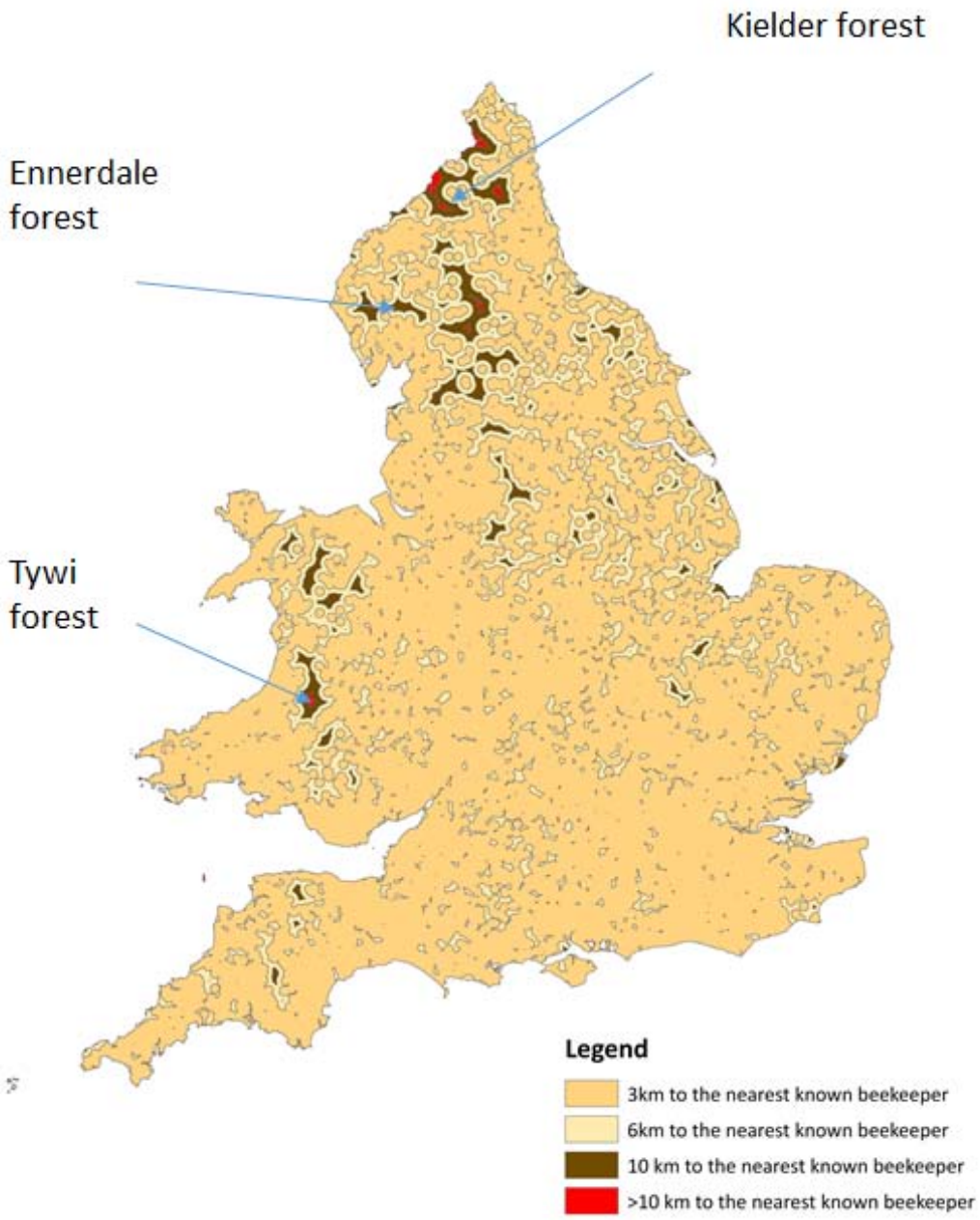


Figure 5.3: A map of areas up to 10 km from the nearest known beekeeper (data compiled from FERA's BeeBase).

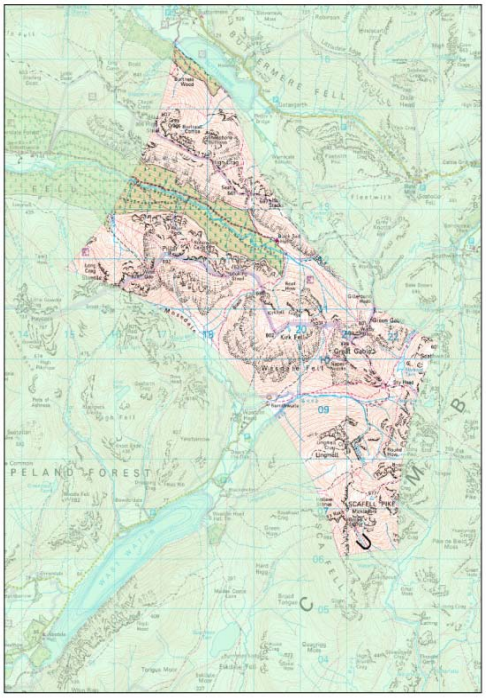


Figure 5.4: Ennerdale Forest study area

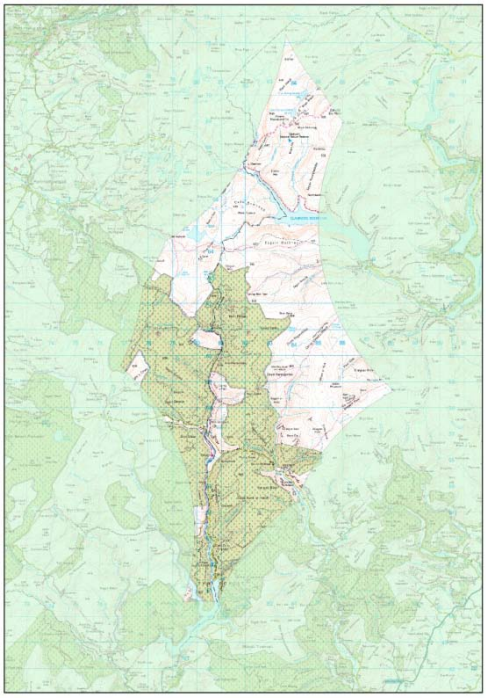


Figure 5.5: Tywi Forest study area

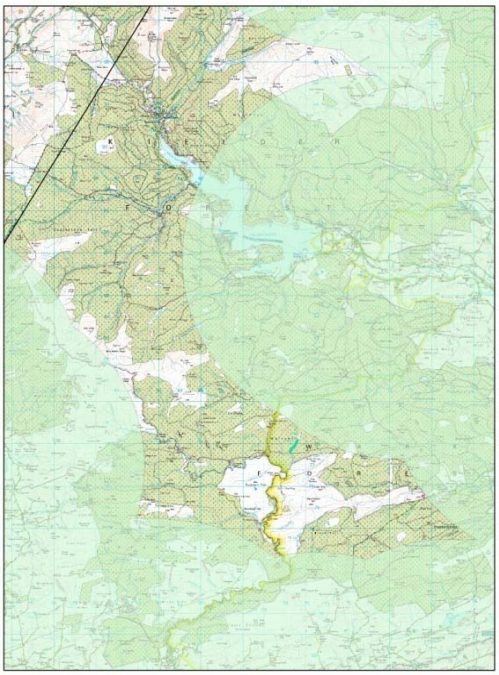


Figure 5.6: Kielder and Wark Forest study area

Table 5.4: Presence of honeybees in remote areas

Site	Honeybees seen during transects?	Honeybees collected in pan traps?	Honeybees successfully lured?	Suitable honeybee habitat?
Ennerdale Forest	No	No	No	Improving
Tywi Forest	No	No	No	No
Kielder	No	No	No	Improving

Are breeding programs successful?

Being part of a breeding program seemed to have a positive effect on *A. m. mellifera* purity compared with background and feral honeybee levels (figure 5.7). A between-groups analysis of variance was conducted to examine the difference in purity levels between breeding programs (Islands n=93, Very remote n=85, Remote n=92, high beekeeper density n=66), and background level data (RAS), n=248. There was a statistically significant difference in levels of *A. m. mellifera* between the groups ($F(4,583)= 25.48$ $p<.001$). Post-hoc comparisons using the Tukey HSD test indicated that the mean *A. m. mellifera* percentage for background level (RAS M=41.90, SD = 26.21) differed from the other breeding programs locations, but that they did not differ between each other (Islands M=66.20, SD = 21.42, Very remote M=59.11, SD = 14.73, Remote M=64.40, SD = 18.36, high beekeeper density M=56.14, SD= 11.00) (see figure 5.8 and 5.9, table 5.6) .

Figure 5.6: The location of *A. m .mellifera* stocks held by beekeepers as part of conservation efforts

Table 5.5: The four *A. m. mellifera* breeding program categories			
Islands	**Very Remote**	**Remote**	**High Beekeeping density**
Colonsay A	St Andrews	Iburndale	Lancaster A
Colonsay B	Gairloch	Morpeth	Lancaster B
Tobermory A	Kinross	Coniston	Sussex
Tobermory B	Rosneath	Fylingthorpe	Stratford A
Orkney A	Rahane	Iburndale	Corbridge
Orkney B	Bryness	Tregena A	Stratford B
Alderney A	Lethangie	Tregena B	
Alderney B	Daligan	Glan-yr-afon	

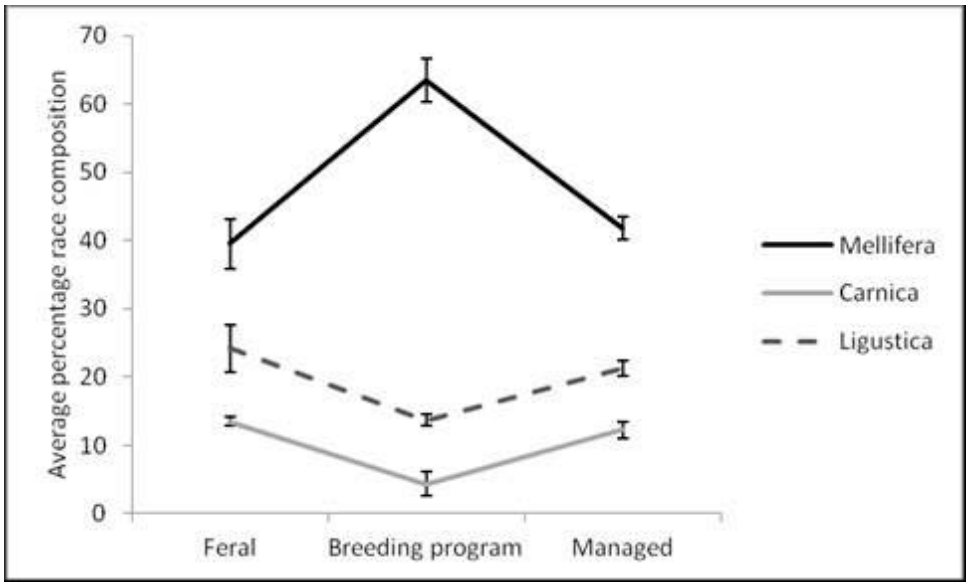


Figure 5.7: Average percentage race composition between groups.

