Demographic and genetic patterns of water voles in human modified landscapes: implications for conservation

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Abstract

The European water vole (*Arvicola amphibius*) has undergone widespread declines across much of the UK and a major consideration for their future conservation is facilitating the admixture of individuals in modified landscapes to promote population growth, restore geographical distribution and ensure long term viability. However implementing remedial management strategies for these purposes requires knowledge of how populations are affected by, and respond to, fragmented and perceptively hostile landscapes. This thesis uses non-invasive genetic tools in conjunction with a live capture study to investigate the population structure, genetic variation and social systems of both natural and reintroduced water vole populations living within modified landscapes in southeastern England. In particular this research determines; a) whether non-invasive hair capture tubes are effective at collecting samples for genetic monitoring of water voles; b) whether there is a quantifiable difference in the demographic performance (survival, recruitment, population growth), neutral genetic variation and kin structure of populations occupying linear habitats compared to unmodified non-linear wetland habitats and c) identify the spatial pattern of variation in microsatellite and mitochondrial DNA amongst water vole populations to investigate natural and human mediated factors effecting historical and contemporary distribution.

Non-invasive hair capture tubes proved to be a successful and efficient method for obtaining hair samples from wild populations of water voles with one genetic sample taking 0.53 man hours and costing £0.80, which was four times more efficient and 13 times more cost effective than live capture methods. However, DNA degradation, low quantities of DNA template and small sample sizes were identified as limiting factors when applying this method to genetic studies on water voles.

Different patterns in demography and kin structure of water voles were observed between populations occupying linear and non-linear wetlands. Water voles in linear wetlands showed higher overall survival and dispersal of

young, but were characterised by a significantly lower proportion of breeding females in spring which resulted in higher levels of female philopatry and incidents of inbreeding. Populations occupying non-linear wetlands were comprised of more breeding females in spring which had a positive influence on population growth. There was also evidence to suggest that when the abundance of overwintered females was high, the sexual maturation of female young was suppressed which concurred with fewer breeding female relatives being observed after the breeding season in populations occupying non-linear habitat types. Tolerance between female kin was reduced in both habitats types with increasing abundance of voles and no spatial avoidance between opposite sex mates was observed.

Genetic diversity was similar between populations occupying linear and non-linear wetlands and significant temporal variation in genetic composition was observed, particularly in linear wetlands, suggesting a high turnover of individuals. Between populations, genetic variance at microsatellite loci was partitioned amongst river catchments, which concurred with patterns in mitochondrial variation across southeast England. Reintroduced populations exhibited significantly higher genetic diversity in terms of heterozygosity and allelic richness, however spatial analyses across the study sites suggest that reintroductions have homogenised the genetic variation at microsatellite loci and evidence of captive breeding between divergent lineages that have no geographical affiliation to the study area highlight that reintroductions can contribute to the loss of genetic heritage and diversity.

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Author's Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed:.....  .....

Dated:.....28th April 2015.....

Chapter One: General Introduction

1.1 Processes of human landscape modification

Human landscape modification is irrefutably a major threat to the world's biological and genetic diversity (Fischer & Lindenmayer, 2007; Tscharntke *et al.,* 2005; Sanderson *et al.,* 2002). Approximately 75% of the terrestrial biosphere has been altered to some extent through processes associated with natural resource exploitation and, at its most extreme, complex natural ecosystems have been transformed into simplified managed landscapes, dominated by agriculture, forestry and urban habitat (Ellis & Ramankutty, 2008; Tscharntke *et al.,* 2005). This process has led to the relative loss of natural habitat whereby formerly large tracts of native vegetation become completely replaced for commodity production or become perforated, dissected or sub-divided by new and often intensively managed land use types (McGarigal *et al.,* 2005). As a result, remnant patches of natural habitat are generally smaller, geometrically altered and fragmented within a matrix of perceptively hostile or unfamiliar land (Fischer & Lindenmayer, 2007).

Alterations to the natural state of landscapes can lead to disruptions to the distribution of natural resources and ecological processes that determine the viability of wildlife populations including, but not limited to, their population growth, survival and dispersal (Csorba & Szabó, 2012; Fischer & Lindenmayer, 2007; Mills, 2007). Deleterious impacts of habitat loss and fragmentation have led to the decline and/or loss of populations or species from all taxonomic groups including birds, mammals, reptiles, invertebrates and plants (Fischer & Lindenmayer, 2007). Of the 18,788 species currently known to be threatened with extinction, negative effects associated with habitat loss and fragmentation are known to affect 86% of all birds, 86% of all mammals assessed and 88% of amphibians (IUCN, 2010).

Understandably, a major priority for global biological and genetic conservation is to identify how human modified landscapes influence the elements of nature under threat so that management strategies can mitigate and prevent any further loss to worldwide biodiversity (Sanderson *et al.,* 2002).

1.2 Threatening processes of landscape modification on wildlife populations

Individual species can be negatively impacted by landscape modification through the direct loss of resources required to sustain an individual and/or population (McGarigal *et al.,* 2005). Based on island biogeography theory, this will result in a reduction in the amount and abundance of species that occupy a particular patch (MacArthur & Wilson, 1967). Firstly, at a patch level, smaller habitat patches are less likely to support larger more viable populations due to the loss of resources required to meet the life history requirements of a species (McGarigal *et al.,* 2005). This is often exacerbated by the proportional increase in edge-influenced habitat, which may provide lower habitat quality and therefore reduce the effective area of undisturbed interior habitat that is suitable for a given species (McGarigal *et al.,* 2005; Fagan *et al.,* 1999). Secondly, when patches of suitable habitat become spatially reduced, the distance between patches generally increases and patches become interspersed by unfamiliar and perceptively hostile land use types (Fischer & Lindenmayer, 2007; Fahrig, 2003). This can reduce the probability of successful dispersal between suitable patches due to the increased time spent within the matrix of non-habitat and/or reduce the number of individuals choosing to disperse due to the increased energetic cost involved in obtaining new resources (McGarigal *et al.,* 2005; Schtickzelle & Baguette, 2003). For instance, landscape features such as habitat type and roads have been found to restrict migration between populations of carnivores (McRae *et al.,* 2005) salamanders (Wang *et al.,* 2009) and toads (Dixo *et al.,* 2009), and increasing distance between resource patches is frequently cited to reduce dispersal success (Le Galliard *et al.,* 2012; Bender *et al.,* 1998).

A small population that is isolated within the landscape becomes increasingly at risk to localised extinctions due to the reduced rate of immigration of individuals that would provide demographic and genetic rescue (Amos & Balmford, 2001). Small populations are particularly vulnerable to environmental and demographic stochasticity, which would exacerbate the risk of localised extinction (Mills, 2007). There is, therefore, likely to be a threshold amount of suitable habitat

accessible to a given species below which there is likely to be a decrease in the probability of species persistence within the landscape (Püttker *et al.,* 2011).

In addition to the deterministic threats to population size and dispersal success associated with the reduction in resource accessibility, changes to the biology and behaviour of species have also been observed in modified landscapes, both of which can exacerbate species decline (Fischer & Lindenmayer, 2007). For instance, sociality, which has evolved in many group-living species to secure fitness benefits relating to survival and reproductive success, can be disrupted in ways that are most likely maladaptive by constraints introduced by habitat loss and fragmentation (Banks *et al.,* 2007). Reduced dispersal and increased isolation of small patch populations can elevate genetic relatedness among individuals and increase the risk of inbreeding (Stow *et al.,* 2001). Furthermore, increased competition for biological resources in small habitat patches can reduce kin tolerance, negating any fitness benefits, such as reduced infanticide or predator defence, that may have otherwise been accrued (Maher, 2009).

Reductions to the size and alterations to the shape of habitat patches have also been shown to reduce breeding success, survivorship, recruitment and individual growth rates in species (Püttker *et al.,* 2011; Fischer & Lindenmayer, 2007; McGarigal *et al.,* 2005; Hokit & Branch, 2003). These changes can result from the reduced physical condition of individuals due to decreasing resource availability (Püttker *et al.,* 2011), inbreeding depression, or as a result of increasing inter-specific interactions including resource competition, parasitism and predation (Fischer & Lindenmayer, 2007; McGarigal *et al.,* 2005). These interactions are generally more prevalent in smaller, more convoluted habitat patches due to the proportional increase of edge influenced habitat (Fahrig, 2003; Fagan *et al.,* 1999; Bender *et al.,* 1998; Stamps *et al.,* 1987). For example, the loss of bird species from small forest fragments is postulated to result from reduced survivorship and recruitment due to the increased rate of predation and brood parasitism that occurs near the edges of each patch (Andrén & Angelstam, 1988).

Small and convoluted patches have also been shown to alter dispersal behaviour. Several theoretical and empirical studies have found that emigration rates increase with decreasing patch size and/or increasing linearity (Chapman *et al.,* 2007; Schtickzelle & Baguette, 2003; Kindvall & Petersson, 2000; Stamps *et al.,* 1987). This is considered to be influenced by the increased probability of an individual encountering an edge and entering into the surrounding matrix (McGarigal *et al.,* 2005). Whilst this would clearly increase movement into the surrounding matrix and potentially provide, if dispersal is successful, demographic and genetic benefits to recipient populations, the effect on population size of the host patch would be negative if recruitment through immigration and births did not exceed the number of individuals leaving (Mills, 2007). However, immigration rate can also be affected by patch size and several studies have found that dispersing individuals are more likely to end up in larger patches than in smaller ones (Chapman *et al.,* 2007; Kindvall & Petersson, 2000).

Disturbances to demographic performance (survival, recruitment, population growth etc), behaviour and dispersal success of species are important factors that contribute to the stability and persistence of populations within modified landscapes. These factors also have important consequences on the genetic structuring of populations which in turn can influence their long-term viability.

1.3 Genetic consequences of habitat modification for populations

A major concern for species conservation in human modified landscapes is the effect that modifications have on the genetic diversity of populations and/or species (Young *et al.,* 1996). Genetic diversity provides the foundations for species to evolve adaptively to environmental conditions, reducing the capacity of deleterious recessive alleles, which influence individual fitness, to become fixed within populations (Frankham *et al.,* 2011; Amos & Balmford, 2001; Lacy, 1987). Fisher's fundamental theory of natural selection states that the rate at which the fitness of a population increases is proportional to the genetic variance in fitness, which must be directly related to how much genetic diversity is present (Fisher, 1930). As such, genetic diversity is inversely proportional to the threat of extinction and is recognised by the IUCN as a form of biodiversity

that requires conservation (Reed & Frankham, 2003). Reductions in genetic diversity and fitness are commonly observed in populations that are small and/or isolated (Frankham *et al.,* 2011). As such, the loss of genetic diversity is likely to be a key threat to species that are vulnerable to the loss and fragmentation of natural habitat (Mills, 2007).

Small and/or isolated populations are typically more at risk of genetic erosion and reductions in fitness through the processes of random genetic drift and inbreeding (Lebois *et al.,* 2006). Genetic drift is the random sampling of genes during transmission from one generation to the next and is hence a stochastic process; gene variants (alleles) will be lost more rapidly when sampling from a small population relative to a large population (Lacy, 1987). This process leads to the gradual deterioration of genetic diversity and can facilitate the exposure of existing or newly arising deleterious mutations which can accumulate and become fixed (mutational meltdown) (Lacy, 1987). This is because selection is less effective in small populations, specifically when $s<1/2N_e$, where s is the coefficient of selection and N_e is the effective population size (Keller & Waller, 2002). As a consequence, genetic drift can compromise the evolutionary flexibility and average fitness of small populations; however, these processes tend to be gradual and as such do not pose an immediate threat to population viability (Lacy, 1987).

In contrast, the effects of inbreeding can be immediate and commonly occur as a consequence of small population size (Reed & Frankham, 2003). The incidence of random inbreeding within populations that have become small or were founded from a small number of individuals ("founder effect") is generally increased due to the accumulation of relatedness between individuals over time (Reed & Frankham, 2003; Amos & Balmford, 2001); when populations reach the state in which individuals are more likely to encounter and mate with a relative than they are a non-relative (more distant than 3rd cousins), the average inbreeding coefficient of the population will begin to increase over subsequent generations (Keller & Waller, 2002). Inbreeding increases the probability that an individual will inherit genes that are identical by descent, leading to an increase in homozygosity within a population and a loss of genetic diversity

(Frankham *et al.,* 2011). Homozygosity is generally considered to threaten individual fitness as it increases the chance that the individual is homozygous for recessive, deleterious alleles that would generally be masked when in a heterozygote state (Charlesworth & Willis, 2009; Slate *et al.,* 2004). Inbreeding can reduce the reproductive success and survival of offspring, an effect known as inbreeding depression, which has been observed in numerous mammal, bird, fish and plant populations that have become small and isolated (Frankham *et al.,* 2011).

The rate of genetic drift and incidence of inbreeding in populations are elevated when the effective population size (N_e) is reduced (Kliman *et al.,* 2008). The effective population size is the number of individuals required to contribute the observed genetic variation to the next generation (Frankham *et al.,* 2011). Specifically, N_e = the size of the ideal population that will result in the same amount of genetic drift as the actual population being studied (N). For this reason $N = N_e$ only under specific conditions; equal numbers of breeding males and females; all individuals having an equal probability of reproducing and equal family sizes; constant N; and discrete generations (Kliman *et al.,* 2008). The fact that most real populations rarely satisfy these conditions results in N_e being typically smaller than the actual number of individuals in the population. In addition, this discrepancy is due to variation in mating systems (monogamous vs. polygamous), individual/population dispersion and demographic stochasticity in response to chance environmental events (Mills, 2007). As such, populations that have become small and confined to suitable habitat patches and have exhibited changes to their behaviour and/or biology in response to the spatial arrangement of resources (as described in Section 1.1) will be more at risk to reductions in the effective population size, genetic variation and the accumulation of deleterious alleles. For example, Kawata (2001) found that more deleterious alleles accumulated and the risks of extinction were larger for simulated populations occupying longer and narrower habitats than those in regular square habitats, due to the reduced mating opportunities presented in linear habitat patches.

The effects of genetic drift and inbreeding depression on populations within modified landscapes will be reduced when migration (gene flow) of individuals between occupied patches is facilitated (Frankham *et al.,* 2011). New individuals allow the introduction of new polymorphisms into a population, on which selection can act, in turn reducing the loss of genetic variation caused by drift and decreasing the potential for inbreeding (Lowe *et al.,* 2007). It is, however, contentious as to how much gene flow is required to prevent the potential for inbreeding depression in small populations. Mills and Allendorf (1996) argue that one migrant per generation is an appropriate minimum; however, Vucetich and Waite (2000) suggest that this may not be sufficient when populations fluctuate in size due to demographic stochasticity or when populations typically undergo extinctions and recolonisations, as in metapopulations (Young *et al.,* 1996). They suggest that >10 or even >20 migrants per generation may be required to prevent the adversities of inbreeding depression.

There are, however, a number of processes associated with human landscape modification that are likely to influence the number of migrants being exchanged between patches. Increasing distance between habitat patches has been found across multiple taxa to reduce the rate of gene flow, resulting in positive correlations between distance and genetic differentiation between populations (isolation by distance effect) (Frankham *et al.,* 2011; Manel *et al.,* 2003). This is predicted to be more pronounced in essentially linear habitat patches (Wright, 1965) where only neighbouring populations exchange migrants. For example, Aars *et al.* (1998) found restricted gene flow in bank voles occupying linear forested habitat in comparison to more naturalistic two-dimensional forested blocks. Habitat barriers such as roads, railways and impermeable land uses within the intervening matrix are also likely to influence whether migratory individuals reach or colonise a patch. For example, Frantz *et al.* (2010) found that motorways restrict Eurasian badger *(Meles meles)* movement causing genetic differentiation between populations in western England and, similarly, Piertney *et al. (*1998) found that unsuitable habitat restricted gene flow between red grouse *(Lagopus lagopus)* populations in Scotland.

Understanding the demographic and genetic patterns and processes of species occupying human modified landscapes is essential to determine whether species may become or are 'at risk' to extinction as a result of habitat loss and fragmentation. The sensitivity of species to these processes will depend on their spatial and resource flexibility and as such there are considerable merits for undertaking species-specific approaches for assessing the deleterious effects of landscape modification (Fischer & Lindenmayer, 2007; Sanderson *et al.,* 2002).

1.4 Genetic tools for conservation

Recent advances in molecular techniques have facilitated the use of genetic theory to provide further insight into understanding populations so that more informed decisions can be made that address contemporary conservation problems (Lowe *et al.,* 2007). The principle behind this approach is that, because genes are inherited from parents to offspring in a predictable fashion, following Mendel's laws of inheritance, contemporary patterns of genetic variation capture the demographic history of the population under study (Bateson, 2007). Specifically, population bottlenecks, expansions, isolation, amalgamation and inbreeding can all be inferred by careful analysis of a variety of genetic markers (Lowe *et al.,* 2007).

Many of the issues that are addressed using genetic tools are applicable to studies aimed at quantifying the patterns and processes of a given species within human modified landscapes. For example, genetic data from current populations allow the prediction of the genetic consequences of habitat loss and fragmentation, such as loss of genetic variability within and between populations and inbreeding depression, allowing the effective management of populations identified to be at increased risk of extinction (Frankham *et al.,* 2011; Lowe *et al.,* 2007; Wan *et al.,* 2004). It can also help to elucidate the underlying processes that have determined the current status of species by identifying historical patterns and processes of populations (demographic fluctuations, colonisations, gene flow) (Moritz *et al.,*1987) and their contemporary behavioural and ecological parameters (social structure, mating systems, reproductive success and dispersal) (Piertney *et al.,*1999). This

information can be invaluable in determining the appropriate scale of management to ensure genetic integrity is maintained between historically divergent populations and in directing *in-situ* and *ex-situ* conservation programmes that incorporate the life history requirements of the species (Frankham *et al.,* 2011; Lowe *et al.,* 2007).

The development of efficient DNA amplification methods using polymerase chain reaction (PCR) and identification of highly variable and informative molecular markers has given rise to the widespread application of genetic information (Frankham *et al.,* 2011; Lowe *et al.,* 2007; Mills, 2007). Molecular markers are heritable DNA sequences that occur in the nuclear and organelle genomes and which vary in their evolutionary characteristics (Lowe *et al.* 2007). These differences are determined by the mode of inheritance and the rate of structural and sequence mutation and, as such, the degree and resolution of variation that is found between individuals, populations and species (Lowe *et al.,* 2007; Mills, 2007). As such, the choice of genetic marker used for a particular study will be determined by the nature of the investigation. Ideal markers for quantifying the patterns and processes of threatened populations in modified landscapes should be able to detect qualitative or quantitative variation, be selectively neutral and show codominant inheritance (both alleles are expressed), if applicable (Lowe *et al.,* 2007). Of perhaps the most widely used molecular markers are microsatellite loci and mitochondrial DNA (Selkoe & Toonen, 2006; Castro *et al.,* 1998).