Data for managed hives come from the RAS survey, data for feral hives comes from chapter 2, and data for breeding programs represent the mean of all programs assessed in this chapter.

Table 5.6: Mean *A. m. mellifera* percentage by breeding program location based on beekeeper density per 10km^2 area.

Location	Average *A. m. mellifera* levels %	**Lower 95% confidence interval**	**Upper 95% confidence interval**	Standard Deviation	Apiary Density (10km^2)
Islands	66.20	61.16	71.25	21.42	0-2
Very Remote	59.11	53.83	64.38	14.73	3-25
Remote	64.40	59.33	69.47	18.36	50-100
High bee keeper density	56.14	50.15	62.12	11.00	101-250
Background data (RAS)	41.90	38.81	44.99	26.21	

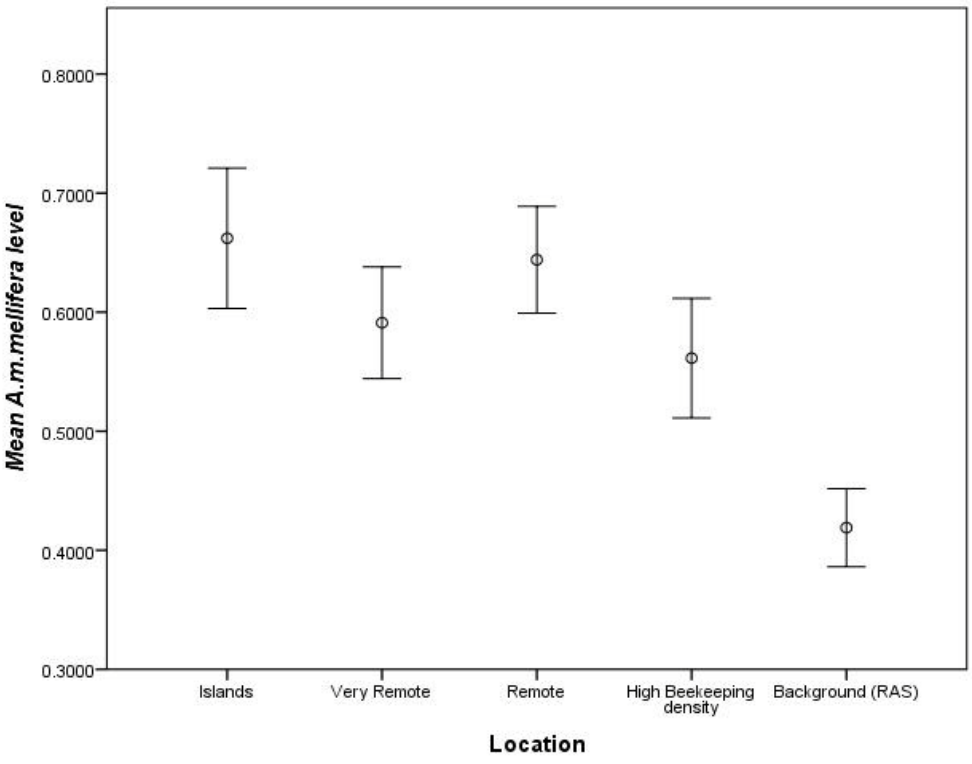


Figure 5.8: Mean *A. m. mellifera* by breeding program location and the background honeybee population *A. m. mellifera* levels provided by FERA's RAS study.

The average *A. m. mellifera* content of the background managed colonies in the UK is 41.90% (Random apiary survey data). All island colonies except Mull B and Alderney A, had significantly higher proportions of *A. m. mellifera* than background levels (figure 5.9)**.** Orkney A and Colonsay A were the purest samples (figure 5.9). Orkney A only showed introgression from Spanish *iberica* and German *carnica* in one individual from the sample (figure 5.10). Colonsay had introgression from a greater number of races (New Zealand *ligustica*, Australian *ligustica* etc) but *A. m. mellifera* levels were less variable between samples A and B.

Figure 5.9 and 5.10 show that there appears to be no effect of beekeeper density on percentage *A. m. mellifera* . Island and very remote site samples are found both with high and lower than background levels of *A. m. mellifera*.

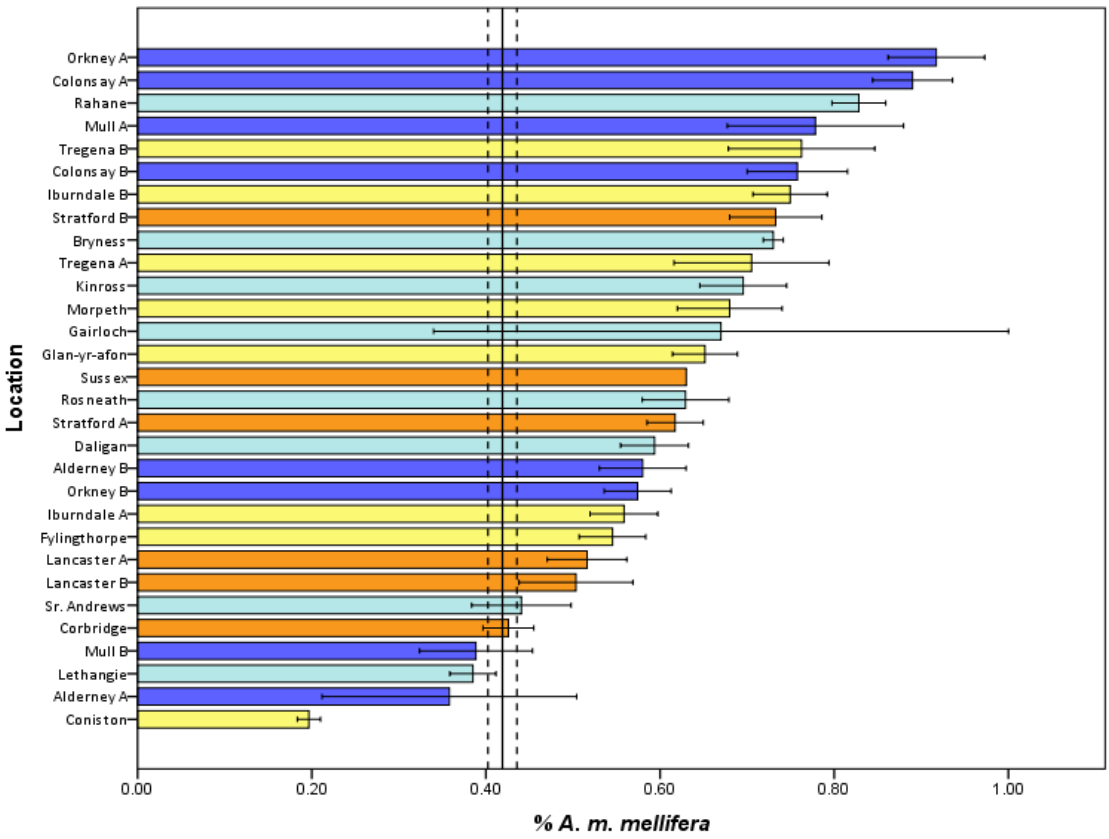


Figure 5.9: Colonies rated by % *A. m. mellifera* levels.

The solid line represents the mean *A. m. mellifera* background levels and the dashed lines

represent one standard error. Blue bars represent island samples, light blue bars represent very remote sites, yellow bars represent remote sites, and orange bars represent areas of high beekeeping density.

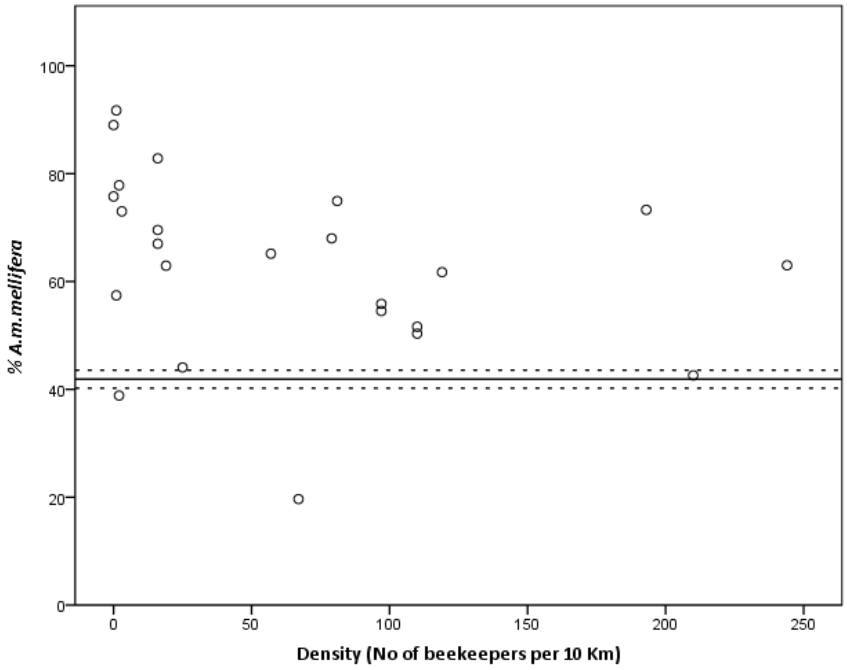
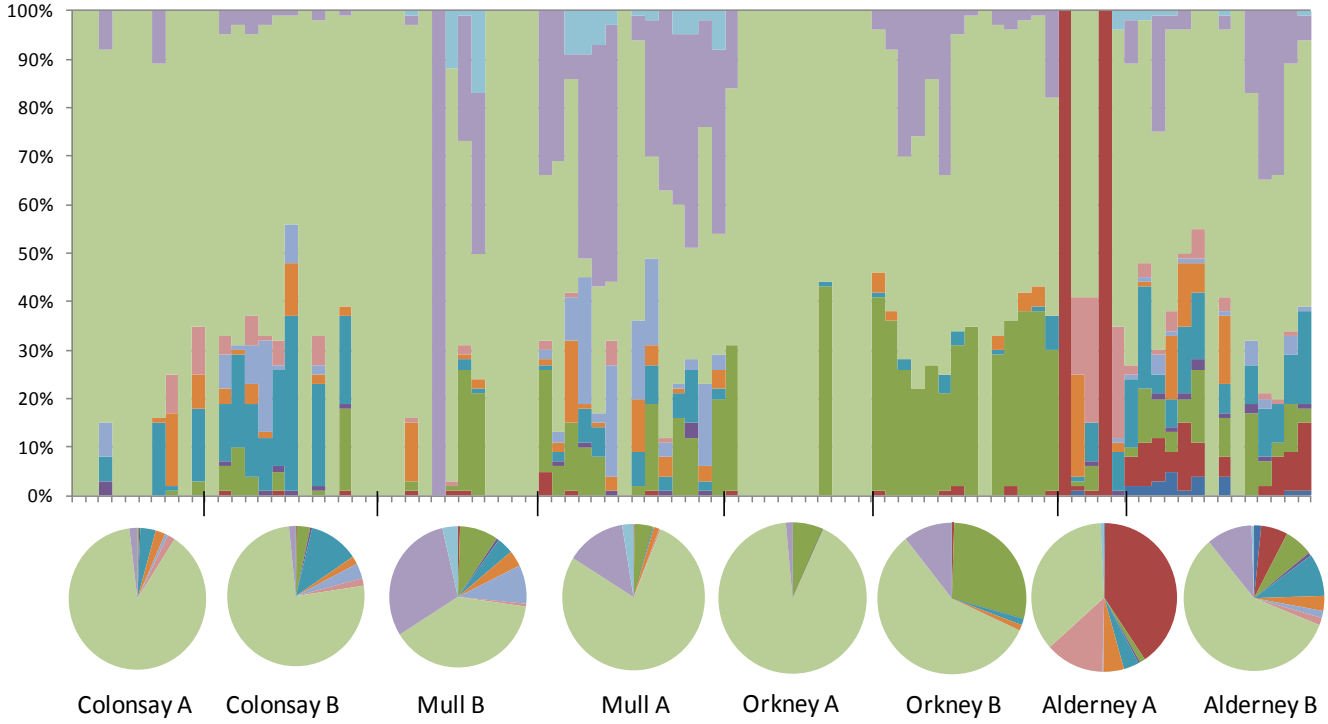


Figure 5.10: The relationship between density and % *A. m. mellifera* for all breeding program samples

Figure 5.11 to 5.14 show the component races of the different breeding program samples. Only workers from the colony Orkney A were predominantly free of introgression from any other race (figure 5.10).


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Colonsay A
Colonsay B
Mull B
Mull A
Orkney A
Orkney B
Alderney A
Alderney B

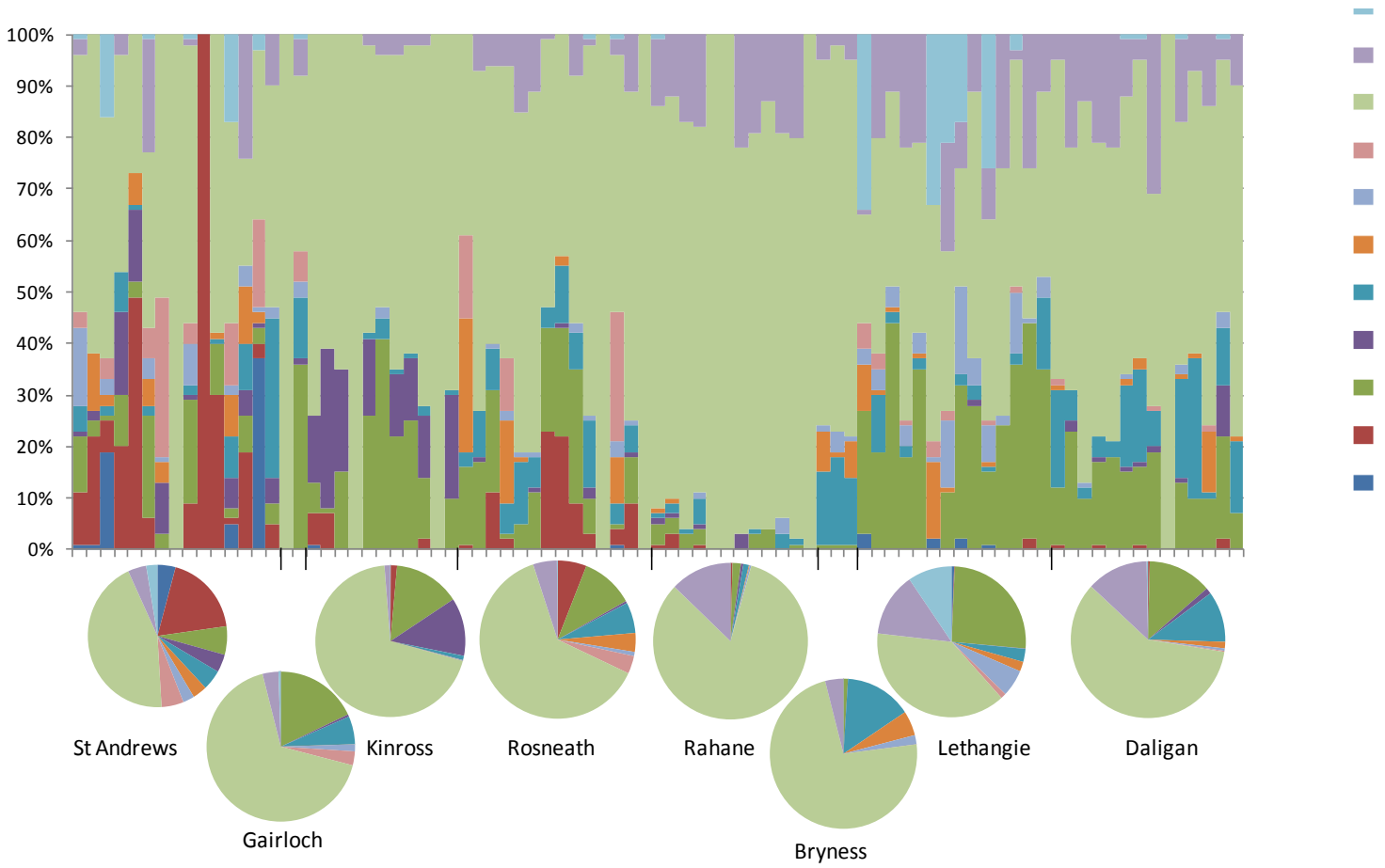


Figure 5.12: Race composition of very remote samples from BAP analysis using FERA s reference queens

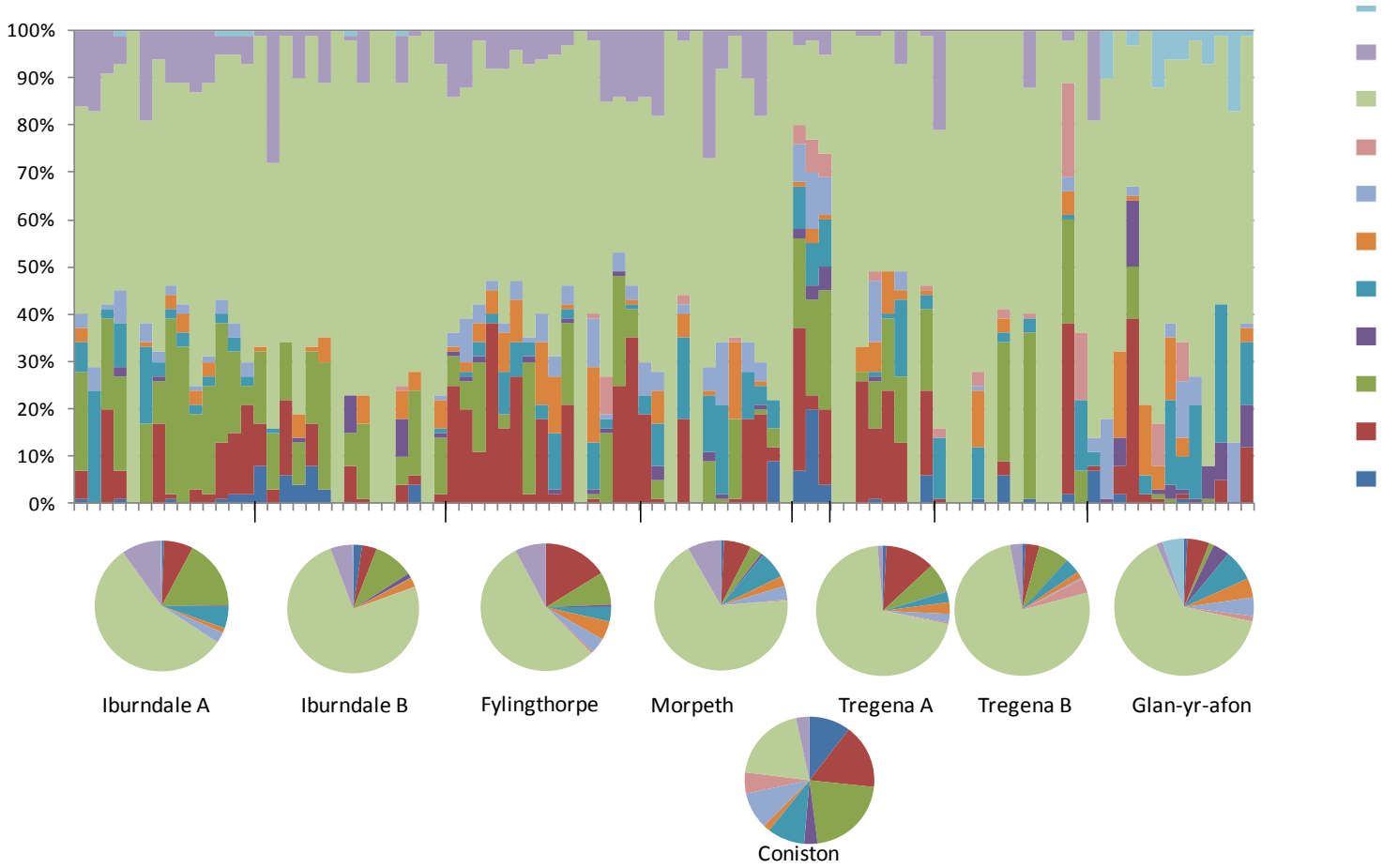


Figure 5.13: Race composition of remote samples from BAP analysis using FERA s reference queens