1.4.1 Microsatellite loci

Microsatellite loci are found at high frequency within the nuclear genome of most species and consist of short motifs (1-6 nucleotide bases) that are repeated in tandem (Frankham *et al.,* 2011; Selkoe & Toonen, 2006). Sequences are typically composed of dinucleotide (eg, $(CA)_n$), where n represents the number of copies, trinucleotide (eg, $(CAG)_n$) and tetranucleotide (eg, $(CAGA)_n$), motifs that occur largely, but not exclusively, within the non-coding region of DNA (Lowe *et al.,* 2007). Microsatellites are commonly used in population genetic studies because they are highly variable between individuals and populations. Each microsatellite marker can be found to have upwards of

ten alleles in a large population allowing for individual identification, hence their development as “DNA fingerprints” now widely employed for forensic investigations (Frankham *et al.,* 2011). The high level of variation possible with microsatellite markers makes them especially valuable for estimating magnitudes of gene flow (migration rates) and the level of inbreeding (Frankham *et al.,* 2011; Mills, 2007). This genetic variation (polymorphism) is a result of mutations that occur at microsatellite loci, that is mostly due to slipped-strand mispairing during DNA replication, repair or recombination, principally due to the fact that the replication procedure lacks fidelity in highly repetitive regions of the DNA (Selkoe & Toonen, 2006). As a result of these mutations, the number of repeat motifs (eg $(CA)_n$) increases or decreases typically to n+1 or n-1 (stepwise mutation) with the consequent alteration in the length of the DNA fragment. Hence each mutation results in a new allele at a particular microsatellite locus (Lowe *et al.,* 2007). New alleles become incorporated within a breeding population over time resulting in a frequency distribution of alleles that can be characteristic of the population, particularly in isolated populations with limited gene flow.

1.4.2 Mitochondrial DNA

Mitochondrial (mt) DNA is a relatively small (~16 k basepairs), double stranded molecule that occurs within mitochondria organelles located in an organism's cell (Moritz *et al.,* 1987). In animals, mtDNA encodes 37 genes essential for normal mitochondria function, which are typically well conserved, whereby intraspecific divergence only arises through infrequent mutations, the frequencies of which are predominantly influenced by migration and drift (Castro *et al.,* 1998). In most animals, mtDNA is typically inherited as multiple copies through the maternal lineage; it does not recombine during meiosis, hence making it a particularly appropriate marker for tracing maternal genealogy and deep phylogenetic splits (Castro *et al.,* 1998). As such, mtDNA is widely used in population genetic studies for elucidating intraspecific and interspecific geographic variation over wide geographic ranges as well as determining population structure, colonization events (founder effects), source populations and recent population bottlenecks (Harrison, 1989).

1.4.3 Non-invasive genetic sampling techniques

A major consideration for studies that utilise genetic tools to elucidate population processes at a landscape level are the inherent limitations in obtaining genetic material from the field that is of sufficient quality and quantity to enable the scientific goals of the study to be met (Taberlet & Luikart, 1999). Traditionally, genetic material is obtained from blood or tissue samples using live capture techniques (Lowe *et al.,* 2007). However, due to technological advancements in DNA extraction, preservation, genetic marker development and PCR, genetic information can be obtained from relatively small amounts of biological material including, but not limited to, hair, faeces, shed skin, urine, saliva and feathers (Periera *et al.,* 2009; Lowe *et al.,* 2007). This has led to the relatively new but rapidly growing and applied methodological approach of obtaining genetic material using non-invasive or remote sampling methods.

Non-invasive genetic sampling (NGS) facilitates the collection of DNA samples from free-living organisms without the need to capture, disturb or even see the individuals (Taberlet & Luikart, 1999). Genetic material can be obtained from either a systematic or opportunistic collection of biological material left *in-situ* by the study organism or by using relatively inexpensive devises that are designed to obtain such samples either passively or through the use of a lure (Periera *et al.,* 2009; Long *et al.,* 2008). As such, NGS provide tangible benefits to genetic studies that include; 1) the collection of DNA from rare or elusive species that would otherwise be logistically or ethically difficult to capture; 2) the capture of samples from large geographical areas that would otherwise be logistically difficult and expensive to obtain and 3) details on fine scale ranging behaviour/habitat use can be obtained that would otherwise be biased due to the induced response that is inevitable when utilising live capture techniques (De Barba *et al.,* 2010; Garcia-Alaniz *et al.,* 2010; Long *et al.,* 2008; Lowe *et al.,* 2007).

1.5 Study Organism: The European water vole *(Arvicola amphibius)*

1.5.1 General background to species biology and ecology

The European water vole *(Arvicola amphibius)* is a relatively small (<350 g) arvicoline rodent whose distribution covers much of continental Europe and

Russia (Shenbrot & Krasnov, 2005). In the British Isles and in other areas of Western Europe, the water vole exclusively occupies wetland environments where individuals form discrete colonies both along riparian riverine systems and within more extensive reedbeds, fens and marshland (Strachan *et al.,* 2011). Water voles are primarily herbivorous and spend the majority of their time between two and five meters from the water's edge where they will either utilise extensive burrow systems or weave above ground nests for refuge and breeding (Moorhouse, 2004). They show high plasticity to different wetland habitat types but generally prefer slow flowing but permanent water, penetrable banks for burrowing and high cover of riparian vegetation that provides food, nest sites (in the absence of banks) and cover from predation (Strachan *et al.,* 2011; Aars *et al.,* 2006; Macdonald & Rushton, 2003).

Like most arvicoline rodents, water vole colonies are structured around breeding females that form exclusive territories during the summer breeding season which lasts from the end of March through till September (Strachan *et al.,* 2011; Stoddart, 1970). During this time, males establish overlapping ranges that incorporate multiple breeding females (Moorhouse, 2004). The territory and home range sizes of females and males respectively are generally resource and density dependent and can range from 20 to 150 m for females and 60 to 300 m for males (Strachan *et al.,* 2011; Moorhouse & Macdonald, 2008). Mating is thought to be primarily polygamous and females can have up to five litters, commonly consisting of between five and eight young, that will be weaned within the burrow or nest for approximately two weeks or until they have reached approximately 30 g (Strachan *et al.,* 2011). After this time, juveniles will emerge from their burrows and after reaching 100 g may either disperse to find new resources and mating opportunities or remain in the natal colony (Telfer *et al.,* 2003). Some individuals may reach sexual maturity within their year of birth; however, studies have found that the majority of breeding is carried out between overwintered adults (Telfer *et al.,* 2003; Stewart *et al.,* 1999).

Water voles do not hibernate but spend the majority of winter within burrows where they are thought to cohabit with other members of the colony (Carter &

Bright, 2003). Demographic studies on water vole populations across the British Isles have demonstrated that water voles can suffer high overwinter mortality and are also short lived, commonly surviving a maximum of two winters (Strachan *et al.,* 2011). As such the turnover of water vole colonies is high and dispersal is crucial for overall persistence (Strachan *et al.,* 2011). This is supported by both ecological and demographic studies that strongly suggest that water voles consist of metapopulations whereby colonies are patchily distributed and have an average lifespan that is shorter than that of the whole network (Moorhouse *et al.,* 2009; Fisher *et al.,* 2009; Telfer *et al.,* 2003; Lambin *et al.,* 1998).

1.5.2 Conservation status and threats

Once widespread across the British Isles, water voles have undergone one of the fastest documented declines of any British mammal, suffering an estimated loss of approximately 90% of populations since 1939 (Strachan *et al.,* 2000) (Figure 1.1). This loss has primarily been attributed to the widespread loss and degradation of wetland habitats, particularly in lowland England, and the predation of populations in regions colonised by the feral American mink (*Neovison vison*) (Jefferies, 2003; Bonesi *et al.,* 2002; Barreto & Macdonald, 2000; Barreto *et al.,* 1998a). These effects have been exacerbated by habitat fragmentation and population isolation (Macdonald & Strachan, 1999; Lambin *et al.,* 1998). As such, there has been a marked decline in the distribution of water voles across the UK and in other areas of Western Europe. To counteract these losses, water voles in the UK became fully protected under European law in 2008 under Section 9 of Schedule 5 of the Wildlife and Countryside Act 1981and conservation initiatives, as set out by the UK Biodiversity Action Plan have been implemented that aim to maintain the current distribution of water voles and to re-establish populations across their historical range through reintroductions and/or natural population expansion (Strachan *et al.,* 2011; Maddock, 2008; White *et al.,* 1997).

Despite conservation efforts, water voles are still threatened by the presence of mink and by activities associated with water course management and agricultural intensification (Strachan *et al.,* 2011). Many water courses remain

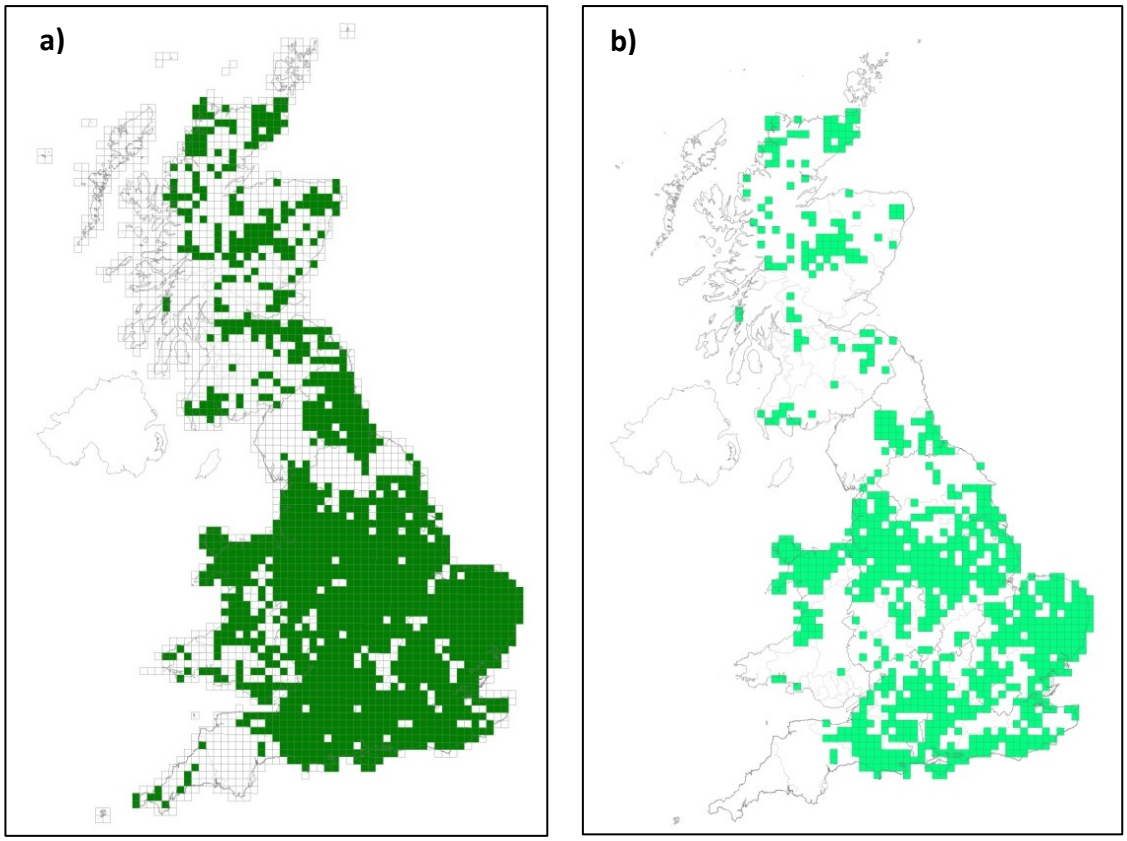

Figure 1.1 *Distribution of water voles within 10km grid squares, between 1900 and 2010 (a) and 2006 and 2010 (b) produced by Hampshire and Isle of Wight Wildlife Trust in collaboration with the Peoples Trust for Endangered Species and the Environment Agency, for the National Water Vole Database and Mapping Project. Datasets used in production of maps and original source are available within Leggett et al., 2012.*

unsympathetically managed by mechanical clearance to alleviate flood risk to agricultural land and urban areas (Telfer, 2000). Many rivers are also heavily engineered and straightened, deeming them unsuitable for water vole colonisation due to increasing water flow and the canalisation of the banks (Strachan *et al.,* 2011). Water courses are also heavily impacted by agricultural land which covers approximately 76% of the land surface of the UK (Macdonald & Burnham, 2011). Diffuse pollution from intensive arable farming, for instance, can contaminate water courses, especially in the absence of buffering vegetation along the banks (Macdonald & Burnham, 2011). Furthermore, intensive grazing along the banks can denude riparian vegetation and cause erosion to the bank which can destroy water vole burrows (Hardman & Harris, 2010). As such, recent estimates have indicated that water voles have

undergone a further 22% decline nationwide since 2011 (The Wildlife Trusts, *pers. comm*.).

1.5.3 Water voles within human modified landscapes

As a consequence of the widespread drainage and modification of wetlands in the UK, water voles have become primarily confined to narrow, linear remnants of formerly more extensive wetland habitat, which is patchily distributed and embedded within perceptively hostile agricultural landscapes (Macdonald & Rushton, 2003). Within these habitats, water vole colonies are typically small and suffer from high overwinter mortality rates, which can result in the loss of over 70% of individuals (Jordon & Chestnut, 1999). This has been partly attributed to the vulnerability of populations in linear fragments to predation, particularly from mink, a hypothesis proposed by Baretto *et al.* (1998b) who argue that predation efficiency increases within increasing linearization. This has been further supported by a study by Carter & Bright (2003) who reported decreased predation of water voles occupying non-linear reedbed habitat and later in a study by Moorhouse *et al.* (2009) that found that water vole survival was positively correlated to increasing amounts of bank-side vegetation, which provides more cover from predation.

As populations in linear habitats can be reduced to fewer than five voles at the beginning of the breeding season (Telfer *et al.,* 2003), discrete colonies are likely to be vulnerable to the loss of genetic variation through drift and inbreeding (Berthier *et al.,* 2005). Furthermore, as water voles operate in distinct units in linear habitats, the biological sampling of genes may be negated, whereby genes are only exchanged with a finite number of neighbouring mates (Wright, 1965). This can cause a reproductive skew, resulting in a reduction in the effective population size and the exacerbation of any effects of genetic drift and inbreeding depression (Frankham *et al.,* 2011). A study by Aars *et al.* (2006), for example, reported that male water voles typically produce more offspring than females and that there are high levels of linkage disequilibrium at microsatellite markers within water vole colonies, suggesting local structure within populations and strong genetic drift. However it was also suggested in the same study, that water voles may adopt a more

monogamous mating strategy in patchy watershed habitats, which reduces the reproductive skew and rate of loss of the effective population size.

Given the demographic instability and risk of genetic erosion in populations occupying modified linear wetlands, water voles are considered to be highly vulnerable to habitat fragmentation. This is because dispersal between colonies is crucial to the overall persistence of populations due to the high turnover of individuals (Telfer *et al.,* 2001; Stewart *et al.,* 1999). Immigration into discrete colonies is therefore integral to provide demographic rescue and would further help to counteract local inbreeding and reduce the loss of genetic variation exhibited in small, isolated populations (Amos & Balmford, 2001). Whilst dispersal rates as high as 25% and consisting of both males and females have been observed in linear systems in Scotland (Aars *et al.,* 2006), dispersal success is likely to be highly influenced by distance, given the small size of the species (Berthier *et al.,* 2005). For instance, the majority of studies that have measured dispersal distances of water voles, have found that individuals are capable of dispersing an average of 1 to 2 km (Aars *et al.,* 2006; Berthier *et al.,* 2006; Telfer *et al.,* 2003; Stewart *et al.,* 1999), thus populations separated by larger distances are at risk of becoming isolated. Furthermore, studies on the patterns of genetic differentiation between colonies in Scotland, have shown that gene flow does not consistently conform to a distance model, suggesting that other factors such as landscape irregularities or habitat type may also influence dispersal success (Aars *et al.,* 2006).

The linearization and fragmentation of wetland habitat are considered to be major threats to the long term viability of water voles due to their impact on survival and dispersal success (Macdonald & Rushton, 2003; Baretto *et al.,* 1998 b). Whilst controlling mink and riparian enhancements are considered important components to reversing the water vole decline in the UK, the scope for management of this kind, at the landscape scale, is costly and, most likely, difficult to achieve (Macdonald & Rushton, 2003). As such, there is an increasing interest in the conservation value of non-linear wetlands for water voles which are postulated to provide suitable refugia from mink predation

(Carter & Bright, 2003; Baretto *et al.,* 1998b). Despite this, however, very few studies of water voles in non-linear habitat types have been undertaken, but given the demographic and genetic threats imposed in linear systems and the limitations on maintaining high levels of connectivity and mink control in watershed systems, non-linear wetlands may prove an important component to the conservation of water voles in the UK.

1.6 Thesis aims and objectives

This study combined genetic and demographic analyses to study water vole populations occupying different wetland habitats in southeast England. The study had two primary aims. The first was to investigate patterns in the demography, kin structure and genetic diversity of water vole populations occupying linear and non-linear wetlands to inform on the conservation value of different wetland systems for water voles. The second was to investigate historical and contemporary factors that have influenced the spatial patterns in microsatellite and mitochondrial DNA variation to inform on suitable units for both human mediated and naturally facilitated recolonisation of the species. To aid in the collection of genetic samples from wild water vole populations, this thesis also presents the results of a pilot study that investigated the effectiveness of non-invasive hair capture tubes for obtaining DNA samples for microsatellite analyses. The thesis is presented in chapters, written in a paper collection form in preparation for publication. Details of each of the aims and objectives, by chapter, are as follows.