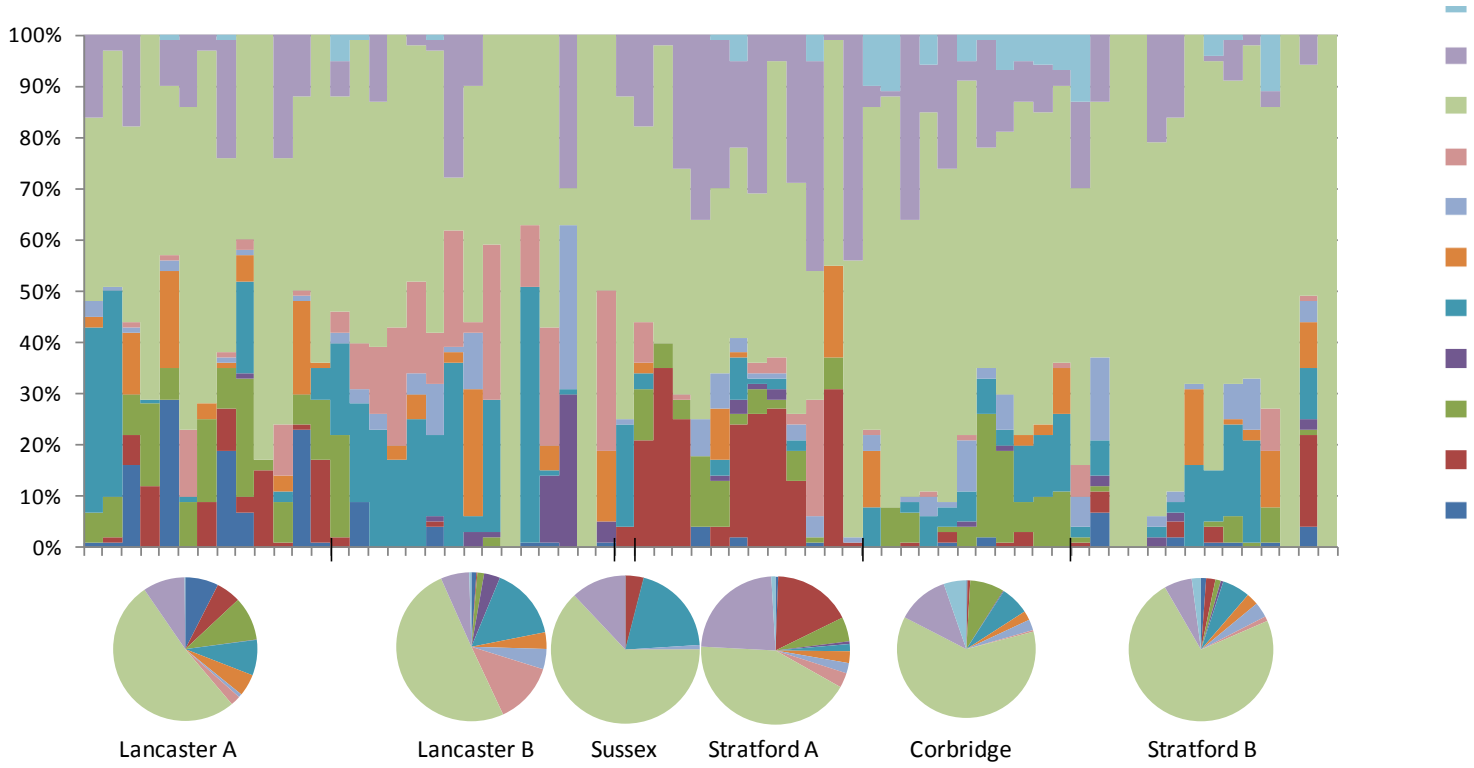


Figure 5.14: Race composition of high beekeeper density samples from BAP analysis using FERA s reference queens

Discussion

Recent examination of the feral and managed populations of the UK have highlighted the urgent need to formally protect *A. m. mellifera* stocks, to safe guard genetic traits and prevent the homogenisation of managed stocks (see Chapter 2, Jensen et al 2005)

Remnant A. m. mellifera populations?

Remote 'survivor' populations of feral honeybees were not found in England or Wales. It is possible that this finding was the result of false negatives i.e the missing of low density colonies in a landscape, but perhaps a more likely explanation is that the absence of honeybees in these areas is likely to be due to the arrival of *Varroa destructor*, which was seen to cause catastrophic losses across Europe and the USA (Meixner et al. 2010; Carreck et al. 2010; Rosenkranz et al. 2010). The *Varroa* mite, leads to a conditions termed Varroosis in colonies due to secondary infection from transmitted viruses (Boecking & Genersch 2008; Rosenkranz et al. 2010). It is possible that there are still some remote populations in Scotland, although they will be increasingly vulnerable as *Varroa* makes its way northwards. Currently *Varroa* is only thought to have reached as far north as Fort William on the west coast but is as high as Helmsdale on the east coast (Ramsay & Atchley 2012). The islands of Islay, Mull, Skye, Orkney and Shetland are thought to be clear of *Varroa* (Ramsay & Atchley 2012).

The feral honeybee population of England and Wales does not appear to be surviving without treatment for *Varroa* (see chapter 3) . Feral colonies have been shown to have significantly higher deformed wing virus levels, due to secondary viral infection from untreated *Varroa* infestation (see chapter 2.) . It seems unlikely that remote populations would be able to survive the arrival of the *Varroa* mite, unless they were sufficiently distant from managed populations to allow the isolated evolution of a stable host parasite relationship (Locke & Fries 2011; Locke et al. 2012; Seeley 2007).

In Sweden, remote colonies have been shown to survive for over 10 years without *Varroa* treatment. Researchers attribute this survival to the evolution of a balanced host-parasite relationship, where reduced honeybee brood production and reduced

Varroa fecundity co-evolve to reduce the negative effect of *Varroa* infestation (Locke & Fries 2011). Parasite a-virulence is selected where vertical transmission of the parasite (from parent to offspring) is more advantageous than horizontal transmission (infectious spread between colonies i.e. by drifting, robbing etc) (Bull 1994). This usually occurs in areas where colonies are at a low density in the environment. Indeed, Ramsay and Atchley (2012) note that the scattered distribution of bee colonies in parts of the west and the north of Scotland restricts the spread of the mite, although more densely bee-populated areas maintain and spread infestations (figure 5.15).

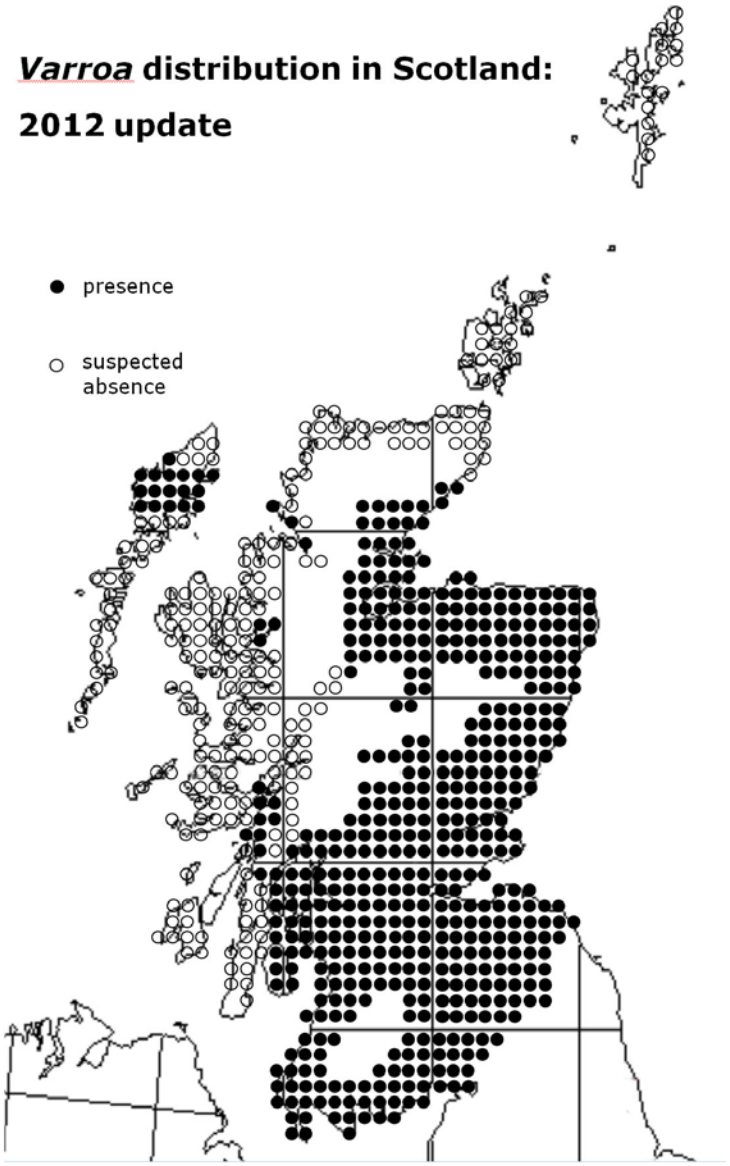


Figure 5.15: The *Varroa* distribution in Scotland (May 2012) taken from Ramsay & Atchley (2012).

Another possible explanation for the apparent lack of remote honeybee populations is that the landscape in these areas is simply not suitable for supporting honeybee colonies (Coulson et al. 2005) . All three sites, (Ennerdale, Tywi and Kielder forest) were owned by the forestry commission and had been used in part as plantation forestry. Trees in intensive forestry of this type are usually harvested around 30 years of age which gives little opportunity for cavities and holes used as nest sites by honeybee colonies to appear in the trees (Pritchard, 2008). The stands of conifers are also very dense, so much of the flowering plants are limited to forest tracks and recently felled areas (figure 5.16) (Coulson et al. 2005).

In the Tywi valley for example, the forestry plantations were large (over 886ha) and dominated the landscape (Cartmel 2001). Over 155ha of this area was clear felled and a further 115ha were recently planted (Cartmel 2001). Where older stands of native trees were found, sheep were allowed to graze beneath the trees removing much of the understory. The tops of the fells were dominated by sheep grazed grassy moorland and bog with few floral resources (figure 5.17). Heather was very scarce due to intensive grazing by sheep, and where found, tended to be restricted to areas along roads and footpaths lower in the valley.

Figure 5.16: Dense forestry stands of predominantly Sitka spruce (*Picea sitchensis*).

Figure 5.17: Sheep grazed moorland and clear fell forestry

Local councils, the forestry commission and local wildlife trusts, aware of the negative public image and poor biodiversity of intensive forestry, attempts have been made in recent years to lessen the impact on the landscape (Yanik 2006; Spellerberg & Sawyer 1996). This has been helped in part by the fact that forestry is now largely uncompetitive in England and Wales (Slee 2007). The UK government is committed under the Rio Principles, Helsinki Guidelines and various other EU initiatives, to conserve and enhance biodiversity in British forests and woodlands (Garrod & Willis 1997; Spellerberg & Sawyer 1996). It seems likely therefore that these locations will become increasingly promising for honeybee populations (table 5.7). Being aware that these areas are remote from beekeepers and currently without honeybee populations, means that these areas could easily be adopted as remote breeding sites or *A. m. mellifera* apiaries. All the sites are currently owned by the forestry commission, and beekeepers wishing to keep their bees on the land have to apply for a permit. If government and forestry commission agreement could be reached on designating these sites and native bee conservation zones, beekeeper movement could be relatively easily controlled.

Table 5.7: Future management strategies for three remote locations

Location	**Management to benefit honeybee conservation programs**
Ennerdale valley	In Ennerdale many of the areas of conifer plantation have been clear felled and are being allowed to regenerate naturally in accordance with the Forestry Commission's 'Wild Ennerdale Stewardship Plan' (Yanik 2006). The valley is uninhabited and remote, with no known beekeepers keeping hives along the valley bottom. There are a variety of habitats with a diverse flora; summer meadows are found along the valley floor and there is an extensive autumn heather crop. The valley is surrounded by Lakelands highest summits Green Gable (801 m), Great Gable (899 m), Pillar (892 m), Kirk Fell (802 m) and Steeple (819 m) which would act as a geographic barrier to Queens and drones (Kraus

	2005).
Tywi forest	Tywi forest is still primarily a farmed landscape. Cartmel (2001) suggests that planting pine species other than Sitka spruce, and allowing natural regeneration would enhance the forests biodiversity. However as the forest is still farmed relatively intensively and does not benefit from the tourist trade to the north of Wales, few direct measures have been taken. Tywi forest is remote and it has previously been used as a remote breeding apiary. It is likely that the lack of forage, and large blocks of forestry could act as a barrier to movement of external drones and queens.
Kielder forest	Management activity over the last 15 years has enhanced the areas biodiversity value through the creation of over 100ha of native woodland , 95% of which is broadleaved (Blackie 2005). Native woodlands have a greater abundance and diversity of pollen and nectar for bees from tree species such as lime (*Tilia cordata*), white beam (*Sorbus sp*), horse chestnut (*Aesculus sp)* and hazel (*Corylus sp)* etc . Unlike, intensive pine plantations they also allow enough light to penetrate for the development of an understory of woodland plants (Cartmel 2001). There is sustained activity to improve the remnants of ancient woodlands in this region and continue to improve the area for biodiversity and visitors (Blackie 2005). Large areas of the landscape are maintained as heather moorland. It seems likely that this area could support a year round *A .m. mellifera* apiary, as it is remote from other beekeepers and importation of honeybee colonies could be controlled by permit.

Maintained A. m. mellifera populations

The absence of remnant *A. m. mellifera* populations means that the future purity of the UK's native honeybee is in beekeepers hands (Meixner et al. 2010). This study

shows that colonies within a breeding program have significantly higher levels of *A. m. mellifera* than background levels. However, *A. m. mellifera* purity cannot be explained by beekeeper density. The relationship between beekeeper density and *A. m. mellifera* purity is unlikely to be straightforward as a low density of beekeepers does not necessarily imply a low level of importation. Importation is not restricted by geographic location and country wise importation has been at a high level for some time. The relationship between beekeeper density and *A. m. mellifera* purity is also unlikely to be linear as when importation levels increase a variation in purity is seen but the majority of colonies represent a hybridised state (Jensen et al. 2005). See figure 5.18 for a projected relationship between density and *A. m. mellifera* purity for breeding program samples.

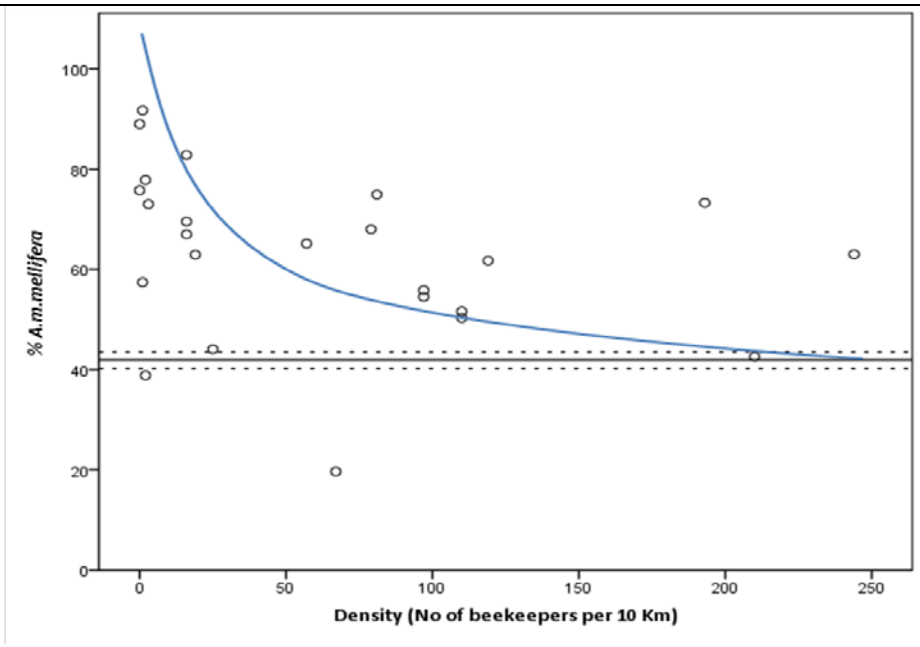


Figure 5.18: A projected relationship between density and percentage *A. m. mellifera* for breeding program samples and the National Bee Unit's BeeBase data.

It is possible that as density of beekeepers increases, so too does the likelihood of local importation of other races, and the hybridisation and reduction in purity of *A. m. mellifera* samples.

Density data in this study may also have been an underestimation as beekeeper density was drawn from the newly established BeeBase for Scottish samples. As beekeepers voluntarily sign up to the BeeBase database it is likely that many beekeepers are still not registered. This can be seen in the case of the St. Andrews sample which is detailed as being in a very remote beekeeping area. Given the higher human population along the east coast of Scotland you would expect there to be a relatively large beekeeping population. The St. Andrews samples were highly introgressed with relatively low levels of *A. m. mellifera.*

Islands give the highest levels of *A. m. mellifera* purity by a very small margin. The mean purity of 66.20% reflects marked difference in situation between the island samples. On Colonsay for example, there is only one beekeeper and importation of other bee races is strongly discouraged. On Alderney importation of other races is not controlled and high levels of introgression are seen with some individuals representing almost pure examples of Hawaiian *ligustica*. Alderney may also be a difficult location to maintain a breeding program as the mild and sunny climate is unlikely to favour *A. m. mellifera* over other continental races. The climate on the Scottish islands is much more likely to favour a hardy and conservative honeybee.

The colonies found on Colonsay were collected from sites across Scotland in the last 30 years as importation levels increased and fears for the genetic integrity of native stocks rose. The high number of component races could reflect this legacy of importing mildly hybridised colonies from a large number of sites.

Remote samples in this study, boasted levels of *A. m. mellifera* similar to that of the average island samples (64% and 66% respectively). Iburndale B and Tregena B were the purest colonies within the remote samples although this may reflect high levels of beekeeper effort rather than a significant location. Colonies in this region were selected both by wing morphology as well as other morphometric attributes and behavioural characteristics (personal comment Dews, John). The purest samples from areas of high beekeeper density, Statford samples A and B , also belonged to a breeding program with high levels of beekeeper effort. This beekeeper adheres to the most stringent selection criteria based on both behavioural and morphometric characteristics (Edwards 2010).

Some importation of other *A. m. mellifera* races may also have taken place to bolster purity. Relatively large proportions of the Stratford A colony (23%) were composed of French type *mellifera* (figure 5.14). These alleles were also found in the Mull population (figure 5.11) (A: 38%, B: 13%).

Conclusions

Breeding efforts in the UK have been shown to boost *A. m. mellifera* levels above that of FERAs average managed honeybee colony level of 42.9%. Purity varies between locations and results suggest that beekeeper effort may be a more important factor than breeding effort location. These data provide a solid foundation for the construction of a more integrated and effective UK wide *A. m. mellifera* breeding program. Future breeding program and conservation suggestions are detailed in the general discussion.

Chapter 6: General conclusions

The future of beekeeping in the UK

Honeybees of the UK exist in a rapidly changing landscape. Agricultural intensification, chemical contamination, novel diseases and parasites contribute to a reduction in colony health and longevity (Genersch 2010b). Most significant of these is the *Varroa* mite and resulting Varroosis. Feral colonies which can be used as an indicator of background honeybee health were shown to have significantly higher levels of deformed wing virus (DWV), a *Varroa* associated virus, than managed colonies. There was also a significantly lower level of DWV in managed honeybee colonies treated for *Varroa* verses those left untreated. As DWV and Varroosis is such a significant cause of mortality for honeybees it is imperative that managed colonies are subject to a comprehensive *Varroa* management program (Danka et al 2011, Harris et al 2011; Dainat et al. 2012; Martin et al. 2012) . Whilst reducing *Varroa* treatment seems intuitively positive; reducing the reliance on chemical treatments and contaminants in the hive, the critical supportive network of bee breeders and researchers selecting for *Varroa* tolerance is not yet in place. Beekeepers acting alone to this end run the risk of losing large numbers of colonies and triumphing methods or treatments that do not have scientific support.

Feral colonies were suggested as a potential life raft of genetic diversity (Kukielka et al 2008; Le Conte et al 2007), but were shown in this study to be genetically similar to local managed colonies . There was a very small but significant genetic difference between the feral and managed honeybee populations of the UK of about 2%. It seems likely, given the high levels of DWV in feral colonies and the high mortality levels seen (47.22 %) that feral colonies do not represent an adaptive *Varroa* tolerant population. However it is possible that the significance of the difference hints at some adaptive mechanism. A certain tolerance for *Varroa* may be present within our managed population but be masked by *Varroa* treatment. Alternatively, the genetic difference may be due to the high levels of importation in managed populations, and foreign maladapted races that would be unable to survive without beekeeper support.

Further research must be carried out to assess how long feral colonies of the UK are able to survive with such high DWV levels, and whether there is any unique genetic, behavioural or environmental factor that enables them to survive. For example although annual swarming is a well documented trait (Seeley 1978; Winston 1980) it is still thought to demand a certain level of health from a colony (Fries et al 2003). Fries et al (2003) suggest that high *Varroa* levels may inhibit swarming by reducing the health of the colony. In a six year study of colonies left untreated for *Varroa* in Gotland, swarming behaviour was shown to increase as winter mortality decreased (Fries et al. 2006). No consistent queen genotype was found in any of the 12 colonies that were continually occupied by honeybees during the course of the 2.5 year period. It is not possible to determine from this research whether swarming could be an adaptive trait to deal with high *Varroa* levels, but it poses an interesting question for future research. Seeley's (2007) study placed feral colonies in bait hives and monitored the behaviour and survival of resulting swarms. He found no evidence that feral honey bee colonies were better at limiting the reproduction of *Varroa*, instead suggesting that the mite may have evolved avirulence in this region (Seeley 2007). This study could easily be replicated with the UK's feral population.