1. To investigate whether non-invasive hair capture tubes are an effective tool for genetic studies of water vole populations

Advances in molecular techniques over the past two decades have increased the accessibility of genetic tools to help ecologists obtain an accurate picture of population patterns and processes. Obtaining genetic samples from wild populations of water voles, however, is logistically constrained by the costs of ecological sampling and the requirement of a protected species license for invasive surveys in the UK. To overcome this, I designed and tested the efficiency of a non-invasive hair capture tube device to facilitate the collection of

genetic material that could be used to study genetic patterns of water voles across large geographical areas. This chapter had three objectives:

1. To test the efficiency of hair capture tubes at obtaining water vole hair in the field.
2. To determine the quantity and quality of DNA obtained from collected hair compared with DNA obtained from tissue and hair plucked from live captured individuals.
3. To evaluate the effectiveness of hair capture tubes in obtaining reliable genotypes from individuals in quantities that would allow for an accurate representation of genetic structure within and between populations.

2. To determine the influence of habitat patch geometry on the demographic processes of water vole populations

There is an increased interest in the conservation benefits of extensive non-linear wetlands, such as reedbeds, for water voles and research has suggested that population viability may increase in non-linear habitats due to the decreased risk of predation from American mink. I proposed that both survival and dispersal rates would vary between populations occupying linear and non-linear wetlands due to differences in resource competition and mortality, which would lead to different patterns in population demography between habitat types. This chapter used data from marked individuals captured over a two year period in linear and non-linear systems to test the two predictions:

1. That the apparent survival rates of water vole populations in linear wetlands would be lower than in non-linear wetlands, which would result in linear populations exhibiting larger fluctuations in population size.
2. That increased competition in linear wetlands would increase natal dispersal of both sexes resulting in an adult and male biased population structure, reduced recruitment and delayed sexual maturation of young of the year compared to non-linear populations.

3. To determine the extent of sociality in water vole populations and whether this is influenced by habitat type.

Social structure exists in most group-living species and can have a considerable influence on demographic rates and genetic structure of natural populations by affecting the physical arrangement, mating behaviour, survivorship and dispersal of specific individuals. I used molecular and demographic data to determine the extent of sociality in water vole populations occupying linear and non-linear wetland habitats to investigate the relationship between genetic relatedness and the spatial organisation of individuals. I expected that the extent of social affiliation would vary between habitat types due to differences in the extent and accessibility of biological resources. Two competing hypothesis were investigated:

1. That increased edge effects and competition for resources in linear wetlands can cause an increased rate of dispersal in both sexes resulting in low levels of genetic relatedness and kin tolerance, relative to more extensive non-linear wetland habitats.
2. That increased edge effects and competition for resources in linear wetlands will select for a greater affiliation amongst kin, reducing dispersal and resulting in higher levels of relatedness and kin tolerance when compared with non-linear wetlands.

I expected that both scenarios would result in differences between mating opportunities and thus incidents of inbreeding and inbreeding avoidance strategies were also investigated and compared between habitat types.

4. To determine spatial and temporal patterns in the genetic structure of water vole populations occupying modified landscapes

There is a recognised risk of genetic erosion for populations occupying modified landscapes where population size, stability and level of migration is expected to be negated by the loss and fragmentation of natural resources. This study used genetic variation at microsatellite loci to investigate temporal patterns in the genetic structure of water vole populations, at a patch and landscape level and had two primary objectives:

1. To determine whether genetic diversity in linear wetlands is comparable to non-linear wetlands, where discernible differences in resource extent and predation risk were expected to influence demographic stability and thus the maintenance of genetic diversity within populations.
2. To identify how the genetic variation amongst populations is spatially structured across river catchments to identify suitable management units for conserving genetic diversity and integrity.

Whether reintroductions and historical bottlenecks have contributed to the patterns of genetic variation within the study area were also considered.

5. The role of phylogenetic relationships in informing conservation management of water voles in southeastern England

There is a widespread conservation effort in the UK to re-establish water vole populations across their historical range by implementing remedial action plans that aim to improve ecological connectivity between extant populations and, where necessary, to reintroduce populations to facilitate recovery. An important consideration when implementing such strategies is to reduce the loss of adaptive genetic diversity and to preserve genetic heritage. The study of phylogeographic patterns in populations can provide practitioners with information on historical gene flow and genetic divergence that can help to identify conservation management units and guide captive breeding programmes. This study investigated the phylogeographic patterns amongst water vole populations using maternally inherited mitochondrial DNA and had two primary aims:

1. To identify geographical and ecological factors that may influence the distribution and diversity of genealogical lineages of water voles in southeastern England, so that suitable units for maintaining genetic heritage and diversity can be determined.
2. To establish whether the phylogeographic pattern observed can be used to monitor the success and recolonisation of populations that have been reintroduced within the region.

Chapter Two: General materials and methods

This chapter contains details of the study area and the materials and methods used to obtain demographic and genetic data for achieving the aims and objectives detailed in Chapter One.

2.1 Study area

This study was carried out in southeast England within the counties of West Sussex, East Sussex, Kent and Greater London which are located between the coordinates 50.4 to 51.4° N and 0.5° W to 1.25° E (Figure 2.1).

Figure 2.1 *Location of study area within England and the southeast region.*

The southeast of England is one of the most densely populated parts of the UK (Environment Agency, 2010) and thus the majority of the landscape is heavily modified for commodity production and urban space. According to the most recent government statistics (Defra, 2013), approximately 53% of land within the study area is used for commercial agriculture and 22% is designated as urban. This poses an inherent threat to the quality and quantity of wetland habitat in the area. For instance, in Sussex alone, 60% of wetlands are estimated to have been drained since the 1960's to accommodate land use change (Southgate, 2012) and, within the southeastern region, over four billion

litres of water is abstracted from the environment per annum for agricultural and public consumption (Environment Agency, 2010). Consequently, suitable habitat for water voles across the study area has become increasingly scarce and the majority of populations are confined to linear remnants of formerly more extensive wetlands that are now embedded within perceptively hostile agricultural and urban dominated landscapes.

2.2 Sampling areas and study sites

Across the study area seven lowland areas that consisted of suitable wetland habitat and were occupied by multiple water vole populations (as advised by non-government organisations and/or the national water vole steering group), were selected as sample areas (Figure 2.2). Each sample area was within a separate river sub-catchment that lay within the South East or Eastern Thames River basins, which are characterised by rural agricultural and urban landscapes respectively (Table 2.1). Within each sampling area, study sites were selected if they: a) supported a resident population of water voles, b) the habitat was either a linear system or a non-linear wetland and c) access to the site was permitted and suitable for the placement and transportation of sampling materials (Plate 1). In total, eight study sites, comprising of four linear wetland replicates and four non-linear wetland replicates, were selected for a live capture (LC) study, six of these were within separate sample areas and two (sites NL-AW and L-HB) were within the same sample area (Figure 2.2, Table 2.1). Throughout this thesis, linear and non-linear sites are identified by the prefix ‘L’ and ‘NL’ respectively. A further 18 sites, across the region, were selected for genetic sampling using non-invasive hair capture tubes (NGS).

Plate 1 *Pre-placed live capture traps on study site.*

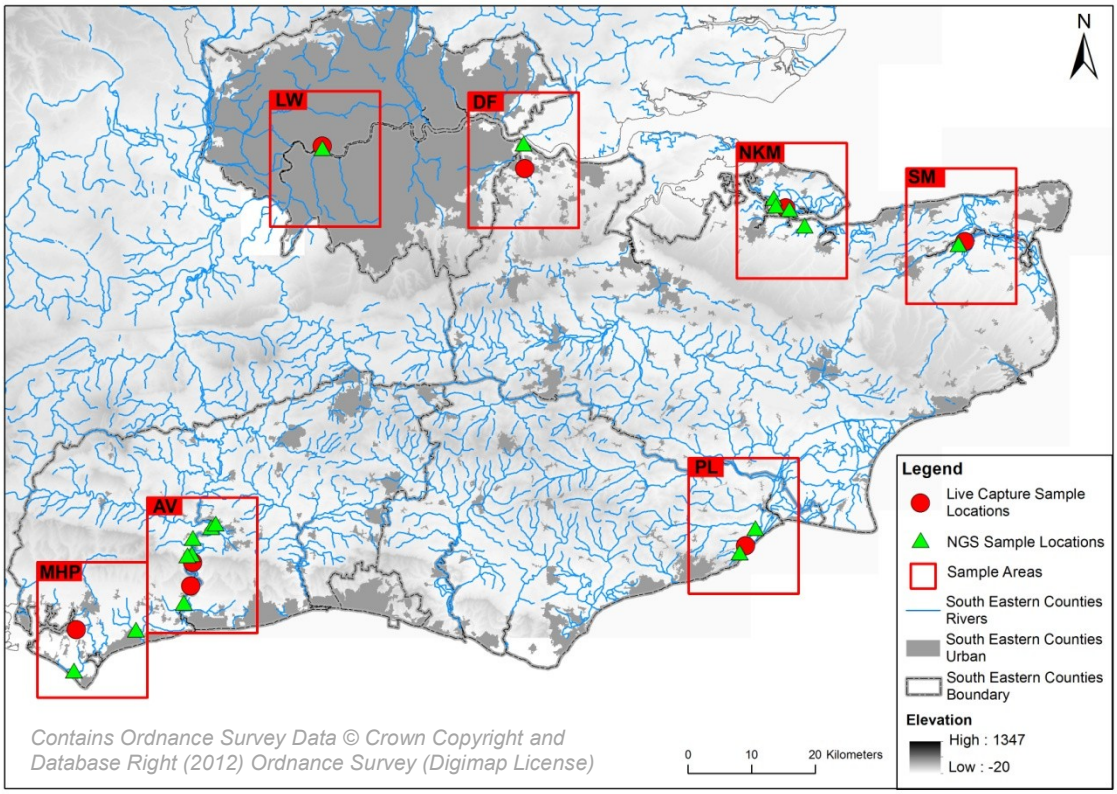

Figure 2.2 *Location of study sites within sample areas and study area.*

Sample Area Code	LC Study Site Code	River (sub) catchment	Landscape type	No. sampled sites by method		Median distance to nearest water vole colony (km)
				NGS	LC	
LW	NL-LW	London	Urban	1	1	0.42
DF	L-RD	Darent	Urban	1	1	1.52
NKM	L-EM	Lower Medway & The Swale	Pasture/ Grazing Marsh	4	1	0.85
SM	NL-SM	Wingham & Little Stour	Agricultural	2	1	0.48
PL	NL-PV	Cuckmere & Pevensey Levels	Pasture/ Grazing Marsh	2	1	2.31
AV	NL-AW L -HB	Lower Arun	Pasture/ Grazing Marsh	6	2	0.64
MHP	L-CC	Western Streams	Agricultural	2	1	1.1

Table 2.1 *Details of water vole sample areas including sample area code, live capture study site code, river catchment, landscape type as determined by the predominant land use within the catchment (>50%) derived from River Catchment Management Plans (Environment Agency, UK); number of sites sampled using non-invasive genetic sampling (NGS) and live capture (LC) and median distance of study sites to nearest known water vole colony (data obtained from local Biological Record Centres).*

2.2.1 Live capture study site descriptions

Site NL-LW: London Wetland Centre, Inner London

London Wetland Centre is a 42 hectare wetland reserve of Special Scientific Interest (SSSI) that is owned and managed by the Wildfowl and Wetlands Trust. The reserve's northern boundary adjoins the River Thames and lies within the London suburban centre of Barnes. A variety of natural habitats within the reserve are suitable for water voles including floodplain grazing marsh, reedbeds, fen and ponds. In 2001, 147 water voles, including 111 captive bred and 36 wild captured individuals were released into the reserve (Strachan & Strachan, 2005). Post release monitoring identified that the population had established and in 2002 and 2006 a further 40 and 25 water voles, respectively, were released to provide demographic and genetic rescue (Strachan & Strachan, 2005; *R. Bullock, pers. comm.*). Live trapping was conducted within the densely fringed (~5 m) ponds of 'Wildside', and within the adjacent 2 ha reedbed which are both protected by fox-proof fencing and mink controlled.

Site L-RD: River Darent, Greater London

The River Darent is a 21 mile chalk river that rises within the rural Kent Downs Area of Outstanding Natural Beauty (AONB) where it flows through the urban area of Greater London into the Thames estuary on the Dartford and Crayford Marshes (Holmes, 2005). Over-abstraction and mink have historically had a dramatic impact on water voles on the River Darent (Holmes, 2005), but during the last decade field surveys and sightings confirmed water voles had returned, particularly in areas close to the Marshes that are identified as a water vole protection zone (Holmes, 2005). Live trapping was carried out between Hawley Meadow and Dartford's Central Park.

Site L-EM: Elmley Marshes, Kent

Elmley Marsh covers a 1214 hectare area of coastal grazing marsh on the southern boundary of the Isle of Sheppey and is designated a National Nature Reserve (NNR), SSSI, Special Protection Area (SPA), Ramsar site and a national key site for water voles. The reserve is privately owned and managed for conservation and until 2013 the Spit End area of the reserve was managed by the RSPB. Well vegetated drainage channels and creeks provide suitable

habitat for water voles and mink control is carried out. A well vegetated (~ 8 m sedge and reed dominated) drainage channel within the Spit End area of the reserve was selected for live trapping which was easily accessible and where disturbance, to and by visitors, would be minimal.

Site NL-SM: Stodmarsh, Kent

Stodmarsh is a Natural England managed, 241 hectare reserve located within the Stour valley. The reserve contains a variety of wetland habitats including the southeast's largest reedbed, open water, grazing marsh and alder carr and is designated a SSSI, NNR, Ramsar site and a national key site for water voles. Previous research on water voles at Stodmarsh has been conducted by Carter & Bright (2003) and Hardman & Harris (2010) which valued the reedbed as a refuge habitat for water voles from predation by American mink. No mink control was being carried out at the time of this study and mink presence was confirmed by visitor sightings. Due to the presence of rare nesting birds, live trapping was undertaken along the reedbed edge north of Harrisons Drove and was extended into the reedbed at the western end of the site.

Site NL-PV: Pannel Valley, East Sussex

The Pannel Valley Reserve is a privately owned 23 ha mosaic of reedbed, *Salix* scrub and open water which is surrounded by farmland and coastal grazing marsh. The reserve is primarily managed for birds and is one of Europe's largest bird ringing centres. Mink are prevalent in the area but an intensive mink control programme is in place. Due to bird ringing activities, access to the reedbed was mostly constrained and an alternative fen dominated by tussocky sedges, rushes and areas of open water habitat (known locally as Carters Flood), was selected for live trapping.

Site NL-AW: Arundel Wildfowl and Wetland Centre, West Sussex

The Arundel Wildfowl and Wetland Centre is a 28 ha reserve located on the banks of the River Arun, in the Arun Valley and is owned and managed by the Wildfowl and Wetlands Trust, UK. Formerly watercress beds, a variety of habitats have been created within the reserve including wet meadows, and reedbeds, of which the latter is a designated SSSI. An unsuccessful

reintroduction of 60 water voles was carried out on the reserve in 1999 and in 2005 a second release of 171 individuals from seven different bloodlines was carried out. Post release monitoring identified established populations both within and outside of the reserve. This research was carried out within the 'Wetland Discovery' area of wet meadow and densely fringed (~8 m) meandering watercourses. The water vole population in this area had been studied by the author since 2010 where live trapping and genetic samples had been obtained for monitoring purposes. The genetic samples were stored and incorporated in the genetic analyses presented in Chapter Six.

Site L-HB: Houghton Bridge, West Sussex

Houghton Bridge is located 6.5 km upstream of site NL-AW in the Arun Valley and is an unprotected area of privately owned floodplain grazing marsh. Water voles in the area had previously been identified as 'on the brink of extinction' (WildCru, 1998) and in 2009 a population was discovered by the author along a drainage ditch that runs parallel to the River Arun. The ditch was, at the time of this research, managed by the Internal Drainage Board for flood alleviation which included vegetation clearance on rotation and de-silting. As with site NL-AW, this site has been monitored since 2010 and genetic samples were stored and incorporated in the genetic analyses presented in Chapter Six.

Site L-CC: Chichester Canal, West Sussex

The Chichester Canal is located south of the city of Chichester on the Manhood Peninsular and is a designated Site of Nature Conservation Importance (now referred to as a Local Conservation Area or LCA) and was formerly part of the Portsmouth and Arundel Canal. Abandoned for navigation in 1906, the 6 km stretch of Chichester Canal is now owned by West Sussex County Council who manage it for conservation with the help of local conservation and heritage groups. Well established marginal vegetation, including reedbeds, provide suitable habitat for water voles that are well established along the canal and across the Manhood Peninsular. An effective mink control strategy is enforced on the canal and across the sampling area. Live trapping was carried out between the Donnington and Birdham roads, where an established water vole population is monitored annually using field surveys by a local group.