The feral population of the UK was not found to be more native type (*A. m. mellifera*) than the managed population and was also highly introgressed. It is possible that hybrid colonies compose of many different races, offering better protective genetic diversity than pure examples of race (Hughes et al 2008). Multiple paternal alleles have been shown to convey a colony advantage through enhanced productivity, and lower disease infections (Seeley & Tarpy 2007; Mattila & Seeley 2007). Hughes et al (2008) predict that genetic diversity is likely to be most relevant in highly variable environments or those subject to rapid anthropogenic change. In the States, populations that have suffered significantly with *Varroa* and CCD have been shown to have undergone a genetic bottleneck through extensive breeding from a small number of mother queens (Delaney 2008). Delaney (2008) showed that 473 breeder queens were used to make replacement queens for 1/3 of all managed colonies in the US. Interestingly, Seeley (1978) noted that the honeybees in the Ithaca region of New York

were hybrids of several European races imported for American apiculture. The US are now trying to recover from this bottle neck by importing semen from old world honeybee races and incorporating it into their *Varroa* tolerance breeding programmes (Sheppard 2012). This flies in the face of 'purist' beekeepers who want to exclusively maintain the integrity of a native race. This conflict between maintaining genetic diversity and maintaining genetic purity is easily solved through adaptive breeding efforts that are not mutually exclusive (see the future of bee breeding programs below).

If the health of future honeybee populations relies on genetic diversity, steps must be taken to prevent the loss of native honeybee races as an extension of wider diversity, not simply for their own merits (Jensen et al 2005; Lodesani & Costa, 2003; Meixner et al., 2010). Working towards the overarching goal all honeybee health is far more likely to gain the necessary momentum and funding than single race breeding programs.

Breeding efforts in the UK do seem to maintain *A. m. mellifera* at a higher than background level of purity, however percentage levels are highly variable between sites. This suggests that beekeeper effort and breeding program protocol is likely to have a greater effect of purity that breeding program location. There is much room for improvement. Breeding programs in other European countries such as Germany and Switzerland have shown significant improvements in the levels of *A. m. mellifera* with concerted effort and the uptake of new genetic technologies (Table: 6.1).

The Swiss *mellifera* breeding society produces mated queens from remote mating yards. These queens are of recorded, good parentage from controlled apiaries. A rigorous 'herd book' is maintained, with the results of hive tests and comparative tests between breeding lines. Beekeepers are compensated for the cost of queen and hive testing (Soland 2012a). In 2010 *A. m. mellifera* was inducted into the 'stockbreeding ordinance of the federal office of agriculture' securing financial support from the government for the breeding program. This and the resulting publicity brought about a resurgence in interest and in 2012 there are 21 test apiaries with 252 queens for grading. A neutral corporation 'apisuisse' has been set up to maintain breeding guidelines, arrange financial support for grading of beehives, manage mating yards and maintain the herd book (Soland 2012a). Queen

grading, inbreeding calculation and support in selection decisions is offered by the Institute for Bee Research in Hohen Neuendorf/Berlin. The data was freely accessible to the public through http://www.beebreed.ue. Overall, the roles of bee breeders, test directors and mating yard managers are clearly defined and training is arranged by mellifera.ch. The result of these efforts is a sufficient stock of purebred, indigenous *A. m. mellifera* (Soland 2012a). The purity is far greater than that seen when systematic wing morphometry was carried out. This method was seen to be insufficient for discerning hybrids which increased rapidly in the breeding program. Genetic testing began in 2007 and became mandatory in 2010 (Soland 2012a).
Table 6.1 The Swiss *mellifera*, a case study.

To improve the fate of *A. m. mellifera* colonies in the UK it seems essential that the following measures are addressed:

A network of beekeepers

Relying on individual beekeepers for breeding programs can be problematic as there is no wider formal conservation plan to follow. This means that selection is unsupervised and in the case of selection by wing morphometry alone, can lead to poor quality colonies that do not represent the selected race genetically (Soland 2012) (see Chapter 4). Furthermore, the stocks maintained by sole beekeepers are in private possession and if that beekeeper retires or dies there is no guarantee that the colonies will continue to be protected. Ultimately breeding programs cannot exist in isolation, as remote or island beekeepers maintaining colonies will eventually need to introduce new favoured lines of further stock to prevent the negative effects of inbreeding (Bourgeois & Rinderer 2009). Inbreeding not only reduces genetic diversity within the honeybee colony (usually the rarest alleles and genotypes disappear first) but also alters the behaviour of the worker bees, causing an inability to thermoregulate the nest, reduced recruitment activity to food sources, reduced hive cleaning and brood rearing and an increased sensitivity to parasites (Solignac, 2005, Kraus, 2005, Zayed, 2009). Kraus (2005) also found that inbred queens were also more aggressive. Inbreeding can be identified by a classic ‘gun shot’ pattern produced on

the brood frames, where larva that are homozygous at the sex alleles are removed by worker bees (Solignac 2005; Zayed 2009; Bourgeois & Rinderer 2009). In essence inbreeding negatively affects the whole super organism of the honeybee colony.

A formal stud book

To create the ideal breeding program, where a high level of diversity is maintained through a high population size, a stud book should be used for transfer of colonies between local beekeepers (Kraus 2005; Zayed 2009). A stud book is the starting point for breeding programs as it allows the documentation of parentage and qualities of the individuals (Glatston 1986). German breeding programs for example following the comprehensive German Stud book regulations for honeybee breeding (DIB, 2002,Van Praagh et al. 2006). An early evaluation of an inbreeding co-efficient allows out breeding to be correctly managed before genetic variability is compromised (Jensen et al. 2005; Selkoe & Toonen 2006; Lodesani 2005). Stud books can be a time consuming and costly procedure for conservation institutions, however in the case of honeybees much of the essential data could be recorded at the time of microsatellite purity screening, (i.e allele frequency, inbreeding co-efficients) and then maintained in a data base for future use (Glatston 1986).

Accurate and reliable assessment of purity and regular testing

The success of any breeding program critically depends on accurate and reliable screening methods for purity. In Sweden, genetic testing is used routinely to assess hybridisation in breeding populations and has enabled a marked reduction in hybrid queens, after generations of reoccurring hybridisation using morphometric methods (Bouga et al. 2011). Breeders are said to now be focusing more on the productivity of their colonies than their cubital index (Bouga et al. 2011).

Routine genetic screening is becoming more common place in European bee breeding programs, however, to date no institution in the UK has offered purity testing and the cost for individual beekeepers seeking testing would be prohibitive. Now that FERA has developed a comprehensive new microsatellite system to assess introgression levels, the methodology is in place to offer a high throughput service for beekeepers through the National Bee Unit.

Genetic screening gives a much more conclusive assessment of racial composition and does not over represent particular races as found by Mortiz (1991). True morphometric analysis requires repeated measures of multiple attributes of workers from a colony, which is both time consuming and prone to human error. Recording these characteristics in a standardised way is a challenge not yet solved by the leading apicultural research institutions of Europe (Bouga et al. 2011). However, morphometry is cheap and accessible to beekeepers so energies should focus on using all wing data to improve discriminatory ability over the current index based system i.e. using all 18 wing venation junctions, associated angles and distances (Tokilski 2008). In this way, a more reliable morphometric measure could be used as a preliminary tool, alongside the more expensive genetic screening.

A formal body to organise and document the breeding program

Once selection guidelines have been agreed and documented a central association or government body should be set up to co-ordinate breeding activities, training, the maintenance of records and selection standards (Meixner et al. 2010). Ideally this would be based at a university or a research institution such as FERA. Beekeeper training is already offered by the National Bee Unit, and the bee inspectorate is a well accepted part of UK beekeeping. It would be a small step to extend the knowledge offered beyond bee husbandry and health care to wider breeding issues. The extrapolation of countrywide data (already possessed by FERA) to advise the selection and improvement of local ecotypes would allow unprecedented beekeeper participation and engagement. The positive feedback and knowledge generated by such a program is likely to far surpass the more academic results from researchers working alone. Using the German breeding system as a guideline, preliminary advice could be rolled out quickly. This would help to mediate the frustration felt by some new beekeepers who are aware of the growing ecological problems facing beekeeping and who reject the ‘dogma’ of ‘old fashioned’ beekeeping. Engagement with these beekeepers through peer reviewed evidence, prevents them falling into the realms of pseudo science and fashionable trends that can offer no meaningful improvement to

long term honey bee health. Such a large scale, forward and freethinking proposal would also help to challenge the unduly negative perceptions that government research is by nature restrictive and autocratic.

Remote breeding locations, instrumental insemination, mainland reserves or island reserves

Even a network of beekeepers, formal stud book, and reliable diagnostic methods are not sufficient to develop a breeding system for the dark UK bee without isolated matings that avoid introgression from neighbouring unwanted gene pools.

Remote mating sites

In 2005, Jensen et al examined the distance males and queens flew on their nuptial flights. Jensen showed that Edale, in Hope Valley in the Peak District would make a suitable location for a breeding apiary (Jensen et al. 2005). Despite this, seven years on, little progress has been made on selecting formal remote breeding sites for *A. m. mellifera* conservation. In chapter 5, remote breeding was seen to increase *A. m. mellifera* levels but not significantly so. Remote beekeeping is more costly in terms of travel and inconvenience but when sites are chosen correctly (see chapter 5) it can assure mating purity.

Instrumental insemination

Instrumental insemination is a way to ensure complete control over mating. In the past its use has been restricted by expensive equipment and a lack of the necessary skill base. However the National Bee Unit now offers government funded education services and the equipment has fallen in price (Budge personal communication). Artificial insemination would be the best method for the maintenance of genetic purity when stocks are isolated from other pure *A. m. mellifera* colonies, i.e. where colonies are likely to suffer largescale introgression from races other than *A. m. mellifera*.

Mainland A. m. mellifera reserves

Kraus (2005) suggests that for purity to be maintained, every beekeeper within 20km of the protected queen should be restricted in the race that they can keep. Moritz et al (1991), states that maintenance of a protective pure belt of bees is a ‘herculean

task', concluding that land based mating stations should be used for breeding work and not racial purity (Moritz 1991). Mainland reserves rely on local beekeeping communities working together to create pools with similar selection protocols. This method has been seen to be successful in Southern Ireland for the Galtee bee breeding group who maintain *A. m. mellifera* in the Galtee/Vee valley. With re-queening of non *A. m. mellifera* stocks and local beekeeper cooperation they have created a protective barrier than allows stocks of high *A. m. mellifera* levels to be produced from breeding apiaries in the centre of the valley (http://www.gbbg.net/).

Island reserves

Islands sufficiently distant from the mainland, with predominantly *A. m. mellifera* stocks and restricted importation allow the luxury of assured pure matings (Moritz 1991; Solignac 2005). Assuring compliance to importation restrictions on heavily populated islands is difficult, so areas with fewer beekeepers or sites already containing strong *A. m. mellifera* populations should be favoured. While islands afford protection from foreign drones, exposure and high wind velocities can prevent high mating frequencies (Kraus 2005; Neumann et al. 1999). To remedy this, a number of different island reserves should be used. It is also essential that the initial stocks are shown to be pure.

The disadvantage of island reserves is that they are remote and exclude the wider beekeeping community. If breeding efforts are restricted to only island reserves *A. m. mellifera* will inevitably become a rarity. There is also a risk of inbreeding, and the associated decline in genetic diversity if other stocks are not introduced.

For this reason, to ensure the future of *A. m. mellifera* in a viable and profitable form a combination of the above methods should be used.

The future of bee breeding programs in the UK

In promoting the cause of *A. m. mellifera* it is sometimes claimed that it is the *best* bee. This assertion is unhelpful, as few comparative studies of honeybee race have been carried out (Costa et al. 2012). It is thought that local ecotypes may be better adapted to local environmental conditions and more resistant to native diseases. For example *A. m. mellifera* is known to fly at lower temperatures and so would spend less time confined to the hive by bad weather. This would mean there were fewer days when workers bees were prevented from taking their cleansing flights, reducing Nosema levels (Simone et al. 2009). However, the honeybee population of the UK is now exposed to a variety of new diseases against which historic honeybee populations could have no natural resistance, namely *Varroa destructor* and *Nosema ceranae*. Furthermore, the UK's landscape has altered dramatically. In areas such as Lincolnshire and Cambridgeshire large swathes of the countryside are dominated by oil seed rape and winter wheat monoculture. In these regions beekeepers tend to favour races like *A. m. ligustica* that have large numbers of workers early in the spring to take advantage of the early nectar flow. In many areas it seems *A. m. mellifera* has evolved to specialise on heather moorland (the characteristic late summer heather flow can be seen along the Atlantic coast from Portugal to Norway), with a peak in workers at the time of heather flowering. These heather adapted colonies are often too small in spring to match the early honey yields of *ligustica* and other races (Ruttner 1988). Favouring one race over all others can antagonise beekeepers as seen on Laeso island in Denmark (Jensen & Pedersen 2005). Here, some beekeepers felt they were being restricted to a race for conservation purposes instead of for beekeeping purposes. The backlash against the enforced ruling prevented meaningful conservation on the island for many years and left a wealth of ill feeling.

To gain the best bee for a particular region, beekeepers should select positive attributes from the local race (Moritz 1991). To preserve genetic diversity for the future, different beekeeping goals and methodologies have to be run along side one another (figure 6.2). For example, in a generalist overview, commercial beekeepers require the most productive race for their local area, a bee that is mild tempered to work with, may replace their queens annually and use regular chemical treatment to reduce *Varroa* levels. Hobbyist beekeepers meanwhile, do not rely on their colonies

for income, do not tend to replace their queens annually and favour queen longevity and disease tolerance (such that reduced *Varroa* treatment may be possible) as equally important traits as productivity.

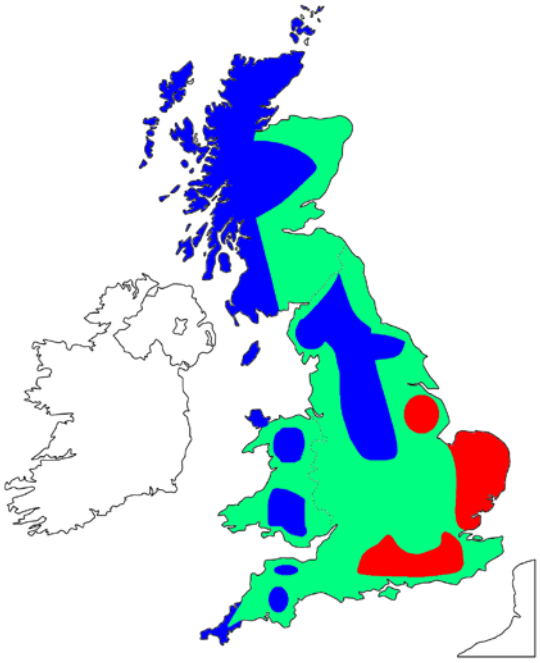


Figure 6.2: Hypothetical beekeeping regions.
Dark blue indicates areas for breeding programs focused on *A. m. mellifera* purity and positive beekeeping attributes, green indicates areas for disease resistance and positive beekeeping attributes from hybrid races, red indicates areas for predominantly commercial beekeeping.

Disease tolerance and fitness may be best served by having a high genetic diversity, so beekeepers close to commercial beekeepers and areas of high importation would be best placed focusing their efforts on disease resistance and other positive beekeeping attributes. Beekeepers in areas with cooler, wetter climates with large expanses of heather can select from *A. m. mellifera* stocks for characteristics that create a good local honeybee. If it could be shown that *A. m. mellifera* represented the best or at least a very positive choice of race for a particular region (such as in the Galtee Valley), beekeepers would be more likely to embrace the selection. Further restrictions, such as those found in Germany, where beekeepers are prohibited from keeping non

carnica bees within 20km of formal breeding stations would then not be as contentious.

If *A. m. mellifera* breeding programs, starting from pure stocks, are assisted by a buffer of pure or near pure *A. m. mellifera* colonies they can remain free to select purely for productivity, temper and disease resistance etc.

In this way, genetic diversity in the UK remains high and our native race is preserved not simply as a museum specimen, but as improved local ecotypes for modern day use. Commercial beekeepers are also able to benefit from a greater knowledge of disease resistance from breeding efforts, and may be able to use the disease resistant local races produced. Locke et al (2012), suggest that interdisciplinary research between apicultural studies and evolutionary biology can provide new insights into parasitic interactions in beekeeping, allow a deeper understanding of how honeybee colonies naturally coevolves with parasites. This is a first step in establishing optimal, long term and sustainable honeybee health management strategies for a diverse and thriving honeybee population (Locke et al. 2012).

In the USA a Coordinated Agricultural Project (CAP) consortium has been set up to deliver sustainable bee management practices to beekeepers (Pettis & Keith Delaplane 2010). This project encompasses all states of the USA, includes a number of leading research institutions and will run for at least 4 years. As detailed in this conclusion, two critical goals of this consortium are a) to identifying geographically discrete pockets of honeybee genetic diversity and b) to deliver research knowledge to client groups. This consists of face to face training sessions and dissemination of the most recent peer reviewed research via a website for beekeepers. This consortium boasts an unprecedented degree of co-ordination and represents the future gold standard of applied honeybee research. Such a scheme could be easily modified for the UK, to embrace local *A. m. mellifera* ecotypes and ensure a healthy balance between conservation and beekeeper needs (Aebi et al. 2012).

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Appendix

Table A.1: Colony 1 estimated queen genotypes for 2009 and 2011.

Some years have multiple estimated queen genotypes due to the presence of un-related workers in the sample.

	1		2		3		4		5		6	
2009	N =	2	5		1		4		1		1	
828/829	118	164	138	138	118	175	118	164	118	177	118	118
836/837	209	209	195	195	191	193	193	209	193	195	185	209
840/841	132	132	134	134	128	132	128	132	128	128	128	138
852/853	106	106	105	106	105	106	106	106	105	106	104	106
864/865	205	205	205	222	205	222	205	222	205	205	205	220
866/867	106	112	106	112	106	116	112	112	106	114	106	108
876/877	180	180	179	180	180	180	181	183	180	180	180	183
882/883												
936/937	157	157	161	161	145	157	157	157	157	157	157	161
938/939	228	228	221	228	221	228	221	228	221	228	228	228
950/951	193	193	191	193	191	193	191	193	193	193	193	193
990/991	168	184	168	168	168	168	166	168	166	168	166	168
2011	N =	7	4		2		1		1			
828/829	160	171	132	171	109	169	160	171	164	171		
836/837	195	195	191	195	185	197	185	195	195	197		
840/841	109	130	128	130	109	130	99	130	128	130		
852/853	106	106	106	106	106	106	104	106	106	106		
864/865												
866/867	118	118	98	106	106	106	104	106	98	106		
876/877	180	180	183	183	180	180	180	180	180	180		
882/883	161	161	145	161	161	161	145	161	161	161		
936/937	118	118	98	106	106	106	104	106	98	106		
938/939	215	215	215	215	215	215	215	215	215	215		
950/951	195	195	191	193	182	182	193	195	191	195		
990/991	162	168	166	182	162	168	168	193	162	168		

Table A.2: Colony 2 estimated queen genotypes for 2009 and 2011.

Some years have multiple estimated queen genotypes due to the presence of un-related workers in the sample.

	1		2		3		4		5		6		7		8	
2009	N=4		2		2		1		1		1		1			
828/829	118	187	118	187	118	187	118	187	118	177	118	169	118	187		
836/837	191	191	199	199	191	199	191	199	191	199	181	191	191	195		
840/841	132	132	109	132	109	132	109	128	99	132	124	132	99	132		
852/853	106	106	106	134	106	149	106	147	106	106	106	106	106	140		
864/865	205	205	205	205	205	218	205	205	205	205	205	205	205	205		
866/867	114	114	106	106	106	106	106	116	106	106	106	106	106	106		
876/877	183	183	179	180	180	183	181	183	179	183	181	183	183	183		
882/883	161	161	161	161	161	161	157	161	145	161	145	161	145	161		
936/937																
938/939	221	228	221	221	221	235	221	221	221	221	221	221	221	221		
950/951	193	205	193	193	193	193	191	193	182	193	193	205	193	195		
990/991	168	194	168	168	168	168	168	180	168	194	166	168	168	168		
2011	N=2		2		4		3		1		1		1		1	
828/829	118	169	118	118	118	118	118	164	118	118	118	187	118	167	118	171
836/837	191	191	191	191	181	213	187	199	191	191	185	191	191	199	191	211
840/841	109	128	109	128	99	109	99	132	99	109	109	109	109	128	109	128
852/853	106	106	106	106	138	138	106	106	106	138	106	149	106	106	106	147
864/865	205	205	205	205	205	218	216	220	205	205	205	205	205	220	205	216

866/867	106	106	106	114	98	106	102	106	106	106	106	106	106	106	106	116
876/877	179	179	179	187	179	179	183	183	179	179	179	183	179	183	179	183
882/883	157	161	145	161	161	161	145	161	157	161	145	161	145	161	157	161
936/937	285	285	285	285	279	285	274	285	285	285	285	285	267	285	279	285
938/939	221	228	221	228	228	228	221	221	221	228	221	235	221	228	221	221
950/951	191	193	181	193	191	193	193	205	193	193	193	193	193	205	193	205
990/991	168	168	168	168	168	178	168	168	168	176	168	194	168	168	168	180

Table A.3: Colony 3 estimated queen genotypes for 2009 and 2011.

Some years have multiple estimated queen genotypes due to the presence of un-related workers in the sample.