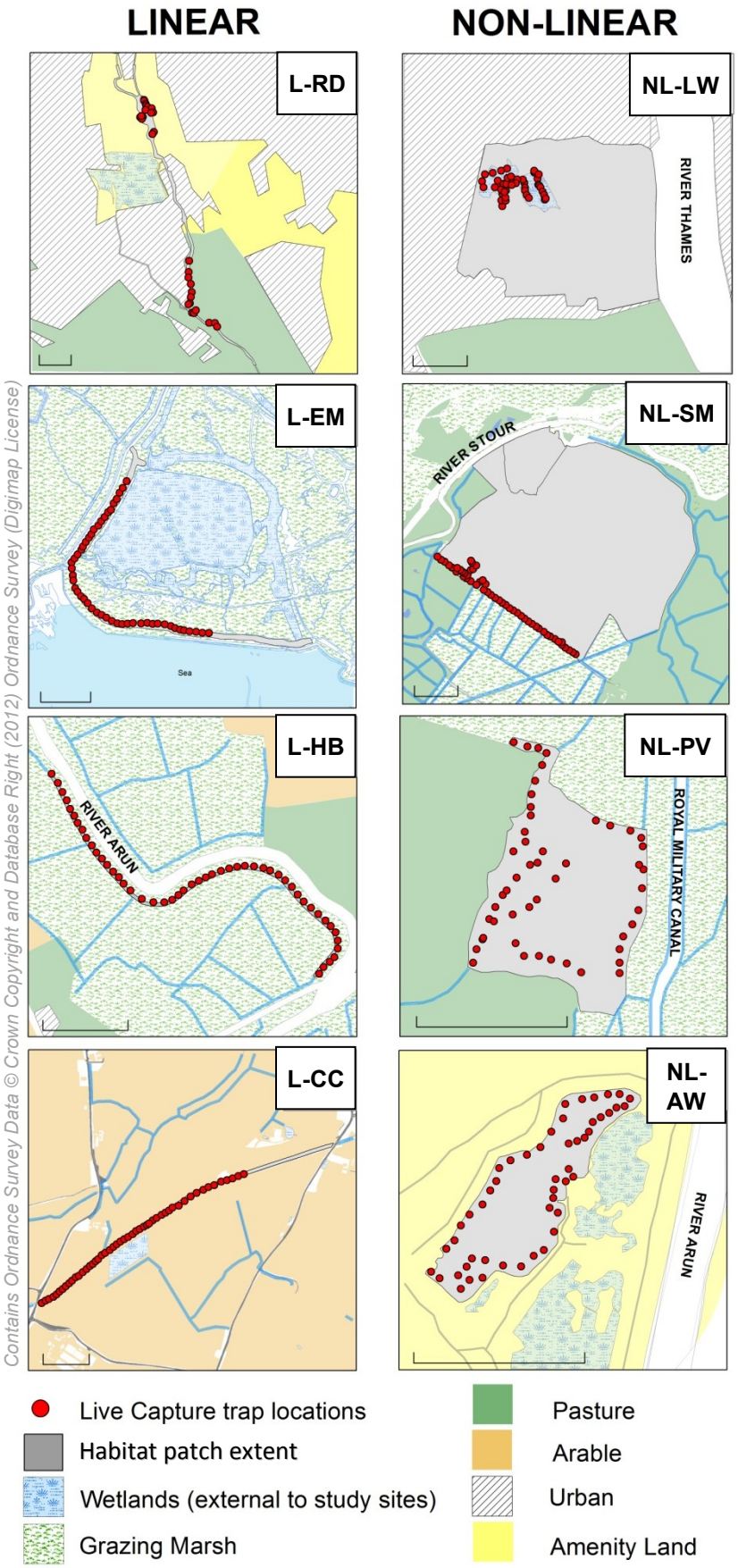

Figure 2.3 *Digitised maps of live trapping study sites showing trap configuration, habitat patch extent and adjacent land use types. Scale bar on maps denote 200 metres. See Table 2.1 for site codes.*

2.3 Sampling materials and methods

2.3.1 Live trapping

Live trapping of water voles was conducted across eight study sites (Figure 2.2) to establish the effects of habitat type (linear and non-linear) on the population demographics and genetic structure of water vole populations. Water voles were live captured, using capture-mark-recapture methods, under a Natural England License (No's. 20120780 & 20123101, provided in Appendix 1) from March 2012 to October 2013. Live capture was carried out for three consecutive days during pre-breeding (March-May) and post-breeding (September-October) seasons across the two years. Live trapping was not carried out during peak breeding season (May-August) for ethical reasons (births in traps and trapping would hinder lactating females feeding dependent young).

At each site, an attempt to place a maximum of 50 Greenalyte water vole cage traps (Wildcare™, UK) was made, although at two of the sites (NL-LW, L-RD) a maximum of only 40 and 30 traps could be placed due to restrictions on space and/or access (trap configuration at each site is shown in Figure 2.3). The traps were placed at approximately 20 m intervals to incorporate the mean length of an adult female territory (Moorhouse & Macdonald, 2005) and were located within 1 m of open water and where water vole signs (cut vegetation, latrines, burrows or nests) indicated activity (Strachan *et al.,* 2011). Each trap's bedding box was stuffed with hay and was baited with 150 g of chopped carrot and ½ an apple which was placed at the entrance to the bedding box located behind the treadle mechanism. Traps were placed perpendicular to the water's edge along dry banks, where present, and secured in place using two canes. At sites NL-SM and NL-PV, where dry banks were not wholly available, water vole traps were floated on specially designed rafts (detailed in Section 2.3.2) which were located within emergent vegetation and loosely tethered to a stake to allow for fluctuating water levels (Plate 3).

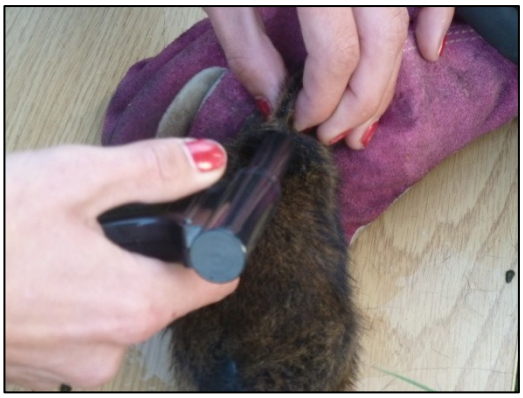

Plate 2 *Author implanting PIT tag into the scruff of a captured water vole.*

Plate 3 *Live capture trap in-situ on floating raft.*

Trap locations were marked using a Garmin ETrex summit GPS, which were accurate to 6 m enabling the same areas to be trapped during each subsequent trapping session.

Traps were checked twice daily at 7.30 am and 4 pm for captured individuals. Each newly captured water vole was released into a handling bag and weighed using 0-500 g Persola scales. Any animals weighing ≤180 g and with glossy, ginger toned pelage were classified as young of the year (Stoddart, 1971). The individual was then transferred head first into a handling tube where the sex and breeding condition could be determined. Sexually active males could be easily determined by identifying testes in the scrotum, whilst females had everted nipples (Stoddart, 1971). Non-sexually active adults and juveniles were determined by identifying the pubic symphysis which is broad and flat in males and narrow and 'keeled' in females (Stoddart, 1971). A small hair pluck (>20 hairs with follicles) was then taken from the rump of each individual and placed in a small 1.5 ml micro-centrifuge tube labelled with the individual's identity code. The animal was then transferred onto a board, and injected between the shoulder blades with a Trovan 2.0 x 32 mm PIT Tag (ID-162B/1.4) (Plate 2), to provide a permanent means of identification. The future identification of marked individuals was determined using a LID572 reader which, when placed over the individual, identifies whether a tag is present and provides the unique identification code of the PIT tag inserted. PIT tagging was done under License from Natural England and with permission from the Home Office. Antiseptic purple spray was applied to the insertion wound to help prevent infection. After

processing, each animal was then released at the same location the capture took place and the trap was replenished with bedding and bait as required. Where the animals showed signs of stress whilst handling, a semi-permanent unique fur clip was used (as illustrated in Gurnell & Flowerdew, 1990) for identification within a capture session and were either PIT tagged if recaptured or were "right censored" from the dataset, indicating that the recapture of the animal between trapping sessions could not be determined. Recaptured individuals across trapping sessions were re-weighed and their breeding condition re-determined.

The capture data derived from each site was then entered into a data base which detailed each individuals PIT tag identity code, their capture history, the capture season, site and locality (12 figure OS Grid Reference), the individuals sex, weight, age (young of the year or overwintered adult) and breeding condition when first captured within each trapping season. Hair plucks were dried on paper, if wet, and stored within 12 hours of collection in a -20°C freezer prior to DNA extraction in the laboratory (described in Section 2.4.1).

2.3.2 Floating raft design protocol for live trapping

Rafts were designed to 1) be buoyant whilst supporting the weight of a water vole and live capture trap, 2) be accessible to water voles in that they were not too steep for a water vole to climb onto when *in situ;* and 3) were large enough to prevent the raft overturning if wildfowl landed on the upper surface. Various raft designs were constructed and compared in terms of meeting the criteria outlined above and in terms of cost efficiency. The final rafts measured 300 x 900 x 57.2 mm and the design was based on an adaption of the Game Conservancy Trust mink rafts (Reynolds *et al.,* 2004) which are frequently utilised by water voles as territorial latrine demarcation sites (Hardman & Harris, 2010). The rafts consisted of two sheets of 3.6 mm external plywood which sandwiched a central piece of 50 mm polystyrene sheet (Figure 2.4). The raft was secured together using 80 mm M8 bolts with nuts and black duct tape was used to cover the polystyrene around the edges of the rafts as styrene, a major

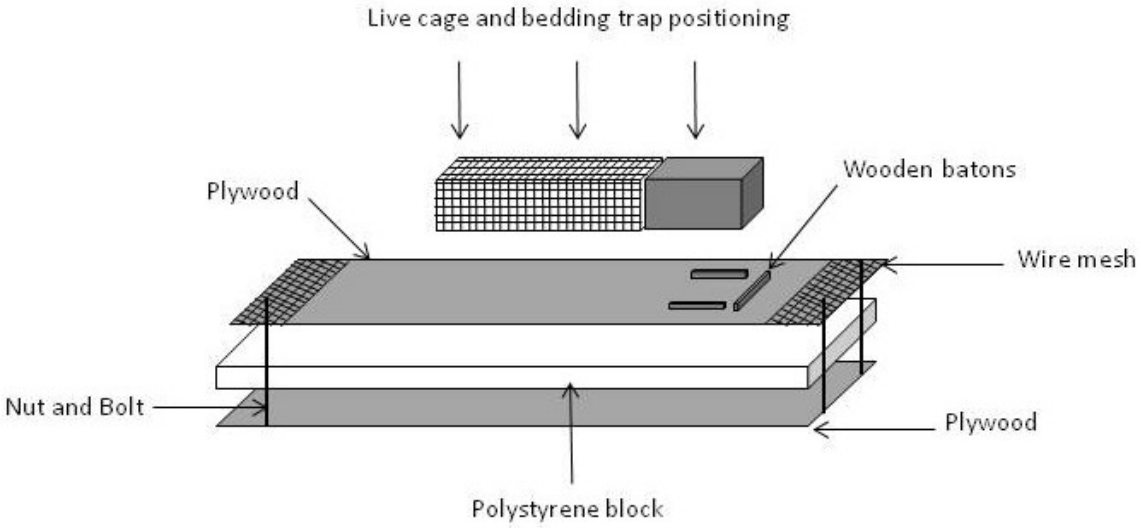

Figure 2.4 *Schematic of floating raft construction.*

component of polystyrene, is moderately toxic to aquatic organisms (Environmental Protection Agency, 1994). Wire mesh netting was stapled over both ends of the raft to facilitate water voles climbing out of the water onto the raft. To ensure the live capture trap did not move whilst placed on top of the rafts, three pieces of wooden baton were screwed onto the top piece of plywood to secure the bedding box in place.

2.3.3 Non-invasive hair capture tubes

Non-invasive hair capture tubes were designed to remotely pluck hair (with follicles) from water voles, providing a tool to obtain genetic information from colonies without the need to capture or disturb individuals. Tubes consisted of 27 cm sections of 3" PVC black piping, which was large enough to allow water voles to move through the tube but too large to impede the free movement of, and hence not obtain hair from, the other smaller mammal species (e.g. *Microtus spp*, *Apodemus sylvaticus & Neomys fodins*) which may occupy the same habitat types (Herzon & Helenius, 2008; Borowski, 2003; Tattersall *et al.,* 1997). A 7 cm wide strip of clear plastic with two 6 x 4 cm pieces of adhesive fly paper (Agralan™ Yellow Sticky Traps) attached, was inserted to form the inside roof of each tube and was secured in place with two elastic bands (Figure 2.5).

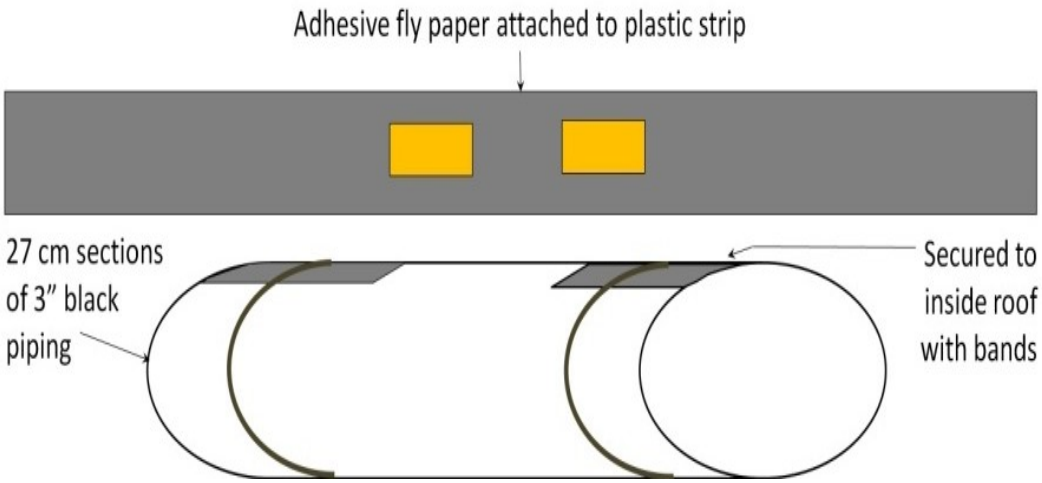

Figure 2.5 *Diagram of non-invasive hair capture tube design.*

Hair capture tubes were used at 18 water vole occupied sites within the seven sampling areas to obtain genetic samples for the purpose of assessing their effectiveness (Chapter Three), to provide genetic data to study regional phylogenetic structure (Chapter Seven) and for ongoing research on landscape genetics of water voles within each sample area (data is not presented as part of this thesis). The locations of each of the hair capture tube sampling sites are shown in Figure 2.2 and a summary of each site is provided in Table 2.2.

At each of the sampling locations, hair capture tubes were baited with a small piece of apple (approximately $^{1}/_{8}^{th}$) to lure water voles to enter the tube. Tubes were placed at 20 m intervals along water vole runways at each site. The number of tubes placed at each site was determined by the length of occupied habitat and ranged from four to 25. Tubes were secured in place using short sections of cane, covered with vegetation and left at each site for a maximum of eight nights between August and October 2012 and in August 2013. These months were presumed to be optimal for sampling non-invasively as this is when the density of water voles is at its highest (Moorhouse, 2004). Due to logistical constraints imposed by the geographical coverage of the sampling areas, hair capture tube sampling was carried out in conjunction with autumn live capture sessions within sampling areas LW, DF, EM and SM resulting in tubes being left for two nights.

Sample Area	No. of tubes per site	No. of sample nights	Total sampling effort	No. of hair samples obtained	Sample site habitat type
LW	20	2	40	1	Fen
DF	25	2	50	3	Drainage Ditch
NKM	20	4	80	12	Drainage Ditch
	20	4	80	11	Drainage Ditch
	16	2	32	5	Drainage Ditch
	12	2	24	2	Drainage Ditch
	25	2	50	11	Drainage Ditch
SM	25	2	50	8	Reedbed
PL	25	7	175	10	Drainage Ditch
	25	7	175	9	Drainage Ditch
AV	21	8	168	7	Drainage Ditch
	16	7	112	8	Drainage Ditch /Fen
	20	6	120	7	Drainage Ditch
	20	6	120	11	Drainage Ditch
	20	7	140	7	Stream
	5	7	35	2	Garden Pond
MHP	22	4	88	1	Nature Reserve Rife
	25	7	175	11	Drainage Ditch
TOTAL	**362**	**86**	**1714**	**126**	

Table 2.2 *Summary of non-invasive capture tube sampling locations by sample area and survey effort and samples obtained by site.*

After collection from the field, all hair capture tubes that had >3 water vole hairs on the fly paper (Plate 4) were placed in glass vials and stored at -20°C until DNA extraction (described in Section 2.4.1). Any hairs which did not match the pelage obtained from water voles were discarded.

Plate 4 *Water vole hair captured using non-invasive hair tubes*

2.4 Laboratory methods

2.4.1 DNA extraction

Hair Plucks

Hair plucks consisting of approximately 20-30 hairs were obtained from each live captured individual. Each sample was digested in 340 µl extraction buffer containing 182.6 µl PCR grade water, 100 mM Tris-HCl (pH 8.0), 100 mM NaCl(1M), 3 mM $CaCl_2$(1M), 68 µl 10% SDS, 40 mM DTT and 250 µg/ml of proteinase K and incubated at 56°C for 2-4 hours until completely lysed, as outlined in Pfeiffer *et al.* (2004). Following the digestion procedure, DNA was washed and eluted using QIAGEN DNeasy™ Blood and Tissue Kit (QIAGEN.com) following the manufacturer's protocol.

Hair Tube Samples

DNA was extracted from all hair tube samples within 1 week of being collected from the field to reduce degradation of DNA quality (Periera *et al.,* 2009). Hair shafts were removed from each patch of adhesive paper using sterile forceps after soaking the patch in mineral oil for approximately 5 minutes. This allowed the glue to soften enough to carefully detach the hair shaft from the adhesive paper with minimal breakage. Hairs were then dried on filter paper to remove excess adhesive and then placed in a 1.5 ml micro-centrifuge tube. Where hair shafts were present on both patches of adhesive paper from the same tube, the samples were pooled and classed as one sample. The amount of hair present per adhesive patch varied from three to >100 hair shafts and the number of hairs used per extraction did not exceed 50. Each hair sample was then digested in 340 µl extraction buffer and the DNA extracted as per the hair pluck protocol described above but with a slight modification involving the elution of samples with 100 µl of Buffer AE rather than the standard quantity of 200 µl. This was to obtain a higher concentration of DNA extract due to the lower quantity of DNA commonly obtained using non-invasive techniques (Periera *et al.,* 2009; Taberlet & Luikart, 1999)

Tissue Samples

Tissue was obtained from four dead animals encountered via live trapping at study sites NL-SM, NL-PV and L-HB. Approximately 25 mg of finely cut ear

tissue from each animal was digested in 180 µl Buffer ATL and 20 µl proteinase K and incubated at 56°C for 3-5 hours until completely lysed. Following the digestion procedure DNA was washed and eluted using QIAGEN DNEasy™ Blood and Tissue Kit (QIAGEN.com), following the manufacturers protocol.

2.4.2 Molecular markers

The use of species specific markers for DNA profiling requires that the flanking region surrounding the repeat motif is identified. From this flanking sequence, PCR primers can be designed that allow for the amplification of the microsatellite or the region of interest in mitochondrial DNA (Lowe *et al.,* 2007; Selkoe & Toonen, 2006). Polymorphic microsatellite loci have been identified in the aquatic form of the water vole (Stewart *et al.,* 1998) and several studies have developed primers (oligonucleotides) that bind to the flanking region (Berthier *et al.,* 2005; Stewart *et al.,* 1999; Stewart *et al.,* 1998), allowing microsatellites to be amplified using PCR and then genotyped for population studies. A highly variable control region of the mitochondrial DNA in water voles has also been identified and the development of both forward and reverse primers that bind to the flanking region have allowed for the determination of phylogeographic patterns across the species' UK distribution (Piertney *et al.,* 2005).

Microsatellite genotyping

DNA profiles ("DNA fingerprints") were obtained for hair pluck and tissue samples using polymerase chain reaction (PCR) amplification for eight water vole microsatellite loci whose core regions, primer sequences and PCR conditions are given in Table 2.3. Each 15µl PCR reaction contained either 3µl of DNA extract from hair plucks or 1µl DNA extract from tissue, 1X PCR Buffer (Invitrogen™) , 0.2mM each dNTP, 1.5mM $MgCl_2$, 0.2 µM of each primer, 1 unit of Platinum Taq DNA polymerase (Invitrogen™), 1.5 µg BSA and 3 µl extracted template DNA . Thermal cycling conditions were 94°C 30s, 35 x [94°C 10s, T_A°C 30s, 72°C 30s] with a final extension of 72°C for 2 minutes (where s = seconds).

Locus	Repeat Motif	Primer Sequences	Size (bp)	K	T_A °C	Ref
AV7	$(GATA)_{12}$	F: AGATGATAAACACGTAGATGC R: TATCCATCTGTCCATCTGTC	177	9	54	Stewart *et al.,* 1998
AV8	$(GATA)_{18}$	F: GGGACAGAAGGAAGTAGAGG R: GCAGTGGTAACAAGAGGATG	310	10	58	Berthier *et al.,* 2005
AV9	$(GATA)_{15}$	F: CACTGGCTCAGATTCAAGACTAC R: GGGAGGGAAAGCTAGGTCACAG	220	10	60	Berthier *et al.,* 2005
AV11	$(GATA)_{13}$ $(GATG)_3$ $(GATA)_{10}$	F: TGGCCTTATCAGGAAACATAC R: GAATAGCTTGCCTGATGGC	336	15	58	Berthier *et al.,* 2005
AV12	$(GATA)_{12}$	F: GGCAGGAGGATAACAAGATTGAG R: TCTCCAAGATGAGTTCCAAACAG	210	11	58	Stewart *et al.,* 1998
AV13	$(GATA)_{13}$	F: CTGGCTCTATCTATCTGTCTATC R: ACAATTACAGCATCCAGAAG	200	7	53	Stewart *et al.,* 1998
AV14	$(GATA)_{16}$	F: TATGTGATATGGCACTAGCATGT R: AGCCTGTCTCAGCAGAAGG	250	8	58	Stewart *et al.,* 1998
AV15	$(GATA)_{14}$	F: TATATGGAAGGTCGTAGATTCAG R: ATTAAAGCATTTGTTGAGAAAGC	205	12	58	Stewart *et al.,* 1998

Table 2.3 *Characteristics of the microsatellites and primer sequences used. K = No. alleles that have been identified and published within the relevant cited studies and T_A = Annealing temperature used in this study.*

T_A refers to the locus-specific annealing temperature. PCR products were multiplexed in two groups (microsatellites: AV7, AV8, AV9, AV12 and AV11, AV13, AV14 and AV15) based on their fragment size and fluorescent reference dye (FAM, HEX or NED). PCR products were sent to Source BioScience for microsatellite genotyping which separates microsatellite fragment lengths using capillary electrophoresis on an ABI 3730xl using a ROX 500 size standard. Microsatellite fragment sizes were resolved using Peak Scanner software (Applied Biosystems™), examples of which are provided in Appendix Two.

Mitochondrial DNA genotyping

Haplotypes were obtained from between one and seven samples collected from each of the sampled populations by amplifying a 736-base pair region of the mitochondrial control region, a particularly variable region of the mitochondrial genome (Piertney *et al.,* 2005). Amplifications were performed using the primer pair described by Piertney *et al.* (2005) (*F15708* 5'-TTAATCTACCFATCCTCCG TGAAACC-3' and *R92* 5'-TKGACACTGGTCTAGGGATATTTGC-3'). Each 15µl PCR reaction contained either 3µl of DNA extract from hair samples or 1µl DNA extract from tissue, 1X PCR Buffer (Invitrogen™), 0.2mM each dNTP, 2.0mM $MgCl_2$, 0.2 µM of each primer, 1 unit of Platinum® Taq DNA polymerase (Invitrogen™), 1.5 µg BSA. Thermal cycling conditions included an initial 3 minutes of denaturation at 90°C, followed by 40 cycles of PCR which consisted of 30 seconds denaturation at 94°C, 30 seconds annealing at 65°C, reducing by 0.5°C per cycle for the first 20 cycles, finishing with a final extension of 30 seconds at 72°C. PCR products were sequenced by Source BioScience using Applied Biosystems 3730 series DNA Analyser, examples of which are provided in Appendix Three.

Chapter Three: Are non-invasive hair capture tubes an effective tool for genetic studies of water vole populations?

3.1 Introduction

Advances in molecular techniques over the past two decades have resulted in the increased accessibility of genetic tools to help ecologists obtain an accurate picture of population patterns and processes in threatened species (Frankham *et al.,* 2011; Lowe *et al.,* 2007; Selkoe & Toonen, 2006). The wide accessibility of molecular markers that isolate highly variable and informative fragments of DNA have proved useful for answering questions on population abundance, bottlenecks, movement, social behaviour, metapopulation dynamics and genetic variation (Lowe *et al.,* 2007; Pearse & Crandall, 2004). Many studies concerned with species conservation now include genetic analysis (Amos & Balmford, 2001). However, such studies can be hindered by the elusive nature, remote distribution, vulnerability and protection of threatened species that can inhibit and logistically constrain the collection of samples from free living species (Garcia-Analiz *et al.,* 2010; Gleeson *et al.,* 2010; Hobbs *et al.,* 2006).

The clear value of non-invasive sampling is driving the development of highly sensitive DNA extraction and PCR techniques, often available in commercial kit form, which have opened up the scope for population studies that have historically had limited success. Common sources of non-invasively obtained genetic material are from hair and faeces (Balestrieri *et al.,* 2010). However, genetic material can also be obtained from feathers, urine, saliva, eggshells, owl pellets and sloughed skin (Buś *et al.,* 2013; Periera *et al.,* 2009). Studies using non-invasive sampling techniques for molecular research have obtained samples despite the absence of observing the focal species and have required no, or very simple and relatively inexpensive, sampling devices (Buś *et al.,* 2013; Clevenger & Sawaya, 2010; De Barba *et al.,* 2010; Gleeson *et al.,* 2010). This cost-effectiveness has made the use of non-invasive techniques particularly attractive for use in the field.

The water vole has undergone a catastrophic decline across the UK (Strachan *et al.,* 2000) due to the widespread loss and fragmentation of wetland habitat. Consequently, many water vole populations have become small, spatially isolated and confined to linear habitat remnants (Macdonald & Rushton, 2003). This can ultimately compromise their long-term viability due to the potential loss of demographic and genetic rescue of discrete colonies, causing them to reduce in size, lose genetic diversity and threaten their ability to react to novel challenges (Amos and Balmford, 2001). Hair capture tubes provide a potentially useful method of obtaining genetic material from wild populations of water voles to facilitate the study of their genetic structure to discern patterns of dispersal, demographic stability and genetic diversity (Macdonald & Rushton, 2003), that is useful to inform conservation management at a local scale.

Hair capture devices have been widely used to obtain genetic material for the identification of species (Garcia-Alaniz *et al.,* 2010; Pauli *et al.,* 2008; Foran *et al.,* 1997) and of individuals for population genetic studies and wildlife monitoring (Drewry *et al.,* 2013; Henry & Russello, 2011; Clevenger & Sawaya, 2010; Anderson *et al.,* 2006). Devices to capture hair are commonly baited to lure target animals to increase their efficiency in the field; however, passive hair traps have also been employed, particularly for addressing fine scale habitat use, as behaviour is not influenced by their presence (Long *et al.,* 2008). A variety of hair capture devices have been employed to target hair from different species. Simple barbed wire structures (snares) placed around bait and located along travel routes and at den entrances have been particularly effective for bears (*Ursus spp*) (Drewry *et al.,* 2013; Clevenger & Sawaya, 2010; De Barba *et al.,* 2010), and for Eurasian badgers, *Meles meles* (Balestrieri *et al.,* 2010). Tubes or boxes fitted with either barbed wire or adhesive have been more widely employed for smaller species including pine martens *(Martes martes)* (Mullins *et al.,* 2010), raccoons (*Procyon lotor)* (Belant & Wolford, 2007) and the American pika (*Ochontona princeps*) (Henry *et al.,* 2012).

Due to the increased use of non-invasive hair capture devices, a number of empirical studies have focussed on determining their efficiency at collecting hair samples in the field and several studies have reported the failure and low capture success of hair on snagging devices. A study by Bartolommei *et al.* (2013) reported that hair tubes failed to collect hair from pine martens (*Martes martes*) and similarly Nichols *et al.* (2008) strongly advised against hair removal traps for detecting the presence of striped skunks (*Mephitis mephitis)* due to inefficiency when compared to other passive methods such as track plates and camera traps. Conversely, studies monitoring bears (*Ursus spp*) have found hair capture devices particularly effective and studies determining minimum population size and recruitment (De Barba *et al.,* 2010), habitat preferences and density (Drewry *et al.,* 2013) and use of wildlife crossing structures (Clevenger & Sawaya, 2010) have been conducted using hair trapping devices.

A number of factors are likely to contribute to the heterogeneity in capture success of hair capture tubes that has been reported across different species. Some species are likely to respond negatively to traps, particularly if human or other species' odours are present (Long *et al.,* 2008). Non-target species may also be attracted to baited devices, resulting in the target species going undetected and negatively biasing population size or distribution estimates (Long *et al.,* 2008). Studies employing non-invasive hair capture tube techniques need to consider the potential limitations to sampling design in the field, so that sampling efficiency is maximised, to enable the scientific goal of the study to be met (Taberlet & Luikart, 1999).

A key consideration of non-invasive genetic sampling (NGS) is the quality and quantity of DNA obtained, which can be inferior to samples obtained invasively (Periera *et al.,* 2009). This can not only lead to genotyping errors and spurious results, but also reduce the sample size and hence ability to detect significant patterns using frequency based population genetic analyses (Periera *et al.,* 2009; Taberlet & Luikart, 1999).

A high proportion of studies using NGS have reported a proportional reduction in sample size due to genotyping failures (Clayton *et al.,* 2011; Gleeson *et al.,* 2010; García-Alaníz *et al.,* 2010; De-Barba *et al.,* 2010; Balestrieri *et al.,* 2010; Periera *et al.,* 2009; Chu *et al.,* 2006). A major cause of this comes from sample degradation which is caused by UV radiation, temperature, moisture and salt concentration (Dean & Ballard, 2001) and is typically a greater concern the larger the target genomic region that is required to remain intact (Periera *et al.,* 2009). Increases in genotyping success rate are expected when the amount of time samples are left in the field is reduced (DeMay *et al.,* 2013; Gleeson *et al.,* 2010), when very short DNA fragments are targeted, such as single nucleotide polymorphisms or microsatellite markers that produce short amplicons (<100bp) and when using more abundant mitochondrial DNA (Periera *et al.,* 2009; Chu *et al.,* 2006). Another common cause of genotyping failure is the presence of PCR inhibitors. These can often be overcome by adopting suitable DNA extraction methods to increase sample purity (Taberlet & Luikart, 1999), or by dilution of the DNA extract to reduce the concentration of inhibitors (Periera *et al.,* 2009), which is particularly problematic when the starting concentration of DNA is low.

The small quantities of target DNA observed in NGS is a major cause for concern in genetic studies as it can often lead to genotyping errors caused by stochastic sampling (Periera *et al.,* 2009). When DNA concentration is very low, the probability that the DNA molecules are amplified during the PCR process according to their relative occurrence is prone to error (Taberlet & Luikart, 1999). One particular genomic region may be preferentially amplified by chance during the early cycles of PCR (Taberlet & Luikart, 1999), resulting in false identification of heterozygote individuals as homozygotes, known as allelic drop-out (Frankham *et al.,* 2011).

Study aims and objectives

In view of the potential limitations in sampling efficiency and inherent problems associated with the quantity and quality of DNA obtained non-invasively, a pilot study was conducted during 2012 with the aim of determining the effectiveness

of non-invasive hair capture tubes for obtaining genetic samples from water voles. This study had three objectives which were to:

1. Test the efficiency of the hair capture tubes at obtaining water vole hair in the field.
2. Determine the quantity and quality of DNA obtained from collected hair in comparison to DNA obtained from plucked hair and tissue samples.
3. Evaluate the effectiveness of hair capture tubes for obtaining viable DNA for the purpose of genotyping water voles to study genetic structure within and among populations.

3.2 Methods

3.2.1 Field methods

Genetic samples were obtained from water vole populations (Figure 3.1) during 2012 using non-invasive sampling and live capture methods detailed fully in Chapter Two, Sections 2.3.3 and 2.3.1 respectively.

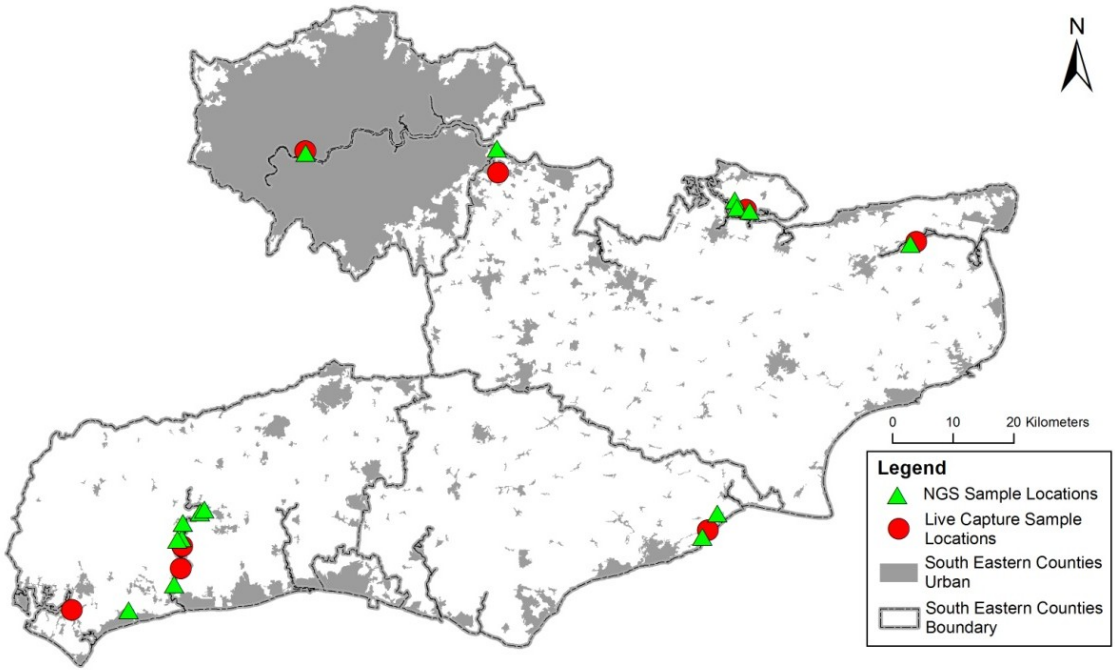

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Figure 3.1 *Map of genetic sampling locations in southeast England, using live-capture and non-invasive hair capture tubes (NGS).*

Non-invasive hair capture tubes

Between August and October, a total of 329 non-invasive hair capture tubes were placed along water vole runways within 15 sample sites. Tubes were left in-situ for between two and seven nights and for any tubes capturing >3 hair shafts, the adhesive fly paper was removed and stored in separate glass vials at -20°C until DNA extraction.

Live Capture

Water voles were live captured for three consecutive days across eight study sites in spring (March to May) and autumn (September to October). A small hair pluck, consisting of approximately 20-30 hairs with shafts, from each newly captured individual was obtained and stored in separate tubes at -20°C until DNA extraction.

3.2.2 Molecular analyses

DNA extraction

DNA was extracted from all hair samples following digestion (described fully in section 2.4.1), using the QIAGEN DNeasy™ protocol (Qiagen.com) with a slight modification involving the elution of NGS with 100 µl of Buffer AE rather than the standard quantity of 200 µl. This was to obtain a higher concentration of DNA extract, due to the smaller yield commonly obtained using non-invasive techniques (Periera *et al.,* 2009). All NGS extractions were done within one week of being collected from the field to reduce the effects of length of storage on DNA quality (Periera *et al.,* 2009).

DNA amplification and quantification

Real time quantitative PCR (qPCR) was used to measure the quantity of target-specific 'amplifiable' DNA extracted from all non-invasive hair samples and from 30 randomly selected hair-plucked samples. This method provides a more accurate quantification of the DNA yield than traditional techniques such as spectrophotometers that do not differentiate between target and non-target DNA. This is of particular relevance when using NGS due to the unknown presence of non-target DNA in the sample. Real time qPCR uses a dye

(SYBR™ Green) that fluoresces when it intercalates to double-stranded DNA. This fluorescent signal is directly proportional to the amount of starting DNA template. Using a five-fold serial dilution of DNA (concentration range of 0.03 – 18.56 ng/µl, measured by spectrophotometry) recovered from water vole tissue as 'Standards', the signal strength of each hair tube and hair pluck sample was compared during the exponential phase of PCR amplification, providing a quantitative measure of each samples DNA yield. All reactions fell between the recommended 90-110% reaction efficiency (Life Technologies, 2012).

The specificity of DNA amplification from each hair sample was assessed using a dissociation curve, which detects the decrease of fluorescent signal as the amplified double stranded DNA is heated; this is referred to as "melting". Non-specific amplification products, that result from shorter fragments (degraded samples), presence of inhibitors and primers annealing to themselves due to the absence of target DNA, can be identified through having different melting profiles from the PCR products of the target region of DNA (Life Technologies, 2012). Samples that dissociated at a temperature different to the standard dilution set were classed as failures and used as a measure of the efficiency of NGS as a methodological technique.

Real time qPCR reactions were conducted in triplicate (standard dilutions) or duplicate (hair samples) on a Rotorgene 6000 real time PCR machine using Qiagen SYBR™ Green kits. Each 25µl reaction consisted of 1µl of sample DNA, 12.5µl of SYBR™ Green Master Mix, 10.25µl of RNAse free H_2O and 1.25µl of AV11 microsatellite primer (10mM). The AV11 primer (Berthier *et al.,* 2005) targets a relatively long (>300 bp), non-coding region of water vole DNA containing a variable microsatellite motif. Due to the higher risk of degradation associated with large fragments (>200-300 bp) of DNA (Periera *et al.,* 2009), this primer should distinguish between samples exhibiting DNA degradation and those where the DNA has remained largely intact. For each qPCR run, a negative control with sample DNA omitted was used.