	1		**2**		**3**		**4**		**5**		**6**		**7**	
2009	N=1		2		1		1							
828/829	162	171	162	162	162	173	118	162						
836/837	191	191	191	191	185	191	191	203						
840/841	128	134	130	130	130	132	99	128						
852/853	105	149	105	105	105	105	105	124						
864/865	205	220	205	205	205	205	205	222						
866/867	106	112	106	114	106	114	106	114						
876/877	179	180	179	179	179	179	179	179						
882/883	145	157	145	157	145	157	145	157						
936/937	279	285	285	285	279	285	279	285						
938/939	221	228	221	228	221	221	221	228						
950/951	193	197	193	195	193	195	193	195						
990/991														
2011	N=3		4		1		2		2		1		1	
828/829	118	177	175	175	118	167	118	118	118	118	118	167	118	177
836/837	185	191	185	191	185	191	191	191	191	217	185	191	185	191

840/841														
852/853	106	106	106	134	106	128	106	106	106	106	106	106	106	128
864/865	205	205	205	205	205	205	205	205	205	205	205	222	205	205
866/867	106	106	108	108	106	108	106	106	106	106	106	114	106	106
876/877	180	180	183	183	179	179	179	179	179	179	179	183	179	180
882/883	157	157	161	161	145	157	145	145	157	157	145	161	157	157
936/937	285	285	279	285	281	285	281	285	281	285	279	281	279	285
938/939	221	221	221	221	221	228	221	221	221	228	221	228	221	221
950/951	193	193	191	191	191	193	191	193	191	193	191	193	182	191
990/991	168	168	168	168	168	170	168	168	168	194	168	193	168	184

Table A.4: Colony 4 estimated queen genotypes for 2009 and 2011.

Some years have multiple estimated queen genotypes due to the presence of un-related workers in the sample.

	1		**2**		**3**		**4**		**5**		**6**		**7**	
2009	N=1		1		2		3		2		2		1	
828/829	167	169	118	169	118	171	169	169	152	169	169	171	118	169
836/837	185	217	191	199	191	217	185	217	191	191	191	195	185	217
840/841	126	130	99	132	109	132	128	132	126	126	99	132	126	126
852/853	106	106	106	106	106	106	104	106	106	106	105	106	106	106
864/865	205	222	205	220	205	205	205	222	205	222	205	220	205	222
866/867	106	112	106	110	106	106	106	106	104	106	106	106	106	106
876/877	179	180	180	180	179	180	180	180	180	183	180	180	180	180
882/883	157	161	157	161	157	161	157	157	157	157	145	157	157	161
936/937	267	285	267	285	267	285	267	285	267	285	267	285	267	285
938/939	221	228	221	221	221	221	221	221	221	221	221	221	221	221
950/951	193	195	193	195	193	193	193	201	193	195	193	193	180	193
990/991	162	168	162	168	166	168	166	168	162	162	168	168	162	166
2011	N=1		1											
828/829	164	171	164	167										

836/837	185	191	185	191
840/841	99	99	99	132
852/853	147	147	106	147
864/865	205	205	205	205
866/867	104	112	104	112
876/877	180	180	180	183
882/883	155	161	145	157
936/937	285	285	279	285
938/939	221	228	221	221
950/951	182	195	182	195
990/991	168	170	168	170

Table A.5: Colony 5 estimated queen genotypes for 2009 and 2011.

Some years have multiple estimated queen genotypes due to the presence of un-related workers in the sample.

	1		**2**		**3**		**4**		**5**
2009	N=2		1		2		2		
828/829									
836/837	191	191	185	191	185	191	185	191	
840/841	99	109	99	128	99	109	99	99	
852/853	106	138	104	138	104	138	138	138	
864/865	205	222	205	222	222	222	220	222	
866/867	106	106	106	110	106	106	106	106	
876/877	179	179	179	179	179	179	179	180	
882/883	157	157	145	157	157	157	157	157	
936/937	281	281	267	281	281	285	285	285	
938/939	228	228	221	228	228	228	221	228	
950/951	193	193	193	193	193	193	193	195	
990/991	180	184	180	180	180	180	180	180	
2011	N=2		5		5		1		2

828/829	118	118	118	177	118	118	118	171	118	118
836/837	191	191	191	191	191	191	191	191	191	191
840/841	109	109	109	134	109	134	132	134	109	109
852/853	106	138	130	138	130	130	106	106	130	130
864/865	205	216	205	205	205	216	205	205	205	205
866/867	106	106	106	106	106	106	106	112	106	112
876/877	179	179	179	179	179	179	179	179	179	179
882/883	157	157	157	157	157	157	157	157	157	157
936/937	279	279	279	279	279	285	279	285	279	279
938/939	221	221	228	228	221	228	221	221	221	221
950/951	195	195	193	193	193	195	193	195	193	193
990/991	168	168	168	168	168	168	168	168	168	168

Table A.6: Colony 6 estimated queen genotypes for 2009 and 2011.

Some years have multiple estimated queen genotypes due to the presence of un-related workers in the sample.

	1		**2**		**3**		**4**		**5**		**6**	**7**
2009	N=4		3		2		3		2			
828/829	167	167	126	175	175	175	118	175	118	118		
836/837	193	193	185	185	185	191	185	191	185	185		
840/841	99	128	99	99	99	99	99	99	99	99		
852/853	106	106	106	108	106	106	106	106	106	112		
864/865	205	218	205	205	205	205	205	205	205	205		
866/867	106	106	104	106	112	112	106	106	112	112		
876/877	177	185	179	180	180	180	179	180	180	180		
882/883	145	145	157	157	157	157	155	155	155	157		
936/937	279	279	267	279	279	285	279	281	279	279		
938/939	221	221	221	221	221	221	221	228	221	221		
950/951	191	193	191	195	193	193	195	195	191	193		
990/991	193	193	168	194	168	168	168	194	168	168		

2011	N=1		2		1		4		2		2		1	
828/829														
836/837	185	193	191	193	187	191	191	191	185	191	185	185	185	191
840/841														
852/853	105	105	105	105	105	149	105	105	105	108	105	105	105	134
864/865	205	205	205	205	205	205	205	205	205	205	205	205	205	216
866/867	106	112	106	112	106	106	106	112	106	112	106	112	106	112
876/877	177	180	177	179	177	180	177	179	177	179	177	179	177	183
882/883	155	157	155	157	155	155	157	161	155	155	157	157	145	157
936/937	267	279	279	285	279	279	285	285	279	279	279	279	279	281
938/939	221	228	221	221	221	221	221	221	221	221	221	221	221	221
950/951	193	195	193	197	193	195	191	193	193	193	191	193	193	195
990/991	168	184	168	168	162	168	168	168	168	168	168	168	168	168

Table A.6: Colony 6 estimated queen genotypes for 2009 and 2011.

Some years have multiple estimated queen genotypes due to the presence of un-related workers in the sample.

	1		**2**		**3**		**4**		**5**	**6**	**7**
2009	N=4		3		4		2				
828/829	118	169	118	169	118	169	169	171			
836/837	191	191	195	195	191	193	185	191			
840/841											
852/853	105	106	106	106	106	112	106	106			
864/865	205	205	205	205	205	205	205	228			
866/867	106	106	106	106	106	114	106	106			
876/877	179	179	180	180	179	179	180	180			
882/883	145	145	157	157	157	161	157	161			
936/937	285	285	281	281	281	285	281	285			
938/939	221	228	221	221	221	221	221	221			
950/951	195	195	193	193	195	195	195	195			

990/991	166	166	166	168	166	168	166	168						
2011	N=3		2		2		3		2		1		2	
828/829	171	171	160	171	118	118	118	118	167	171	118	167	171	171
836/837	185	191	191	213	191	193	185	191	191	191	191	191	185	191
840/841	99	130	109	130	130	130	130	130	130	130	128	130	130	147
852/853	106	106	105	106	105	106	106	106	106	128	105	106	106	130
864/865	205	205	205	205	205	205	205	228	205	205	205	228	205	205
866/867	106	114	106	114	112	112	106	114	112	112	106	114	112	114
876/877	180	180	179	181	180	181	179	181	181	181	180	180	180	180
882/883	157	157	145	157	157	157	150	157	157	157	157	157	157	157
936/937	267	285	267	285	285	285	267	285	285	287	279	285	279	285
938/939	221	221	221	228	221	221	221	221	221	221	221	221	221	221
950/951	195	195	193	195	193	195	195	195	195	195	195	195	193	195
990/991	166	166	166	168	166	168	166	166	166	168	166	168	166	168

Table A.7: Colony 7 estimated queen genotypes for 2009 and 2011.

Some years have multiple estimated queen genotypes due to the presence of un-related workers in the sample.

	1		**2**		**3**		**4**		**5**	
2009	N=1		5		3		2		4	
828/829	169	175	118	169	118	171	118	156	156	169
836/837	185	191	207	207	185	201	185	185	207	207
840/841	124	128	136	136	128	132	128	128	128	128
852/853	106	136	106	130	106	136	106	106	106	106
864/865	205	214	205	220	205	205	205	205	205	205
866/867	106	106	106	106	106	116	106	106	106	106
876/877	180	183	180	180	180	180	180	180	180	180
882/883	157	157	145	157	145	155	157	157	161	161
936/937	279	285	281	281	279	279	279	285	285	285
938/939	228	228	228	228	221	228	221	228	221	228

950/951	193	195	193	193	181	197	181	193	182	182
990/991	166	168	168	170	168	181	168	170	168	170
2011	2		1		2					
828/829										
836/837	191	219	181	219	193	193				
840/841	99	124	99	136	128	136				
852/853	106	106	106	106	106	106				
864/865	205	218	218	218	218	218				
866/867	106	106	106	106	106	106				
876/877	180	180	180	183	183	183				
882/883	157	161	157	161	157	157				
936/937	279	279	279	281	279	281				
938/939	215	215	215	215	215	215				
950/951	193	197	193	193	193	193				
990/991	168	168	166	168	168	178				

Table A.8: Colony 8 estimated queen genotypes for 2009 and 2011.

Some years have multiple estimated queen genotypes due to the presence of un-related workers in the sample.

	1		**2**		**3**		**4**		**5**		**6**	
2009	N=3		2		2		1		2		2	
828/829	167	169	167	167	167	167	169	171	167	171	169	171
836/837	185	195	191	199	185	191	191	195	191	205	189	191
840/841	130	130	126	130	99	130	130	160	130	130	99	130
852/853	104	105	104	104	104	104	104	105	104	104	104	104
864/865	205	216	205	205	216	218	205	216	205	216	205	216
866/867	106	116	112	112	106	112	112	118	106	112	112	112
876/877	180	183	180	180	180	183	180	180	180	180	180	183
882/883	145	145	145	157	161	161	145	145	145	157	145	145
936/937	279	287	279	279	267	279	279	285	279	279	279	279

938/939	221	221	221	221	221	221	221	221	221	221	221	221
950/951	181	193	193	193	193	193	181	193	181	195	181	193
990/991	166	181	162	194	181	193	166	181	181	181	166	193
2011	N=1		1									
828/829	158	158	158	169								
836/837	191	191	191	193								
840/841	109	136	99	134								
852/853	104	106	106	106								
864/865												
866/867	112	112	106	112								
876/877	179	180	179	179								
882/883	145	145	145	161								
936/937	281	285	281	281								
938/939												
950/951	193	199	193	195								
990/991	193	198	193	196								