An additional problem common to DNA-profiling from NGS is where smaller alleles are preferentially amplified over larger alleles (Taberlet & Luikart, 1999). Hence, if an individual is heterozygous at a given marker – with both a large and small allele – the genotype can be erroneously recorded as having just the smaller of the two; this is known as allelic drop-out. To determine the yield of DNA that is required to reduce the risk of 'allelic drop-out' at the AV11 microsatellite, the DNA from a heterozygous individual was quantified, serially diluted and genotyped (Figure 3.7). The point at which the smaller allele measured <100 fluorescence intensity and detection of the larger of the two alleles was below 50% of the fluorescence intensity of the smaller allele, was used to determine the minimum threshold of DNA concentration required to reliably genotype samples. This validation procedure was determined by calculating the peak height ratio of 55 heterozygote water voles genotyped at the AV11 microsatellite.

3.2.3 Data analyses

Trapping effort for hair tubes was expressed as the number of working (trap) nights needed to obtain water vole hair samples and amplifiable DNA. This was compared to the trapping effort required to obtain hair plucks from different individual water voles using live capture. The statistical significance of the differences between trapping effort using each method was calculated using a standard two tailed T-test. Pearson's Correlation Coefficients were calculated to test whether the proportion of tubes capturing hair and the proportion of samples failing to amplify correlated to the number of nights the tubes were left in the field. A two tailed T-test was used to determine the statistical significance of the mean differences between the DNA concentrations obtained using both methods. Fisher's Exact Test was performed to investigate whether sample month had an effect on the trapping effort for obtaining NGS.

To determine whether the successful amplification of samples using qPCR could be predicted using DNA concentration and purity (ratio of absorbance 260 nm/280 nm) readings obtained using spectrophometry, I adopted a generalized linear modelling (GLM) approach, which is less sensitive to the distribution of

error and variance around the response variable (Crawley, 2014), for qPCR success/failure with fixed effects, implemented in Programme R (www.R-project.org). Binomial error distributions were assumed and logistic regression used to select the best-fitting model. I started with a maximal model with all main effects and interactions: $Y = \beta 0 + \beta 1x1 + \beta 2x2 + ...$, where the parameters $\beta_0 \ldots \beta_n$ are estimated by maximum likelihood. The significance of each effect and first order interaction was assessed by comparing the difference between the deviance values of the model before and after the term was fitted; this produced a minimal adequate model (Crawley, 1993; McCullagh *et al.,* 1989). The significance level of all tests was set at 0.05.

To determine the sampling efficiency of non-invasive hair capture tubes, an analyses of power was conducted to determine a) what sample size would be required to effectively sample 95% of 'informative' alleles present in a population and b) what number of genotypes are required from a population to give an accurate representation of its genetic composition. For each test, simulated datasets, consisting of 100 replicates, were constructed from 50 water vole hair pluck samples that were genotyped across eight microsatellite loci (Berthier *et al.,* 2005; Stewart *et al.,* 1999). This was done using a specially designed macro implemented in Excel (Hale *et al.,* 2012), with sample sizes between five and 50, generated in increments of five. Sampling was done without replacement to ensure that no simulated individual was present more than once in a population.

To determine how many samples would be required for effective detection of informative alleles (alleles that allow for the estimation of population parameters relevant to conservation studies), all alleles occurring at a frequency of ≥ 0.05 in the empirical dataset were used. The frequency of these same alleles within each of the simulated populations at each population size was then calculated (Hale *et al.,* 2012). To determine how many samples would be required to accurately measure genetic composition within and across loci, pairwise F_{ST} values were calculated using GenALex 6.2 (Peakall & Smouse, 2006) between the empirical dataset and simulated datasets, where F_{ST} is a measure of genetic

distance between populations. This simulation study identified the sample size needed, given the magnitude of variation at the markers used in this thesis, in order to maximise the probability of identifying significant genetic differences between colonies.

3.3 Results

3.3.1 Hair capture success and trapping effort

Hair samples were obtained from all 15 water vole colonies sampled using non-invasive hair capture tubes. The number of tubes successfully capturing hair ranged from one to 11 amongst sites and was positively correlated to sampling effort ($r = 0.55$, $n = 16$, $p = 0.028$) (Figure 3.2).

In total, 91 non-invasive hair samples were obtained over 1372 trap nights. Overall, trapping effort was 15 trap nights to obtain one hair sample (range: 4.4 - 88 trap nights / site). The proportion of NGS obtained was significantly different between months ($p = 0.006$, Fisher's exact test), with September yielding the highest proportion of samples (Table 3.1).

Month	No. Trap Nights	No. Samples
August	322	17 (0.05)
September	852	70 (0.08)
October	188	4 (0.02)

Table 3.1 *Total number of trap nights and samples obtained per month (proportion of tubes yielding hair is shown in parenthesis).*

In comparison, a total of 190 hair plucked samples were obtained over 3510 trap nights of live capture. Hair plucked samples were obtained from all eight sites and ranged from four to 32 samples between sites. Overall, the trapping effort for hair plucked samples was 18 trap nights to obtain one hair sample (range: 8.8 - 55.4 trap nights / site). The mean trapping effort to obtain NGS did not vary significantly from the effort needed to obtain hair plucked samples ($t = 0.57$, $df = 26$, $p = 0.574$, Figure 3.3).

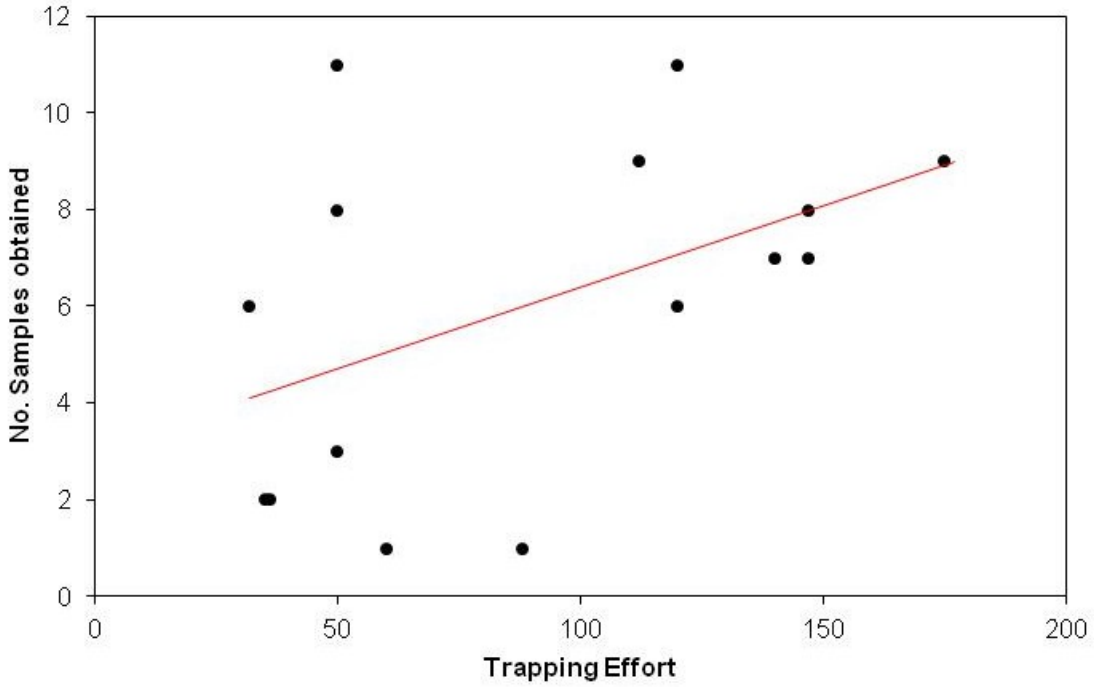

Figure 3.2 *Scatterplot, with fitted line, of NGS against trapping effort (No. tubes x No. trap nights).* $R^2 = 0.225$.

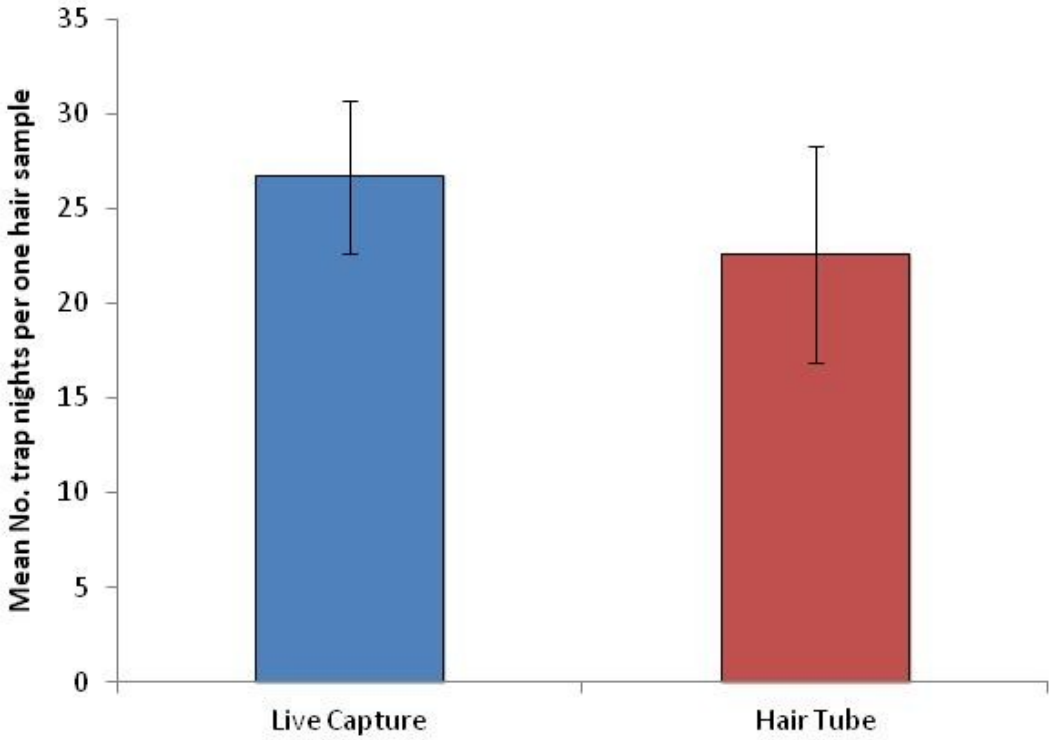

Figure 3.3 *Mean number of trap nights ± standard error required to obtain one hair sample from across the study sites using live capture and non-invasive hair capture tubes.*

3.3.2 DNA sample specificity and quantification

A total of 40 NGS were identified as exhibiting DNA degradation (see example, Figure 3.4). The remaining 51 samples yielded target specific PCR products; which corresponds to a trapping effort of 26.7 trap nights to obtain one amplifiable NGS from hair tubes. The proportion of NGS failing to amplify target specific DNA did not vary significantly between sampling month ($p = 0.07$, Fisher's exact test) and was not correlated to the length of time that the tubes were left in the field (Pearson's correlation coefficient, $r = -0.15$, $n = 15$, $p = 0.58$). In comparison, none of the 30 hair plucked samples analysed were found to yield non-specific products during qPCR.

The real time amplification of the minimum and maximum target DNA yields extracted from NGS and hair plucked samples, is shown in Figure 3.5. The DNA concentration from NGS was more variable in comparison to hair plucked samples, which produced relatively consistent DNA yields.

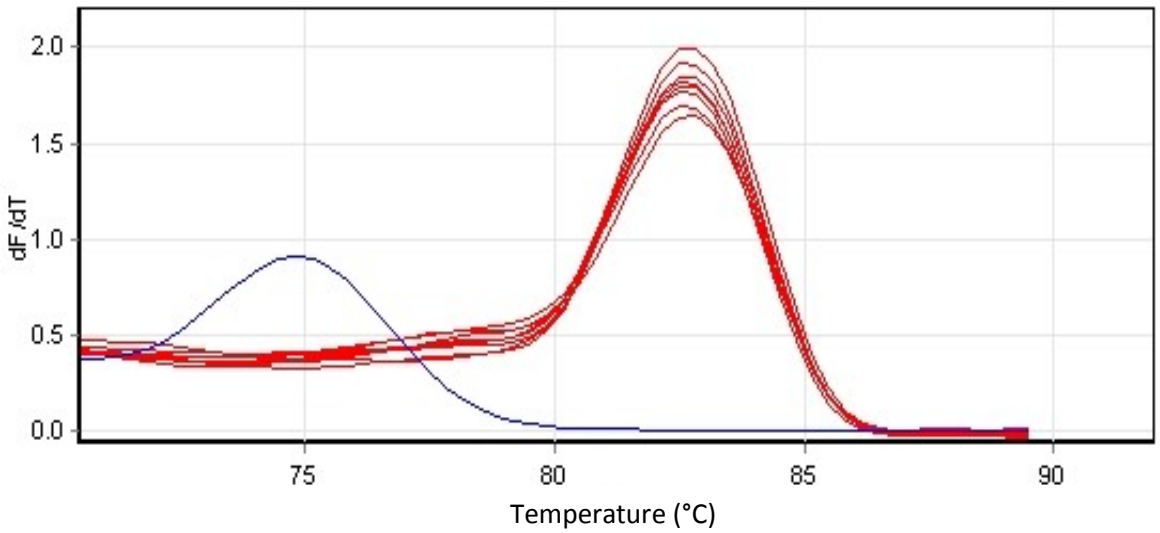

Figure 3.4 *Melting curve showing the detection of one non-specific PCR product generated from NGS (blue). Y-axis explains the rate of change of fluorescence detected. Samples in red are from tissue and show the target specific melt profile for reference.*

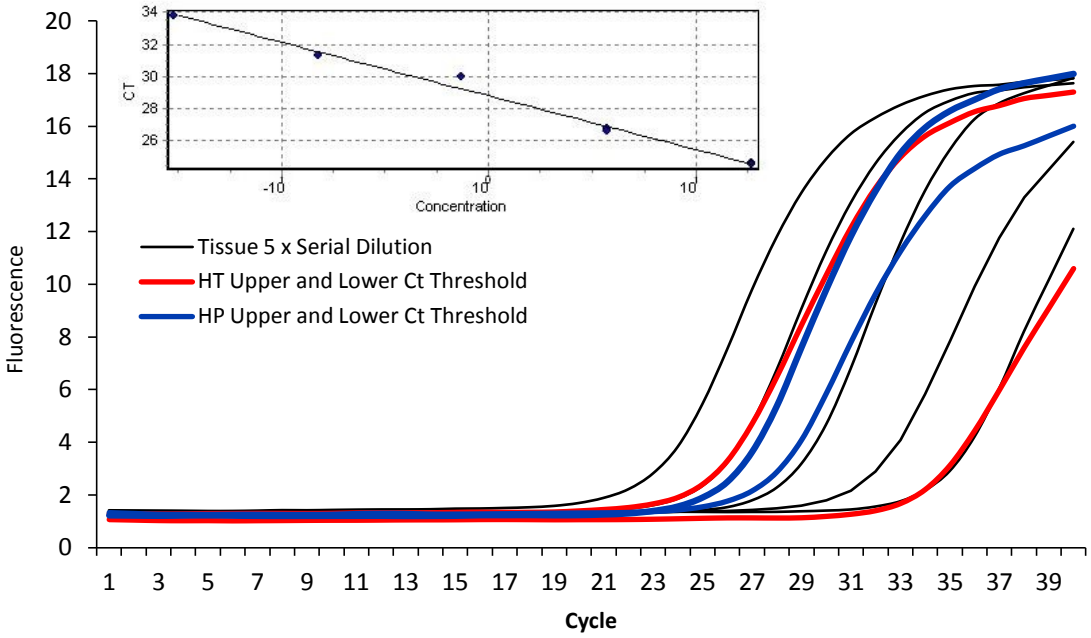

Figure 3.5 *Amplification plot of AV11 target locus in real time. Plotted samples of upper and lower threshold Ct values for NGS (red curves, n = 51, HT = hair tube) and hair pluck (HP, blue curves, n = 31) DNA against standard dilutions (grey curves). Ct values are inversely proportional to target DNA quantity.*

The real time quantification of target DNA recovered from hair tube and hair plucked samples showed that hair tube extracts contained an average of 0.52 ng/µl DNA and that the majority (76%) contained 0.06 - 0.5 ng/µl DNA. In comparison, none of the extracts from hair plucked samples contained <0.5 ng/µl DNA (Figure 3.6) and the mean DNA recovered was 1.84 ng/µl (range: 0.74 - 3.45) which was signficantly higher than the mean yield obtained from NGS ($t = 8.77$, $df = 70.3$, $p = 0.000$). NGS samples yielding unspecific products were recorded as having a mean total DNA concentration of 0.24 ng/µl (range: 0.002 - 0.78 ng/µl).

Both DNA concentration and DNA purity, as determined by spectrophometry (Nanodrop 2000) were significant factors in determining the success rate of samples amplified using qPCR (Table 3.2). DNA purity explained twice as much of the model deviance in comparison to the DNA concentration readings; however both terms only explained a total of 7% of the model deviance.

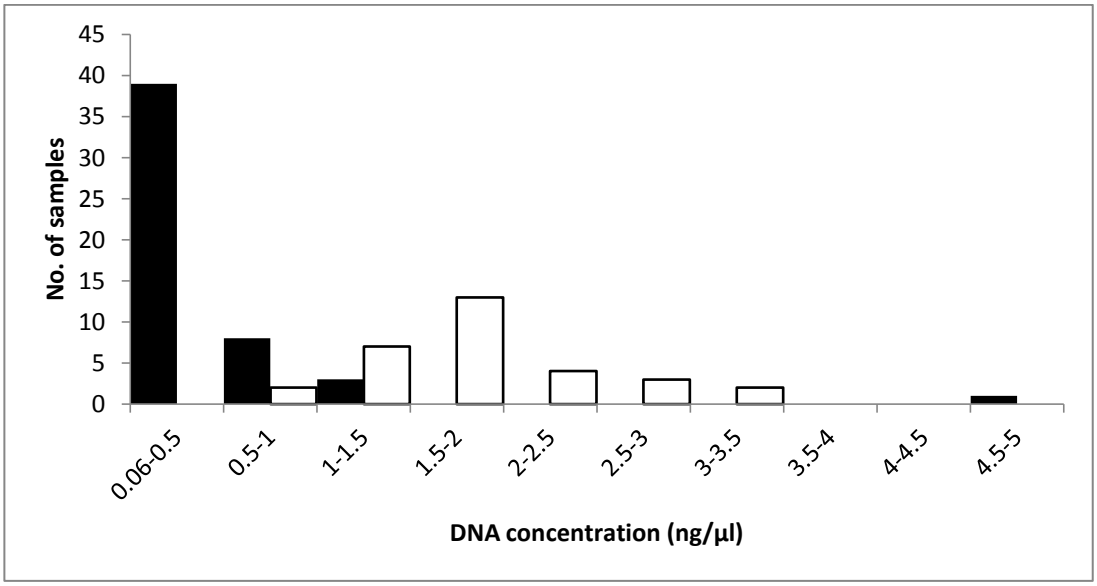

Figure 3.6 *Frequency chart of DNA concentration (ng/µl) in 51 extracts from non-degraded hair tube samples (outlined bars) and 30 extracts from water vole hair pluck samples (solid bars).*

Term	Estimate	Df	% Deviance	*Pr (>Chi)*
Intercept	1.167			
DNA Purity	-0.817	1	4.5	0.0004
DNA Concentration	0.073	1	2.7	0.006

Table 3.2 *Final GLM for qPCR success rate (binomial response, N = 212 against DNA purity and concentration measured by spectrophotometry. For each term, the deviance explained refers to the change in deviance attributed to the term in question when fitted last, as a proportion of total deviance explained by the main effects in the minimum adequate model. P-values were estimated by comparison with the reduced model not containing the term in question.*

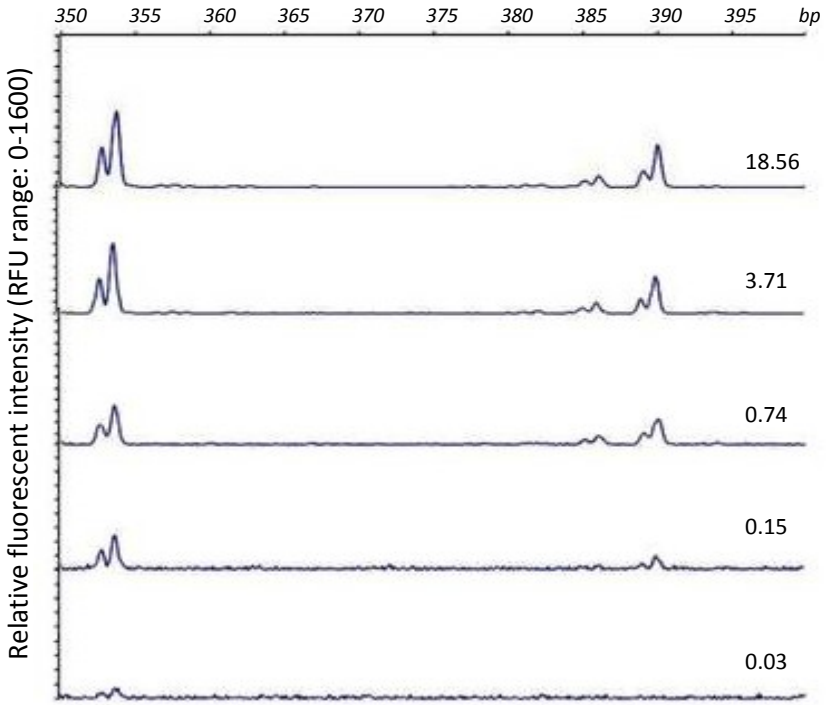

Figure 3.7 *Electropherograms showing alleles of a heterozygote individual at AV11 locus with relative fluorescent intensity (RFU) shown on y-axis and size range in base pairs shown on x-axis. Vertical panels show reduction in RFU of alleles with decreasing DNA concentration (shown as ng/µl on right). Top panel clearly shows two alleles (354 and 390 of the true heterozygote and allelic drop-out occurs at <0.15 ng/ul concentration, producing a false homozygote.*

The minimum threshold of DNA concentration required to reduce the allelic drop-out for heterozygote samples using the AV11 locus was 0.15 ng/µl (Figure 3.7). Of the 51 NGS yielding target specific PCR products, 47% were calculated to have a DNA yield below the minimum threshold and as such are at risk of producing false genotypes. In comparison, 14% of hair pluck samples were below this threshold.

3.3.3 Sampling efficiency of methods for genetic analyses

The sample size required to detect all informative alleles present in the 'real' population at a frequency of $\geq$0.05, was calculated to be 35 when using eight microsatellite loci (Figure 3.8). However, this sample size was not obtained at

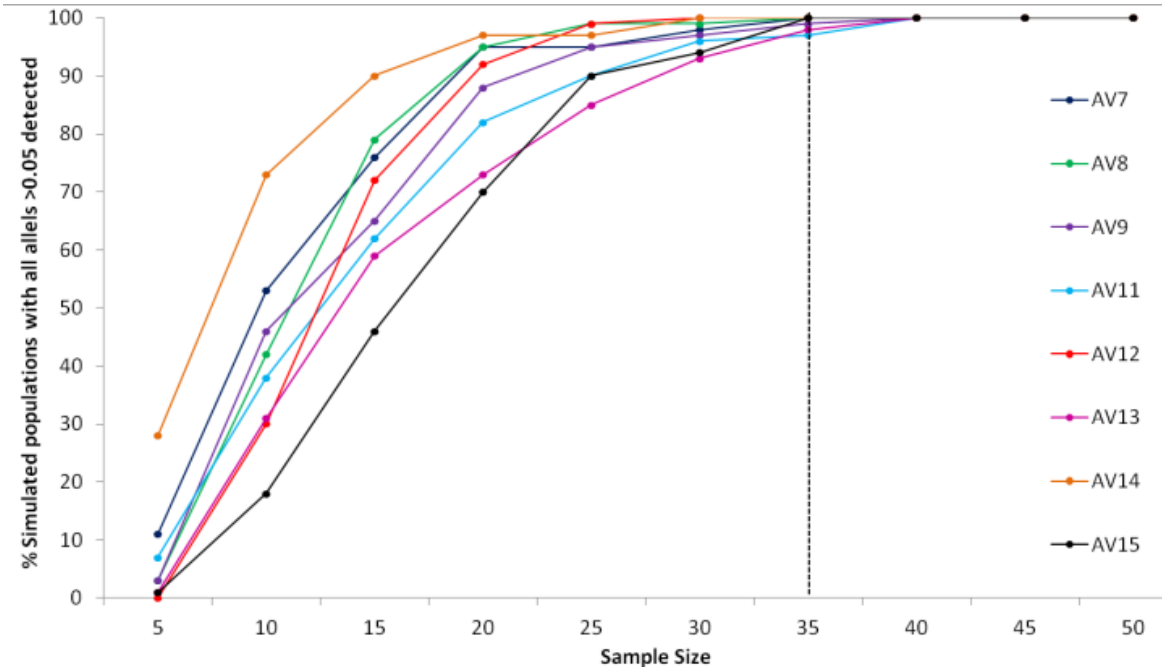

Figure 3.8 *Percentage of samples in which all alleles, at a real frequency of ≥0.05 at eight microsatellite loci, were detected at each sample size.*

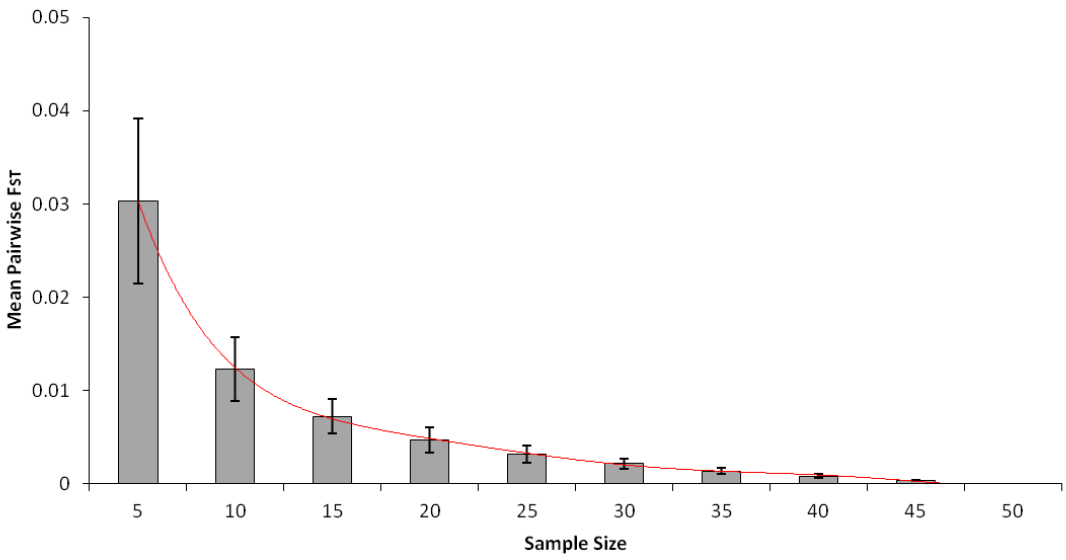

Figure 3.9 *Mean pairwise F_{ST} between 100 random replicate datasets at each sample size and the empirical dataset.*

any sites that were sampled either using NGS or live capture (maximum sample size of 11 and 32 respectively). A minimum sample size of 20 was, however, suitable for detecting all informative alleles at three of the eight microsatellite loci (AV7, AV8 and AV9). This sample size was achieved using live capture only at two of the eight sites (NL-AW, n=31 and L-EM, n=32, see Chapter Four, Table 4.2).

The genetic distance (pairwise F_{ST}) among the simulated datasets and the empirical dataset decreased with increasing sample size (Figure 3.9), which is to be expected as the larger the sample, the more representative it should be of the empirical dataset, hence lower F_{ST}. A sharp decline is observed in the dissimilarity between the empirical dataset and datasets containing five to 20 samples, after which there is only a small incremental decrease in F_{ST} values for datasets containing 25 to 50 samples. Twenty-five samples would be required to maximise the probability of identifying significant genetic differences between colonies, given the magnitude of variation across eight microsatellite markers. This sample size was not obtained at any of the sites sampled using NGS (see Chapter Two, Table 2.2) and only two sites sampled using live capture.

3.4 Discussion

Non-invasive genetic sampling is becoming an increasingly used research tool for monitoring wildlife populations, providing researchers with a means of collecting genetic data from species across large geographical areas without the need to capture or disturb individuals. However, a number of studies that have employed NGS methods have reported high rates of genotyping errors associated with low quantity and quality DNA (Gleeson *et al.,* 2010; García-Alaniz *et al.,* 2010; De-Barba *et al.,* 2010; Balestriera *et al.,* 2010; Periera *et al.,* 2009). As such, the effectiveness of non-invasive methods at obtaining reliable data needs to be considered prior to its extensive use within any study (Taberlet & Luikart, 1999).

Sampling Efficiency

The results of this study demonstrate that non-invasive hair capture tubes are a feasible method for obtaining hair from wild populations of water vole. The overall trapping effort of 15 nights to obtain one hair sample is comparable to studies elsewhere that have used hair capture devises for obtaining genetic samples from other species. For instance, Gleeson *et al. (*2010), reported 17.2 trap nights to obtain one hair sample from stoats (*Mustela ermine*) in New Zealand, and an average of 17.8 trap nights were required to capture one hair sample from brown bears (*Ursus arctus*) in the Italian Alps (De Barba *et al.,* 2010). However, in line with other studies employing similar techniques, this study identified a substantial proportion of samples (n=40) that were unusable due to DNA degradation, which increased the trapping effort to an average of 26.7 trap nights/one sample. This exceeds the trapping effort employed to obtain hair samples using live capture methods (18 trap nights per sample), yet this method requires a protected species license in the UK and uses considerably more resources in respect of field equipment, costs and man hours. This is an important consideration when project sampling units need to cover extensive areas in order to meet the objectives of the study. In the context of this study, a live capture method using 50 traps would, on average, utilise 12 man hours to set up and collect in the traps and a minimum of four man hours, per session, to check the traps and process captured individuals.

This equates to 36 man hours per site when trapping over a three day period. In comparison, non-invasive hair capture tubes would utilise four man hours to set up and collect-in equipment and two man hours to process the samples ready for DNA extraction. This equates to six man hours, considerably less than live capture. The cost of equipment is also relatively inexpensive in comparison to live capture where 50 traps would cost £1750 (+VAT) whilst the production of 50 hair capture tubes would cost approximately £40. Thus, within the context of this study, one genetic sample obtained using non-invasive hair capture tubes took approximately 0.53 man hours (based on 300 trap nights) and cost £0.80, which was four times more efficient and 13 times more cost effective than live capture (£10.62 and 2.15 man hours per sample).

DNA quality and quantity

The utilisation of non-invasive hair capture tubes was more cost effective than live capture when considering field resources and accessibility to unlicensed volunteers; however, the magnitude of samples that were deemed 'unreliable' presents a major limitation to the implementation of this non-invasive technique. A total of 43% of samples obtained using the non-invasive hair capture tubes were degraded, in that their melting profile differed from that of samples containing non-degraded target DNA (Figure 3.4). As the total DNA would have been similar in both cases, these samples would not have been differentiated using traditional techniques such as spectrophometry, which does not distinguish between target and non-target DNA, and, as such, cannot identify degraded samples. Furthermore, the DNA concentration and purity readings obtained via spectrophometry, despite being significant, were not reliable predictors of the successful amplification of target DNA using qPCR in that they only explained 7% of the deviance present in the data (Table 3.2). This highlights the value of real time PCR techniques for NGS DNA quantification over traditional techniques such as spectrophometry. Although qPCR is a more expensive procedure than spectrophometry, it only needs to be conducted once per sample and therefore saves costs by identifying which samples are likely to result in failed genotyping.

The proportion of samples that failed to amplify was unsurprising, as similar genotyping failure rates have been reported in studies elsewhere that have used non-invasive techniques. De Barba *et al. (*2010) reported an average genotyping failure of 56% when sample collection was carried out after hair capture devices were left unchecked for 21 days in the field, and Gleeson *et al. (*2010) reported that 8% of samples collected from the field after 7 days were successfully genotyped at six microsatellite loci. In this current study however, the application of real-time qPCR allowed for the removal of 'unreliable' samples from the dataset prior to genotyping, saving valuable time and resources within the laboratory.

The utility of qPCR was additionally valuable in this study for comparing the quantity of DNA extracted from samples as well as for identifying samples at risk of non-target amplification and allelic drop-out. The number of hairs and quality of samples obtained from non-invasive hair capture tubes was highly variable and contained significantly less target DNA than hair samples obtained from live capture hair plucks. This was expected as the amount of hair obtained from hair pulls is more controlled in comparison to hair capture tubes, where the capture of hair is in part determined by the activity and size of the animal passing through the tube. However, due to the variation in DNA yields obtained non-invasively, 47% of samples were identified as 'at risk of non-specific amplification and allelic drop-out'. These contained low quantities of target DNA that fell below the minimum threshold of 0.15 ng/µl that was identified in this study to reliably detect both alleles in heterozygote individuals at the AV11 locus (Figure 3.7). Although the amount of template DNA can be increased per PCR reaction, to account for samples containing low DNA yield, this can result in amplification failure due to the presence of inhibitors in the sample template which would otherwise be too diluted to affect the PCR reaction (Periera *et al.,* 2009). Although the presence of inhibitors are not exclusive to non-invasive samples or to samples with lower DNA yields, a trade-off between the amount of DNA and the amount of PCR inhibitors is required (Periera *et al.,* 2009).

Advancements to NGS methods to overcome limitations

Due to the number of studies encountering low genotyping success rates from NGS, much work has been carried out to try and reduce these limitations (Periera *et al.,* 2009). One of the most commonly employed methods to overcome DNA degradation and fragmentation is to use very short fragments of DNA such as mini short tandem repeats or single nucleotide polymorphisms. These are typically less than 100 bp in length and as such are more likely to remain intact once the sample begins to degrade (Periera *et al.,* 2009). A long target DNA fragment (>300 bp) was used in this study to identify a minimum magnitude of DNA degradation occurring in samples obtained from non-invasive hair tubes, which allowed the effectiveness of this method to be determined for studying the conservation genetics of water voles. Additional microsatellite primers currently available for water voles (Berthier *et al.,* 2005; Stewart *et al.,* 1999) are typically shorter than the AV11 microsatellite (126 - 300 bp) that was targeted in this study and may provide amplified products despite the identified DNA degradation.

Other considerations for reducing the magnitude of DNA degradation, when employing non-invasive techniques, is to decrease the time that NGS are left in the field and ensuring that sampling is carried out when the focal species is not undergoing seasonal pelage changes (Periera *et al.,* 2009; Long *et al.,* 2008). Firstly, both moisture and ultraviolet light can degrade DNA (Long *et al.,* 2008) and samples left in warm and wet environments are more likely to be affected by degradation. For instance, Gleeson *et al. (*2010) reported a 65% increase in genotyping success when samples were collected on a daily basis, in comparison to samples left in the field for multiple days, which increased the occurrence of degraded DNA. However, the results of this study found no correlation between the time samples were left in the field (2 - 8 nights) and the PCR failure rate for AV11 (Section 3.2.2). This may, however, be masked by variations in climatic conditions that were not recorded across the different sampling areas and which may have had more of an influence on DNA degradation than the sampling duration. Secondly, sampling during seasonal moults may increase the capture of shed hairs rather than plucked hairs. Shed

hairs generally have fewer follicles attached than plucked hairs and so yield lower quality and quantities of DNA (Periera *et al.,* 2009; Long *et al.,* 2008). Water voles are recorded as undergoing their first pelage change aged one year and adults are thought to moult during November and December (Stoddart, 1971). It was, therefore, unsurprising that sampling month did not significantly predict the proportion of samples obtained that failed to amplify target specific DNA in this study (Section 3.2.2).

Using NGS to study genetic structure of water voles

For the purpose of studying the genetic structure of water voles, the capture heterogeneity of non-invasive hair sampling needs to be minimized and samples obtained need to be representative of the true population (De Barba *et al.,* 2010; Long *et al.,* 2008). The non-invasive hair sampling design, employed in this study, aimed to maximise trapping efficiency by sampling during high population density (August-October) and a significantly higher proportion of captures were obtained during September compared with August and October. However, the sample size obtained using non-invasive tubes was relatively small in comparison to live capture techniques undertaken during comparable months. Using the live capture data, it is apparent that there is a far greater proportion of young of the year within populations at this time (see Chapter Four, Figure 4.3). These individuals are smaller in body size and may be undetected when using non-invasive hair capture tubes. This is an important consideration as capture heterogeneity will introduce bias to any subsequent analyses of the data, such as effective population size and gene flow (Long *et al.,* 2008). For water voles in particular, the potential reduction in detection of young of the year is likely to have a large impact on the estimate of genetic structure and migration amongst populations, as this is the age cohort most likely to disperse between colonies (Fisher *et al.,* 2009; Aars *et al.,* 2006; Telfer *et al.,* 2003).

The inability to capture individuals within a population, leading to a reduction in sample size, will clearly reduce the accuracy of any subsequent analyses. Given the degree of genetic variation likely to be found within sub-divided water

vole populations (Aars *et al* 2006), this study identified that the number of samples needed to detect all informative alleles (>95%) and to accurately determine the 'true' genetic composition of the sampled populations was 35 and 25 respectively. This exceeds the sample size obtained using both non-invasive hair capture tubes and live capture methods at six of the eight sites (detailed in Chapter Two, Table 2.2 and Chapter Four, Table 4.2). Although this only reflects a loss in data accuracy if a population of that size is being sampled, it is an important consideration given the scientific goal of this research and the conservation status of the species. An inability to accurately determine the genetic structure of populations could lead to incorrect assumptions being made about a population's status and the failure to correctly allocate resources that are aimed at ensuring the persistence of water voles within a given area. These points are considered throughout the remaining analyses.

3.5 Conclusions

This study has identified that remote hair capture tubes are effective at obtaining hair from wild water vole colonies; however, current limitations to the sampling design and molecular techniques employed have reduced their capability in sampling DNA for quantifying the genetic structure within and amongst populations. However these limitations can be reduced through careful study design and collection methods. For instance, increasing the sampling intensity, whilst decreasing the time samples are left in the field would be valuable for reducing the risk of sample degradation, whilst increasing the sample size for more accurate analyses of genetic structure. Furthermore, selecting shorter hypervariable fragments of water vole DNA and using alternative DNA extraction methods such as phenol-chloroform, which has been found in other studies to increase DNA purity and PCR efficiency (Ząbek *et al.,* 2005), may serve to increase the quality and quantity of DNA. Thus, by making small modifications to the field and laboratory methods, the effectiveness of remote hair capture tubes would be improved, allowing for their utility in future research into the landscape ecology and genetic structure of water voles.

Chapter Four: The influence of habitat patch geometry on the population dynamics of water voles in southeastern England

4.1 Introduction

It is well recognised that the loss and fragmentation of natural habitat in modern landscapes is a major threat to wildlife populations, reducing once numerous and widespread species to remnant patches of suitable habitat that is interspersed by unfamiliar or hostile environments (Fischer & Lindenmayer, 2007). These changes can alter ecological processes that affect the demography and dynamics of populations and potentially lead to reductions in population growth and stability and an increase in a population's risk of extinction (Diffendorfer *et al.,* 1995). For instance, both interspecific (e.g. predation, parasitism, competition) and intraspecific (e.g. dispersal, social interactions, resource use) mechanisms of demographic parameters, such as survival and recruitment, can be influenced by fragmentation and changes to the size and shape of habitat patches (Mills, 2007; Dooley & Bowers, 1998; Gutzwiller & Anderson, 1992).

In many intensively used landscapes, habitat patches are often reduced to linear elements that are likely to play a key role in the dispersal, survival and reproduction of many species (Schippers *et al.,* 2009; Gutzwiller & Anderson, 1992). However, these patches are often regarded as sub-optimal in their ability to sustain viable populations due to the inherent reduction in patch size and the increased area of edge influenced habitat (Schippers *et al.,* 2009). This can alter the quality and dispersion of resources (Murcia, 1995) which can increase competition and cause changes to dispersal rates (Le Galliard *et al.,* 2012; Schippers *et al.,* 2009; Johannsen & Ims, 1996), ranging behaviour (Yletyinen & Norrdahl, 2008; Bowers *et al.,* 1996), recruitment (Hokit & Branch, 2003) and survivorship (Smith & Batzi, 2006). Each of these factors can adversely influence population growth, reducing population size over time and leading to populations becoming increasingly susceptible to stochastic processes, catastrophic events and localised extinction (Mills, 2007; Lande, 1993). Because a consequence of this scenario is localised extinction, a major

goal for conservation biologists and wildlife practitioners has become one of understanding how linear landscape elements influence the demographics and dynamics of populations so that adequate predictions on their effect on species viability can be made.

Water voles have a wide ecological tolerance within wetland systems in the UK, occupying riparian habitat both along linear watershed networks and within more extensive wetland habitat types such as reedbeds, fens and marshland (Lambin *et al.,* 2004; Macdonald & Rushton, 2003; Bonesi *et al.,* 2002). However, the widespread drainage and modification of wetlands for agriculture and urban development has resulted in many wetland habitats persisting as long linear features associated with riverine and drainage networks (Macdonald & Rushton, 2003; Rushton *et al.,* 2000). Consequently, these habitats have become an important refuge for water voles, a species that has declined rapidly across the UK as a result of both habitat loss and predation of populations by feral American mink (Strachan *et al.,* 2011). However, suitable riparian habitat along watershed networks, particularly in agricultural landscapes, is often patchily distributed due to the progressive intensification of adjoining land uses. This often reduces suitable habitat to narrow strips that vary in their quality and dispersion (Macdonald & Rushton, 2003) and are argued to facilitate the predation of water voles by mink that utilise main waterways to hunt (Baretto *et al.,* 1998b). As such there has been an increased interest in the conservation benefits of more extensive wetlands for water voles, such as reedbeds, and research has suggested that population viability may increase in non-linear habitats due to the decreased risk of predation from mink (MacPherson & Bright, 2009; Carter & Bright, 2003). However, the possible role of habitat shape, in terms of linear vs. non-linear, on demographic processes that influence the viability of water vole populations has not, to date, been examined.

There are two main mechanisms that shape demographic patterns in water voles that are likely to vary between linear and non-linear systems; the first is dispersal. Within linear systems, water voles typically consist of small density-dependent colonies (Aars *et al.,* 2006) that are limited by female summer breeding territories (Lambin & Krebs, 1991) that increase in size with decreased

vegetation abundance (Strachan *et al.,* 2011). Thus the dispersal rate of pre-breeding juveniles (<180 g) of both sexes is generally high (Fisher *et al.,* 2009; Aars *et al.,* 2006) and thought to be triggered by resource and competitive exclusion (Le Galliard *et al.,* 2012). This may also be exacerbated by edge-mediated emigration in which the propensity to emigrate is higher due to more frequent encounters with patch boundaries (Schippers *et al.,* 2009; Kindvall & Petersson, 2000; Fauske *et al.,* 1997; Stamps *et al.,* 1987). It can be hypothesised, therefore, that dispersal from non-linear wetlands is undertaken less frequently, particularly by females, due to there being more abundant resources, smaller territories and thus less competitive exclusion by breeding individuals with pre-breeding young of the year. This would not only reduce incidences of dispersal related mortality, but may also improve the reproductive success of territorial females, which can be limited by aggressive encounters with unfamiliar neighbours and enhanced when kin clusters are formed for raising young (Lambin & Krebs, 1991). The second influential mechanism is survival. Water voles are characteristically short-lived with low survival rates in watershed systems that have been associated with flooding (Moorhouse, 2004), human disturbance (Telfer, 2000; Baretto *et al.,* 1998a) and to vegetation abundance, which in turn, determines predation risk (Moorhouse *et al.,* 2009; Carter & Bright, 2003), winter food source availability (Potapov *et al.,* 2004) and can indirectly influence dispersal and thus influence dispersal-related mortality. It is, therefore, fair to assume that in non-linear wetland systems characterised by extensive non-linear wetland vegetation, managed water levels and minimal disturbance, the survival of water voles should increase.

Both mortality and dispersal rate can have important consequences on the demographic rates of populations, altering sex ratios, age structure, social interactions and natality which can ultimately determine population growth, stability and long-term viability (Mills, 2007). Because of this, an understanding of how habitat geometry influences the demographics and dynamics of water voles is important for securing their long-term viability within fragmented agricultural landscapes.

Study aims and predictions

The overall aim of this study was to identify how habitat geometry influences the demographic processes (population structure, breeding, population growth and survival) of water vole populations by using data obtained from marked individuals, captured within linear watershed systems and non-linear wetlands over a two-year period. It was predicted that within linear watershed systems, water voles would suffer higher emigration and/or mortality compared to non-linear wetland sites. This would be due to edge-mediated factors, including increased predation and resource competition, thus resulting in differences in the age structure, sex ratios, recruitment and population stability and growth between habitat types. Consequently, relative to non-linear wetland habitats, linear watershed systems were predicted to exhibit:

1) Lower apparent survival rates (mortality and emigration cannot be distinguished) and thus a higher variability in the rate of change in population sizes over the study period.
2) Increased matrilineal defence of resources which would result in:
    a. an adult biased age structure, due to higher emigration of young
    b. male bias in operational and young of the year sex ratios, due to increased rates of female dispersal and male biased recruitment into the breeding cohort.

Plate 5 *Water vole in handling tube*

4.2 Methods

4.2.1 Study sites

Water voles were live captured within eight study sites located in southeastern England (Figure 4.1). Sites were selected within this region on the basis of geometric composition of resources for water voles and included four study sites representing linear watershed systems and four study sites representing non-linear, more extensive natural wetlands. Linear and non-linear site codes are denoted by prefix L- and NL- respectively. Mink were known to be active in all sampling areas but were excluded by a predator proof fence at sites NL-LW and NL-AW. Approximately 1 km sections of linear wetlands were sampled based on the presence of water vole field signs and included two drainage ditches located within floodplain grazing marsh (L-HB & L-EM), one canal adjacent to agricultural land (L-CC) and one river section that was located within suburban green space (L-RD).

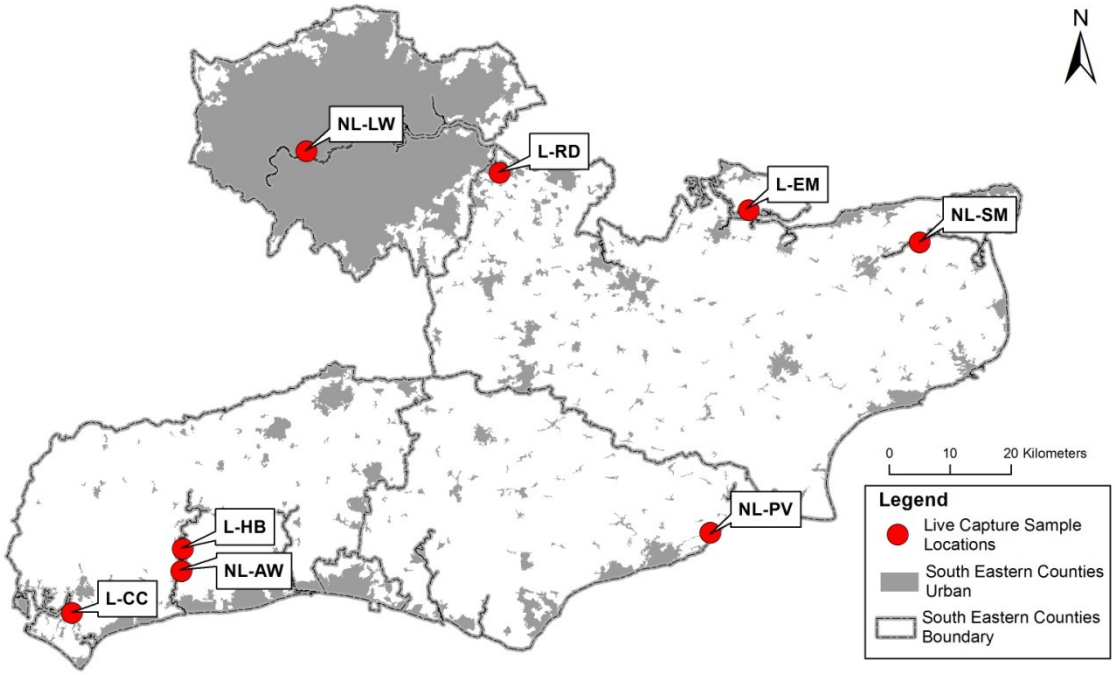

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Figure 4.1 *Map showing location of live capture study sites within southeastern England. Site code prefix denotes linear = L and non-linear = NL habitat types.*

The vegetation composition across all linear sites was similar, comprising of perennial grasses, reeds, sedges and rushes which provided a continuous

distribution of cover along the bank edges. For each linear site, the habitat patch boundaries were defined by a marked gradient of intensive land use (agricultural or urban) at their edges, which were perceived to be unsuitable for use by the resident population of water voles. The four non-linear wetlands were characterised by extensive wetland vegetation that covered a non-linear surface and included two reedbeds (NL-SM & NL-LW), a fen dominated by rush, sedge and tussock grassland with permanent pools (NL-PV) and a sedge dominated wetland with meandering waterways and islands (NL-AW). Patch boundaries were defined by a change in vegetation community to terrestrial species and included the entirety of the wetland and not just the area sampled. Patch boundaries are illustrated in Chapter Two, Figure 2.3. This is because sampling in non-linear wetlands was not systematic (i.e. grid based) due to the presence of sensitive species, such as Great Bitterns (*Botaurus stellaris)* and Hen Harriers (*Circus cyaneus*), thus sampling was restricted to areas where disturbance would be minimal.

For both linear and non-linear wetlands, the area (metre square) and perimeter (metres) of the patch (as described above) was calculated within ArcGIS V10.2.2 (ESRI, 1999-2014) and was used to calculate the perimeter area ratio (PAR) whereby $\frac{Perimeter\ (m)}{Area\ (m^2)}$ provides a value that increases with increasing perimeter relative to the area.

4.2.2 Live trapping

Live trapping was undertaken between March 2012 and October 2013. Trapping sessions at each site lasted for three consecutive days and consisted of six capture occasions whereby each trap was checked each morning and evening at 7.30 am and 4.00 pm respectively. Trapping sessions were carried out at the start of the breeding season in spring, when populations typically consist of overwintered resident adults (Telfer, 2000) and in autumn, after the majority of breeding has occurred. For seven of the eight sites, trapping was carried out in both spring and autumn in at least one of the study years.

			LINEAR				NON-LINEAR			
		Dates	**L-EM**	**L-HB**	**L-CC**	**L-RD**	**NL-AW**	**NL-LW**	**NL-SM**	**NL-PV**
2012	Spring	Start	28/3	11/4	08/5	NT	16/4	NT	22/4	28/4
		End	31/3	13/4	10/5		18/4		24/4	30/4
		Trap Effort	239	295	283.5	NA	282.5	NA	283	283.5
	Autumn	Start	06/9	19/9	10/10	15/10	23/9	11/9	29/9	05/10
		End	08/9	21/9	13/10	18/10	26/09	13/09	02/10	08/10
		Trap Effort	282	283	280.5	161	277.5	254	290	293
	No. weeks between summer trap sessions		24	23	23	NA	23	NA	23	23
	No. weeks between overwinter trap sessions		30	29	52	NA	29	54	30	55
2013	Spring	Start	05/4	09/4	NT	NT	15/04	NT	03/05	NT
		End	08/4	12/4			25/04		06/05	
		Trap Effort	289	284	NA	NA	289	NA	295	NA
	Autumn	Start	03/9	10/9	09/10	NT	16/09	24/09	04/10	29/09
		End	06/9	13/9	12/10		19/09	27/09	07/10	02/10
		Trap Effort	282.5	277.5	281.5	NA	280.5	254.5	288	254
	No. weeks between sessions		22	22	NA	NA	22	NA	21	NA

Table 4.1 *Details of sites trapped per trapping season and year, dates of trapping, trap effort per session as number of trap nights minus false triggers ($^1/_2$ a nights effort) and number of weeks separating capture sessions. NT = Not Trapped and NA = not applicable due to absent data.*

However, for one site (L-RD), trapping was only carried out once in autumn and ceased after this time due to low capture numbers (N=4). Trapping sessions ran consecutively between study sites to reduce any seasonal influences on demographic parameters and, where feasible, the order at which the sites were trapped remained constant between seasons and years so that the same period of time elapsed between trapping sessions for each site. Details of sites trapped and dates of each sampling session are shown in Table 4.1.

At each site traps were baited and placed at approximately 20 metre intervals on the bank or on floating rafts, within 5 m of open water and where water vole signs (cut vegetation, latrines, burrows or nests) indicated activity (Strachan *et al.,* 2011).

Each newly captured individual was examined in order to identify sex, reproductive condition (as described in General methods Section 2.3.1) and weight to the nearest gram. Water voles were further noted as either overwintered adult (≥180 g, worn teeth and dull pelage) or as young of the year (YOTY) (<180 g with glossy pelage) (Stoddart, 1970). New individuals, where possible, were injected with a uniquely coded sterile Passive Internal Transponder (PIT tag, Trovan ID162) beneath the loose skin around the scruff and satisfactory insertion was checked using a portable PIT tag reader before release. Where the animals showed signs of stress whilst handling, a semi-permanent unique fur clip was used (as illustrated in Gurnell & Flowerdew, 1990) for identification within a capture session and were either PIT tagged if recaptured or were "right censored" (removed) from the dataset used for estimating open population parameters (survival, population growth, recruitment). Recaptured individuals within a capture session were identified and released at the point of capture. If individuals were recaptured during subsequent capture sessions, they were re-weighed and their breeding condition determined.

4.2.3 Demographic analyses

Analyses of demographic parameters were conducted using the statistical package R (R Development Core Team, 2008) or the computer programme MARK (White & Burnham, 1999) and are described in detail below. Generalized linear models (GLM), which are less sensitive to the distribution of error and variance around the response variable (Crawley, 2014), were used to identify significant models of relative abundance, sex ratio and the number of young of the year. Analyses started with a global model which included all main effects and interactions: $Y = \beta 0 + \beta 1x1 + \beta 2x2 + ...$, where the parameters $\beta 0 ... \beta n$ were estimated by maximum likelihood. The significance of each of the terms was assessed by comparing the difference between the deviance values

of the model before and after the term was fitted to provide the minimum adequate model of significant effects (Crawley, 1993; McCullagh *et al.,* 1989). For each significant term, the deviance explained refers to the change in deviance attributed to the term in question when fitted last, as a proportion of the total deviance explained by the main effects in the minimum adequate model. P-values were estimated by comparison with the reduced model not containing the term in question. For each analysis, only estimates of coefficients having a significant influence on the model are provided in the results. Where appropriate Tukey's all-pairwise comparison tests, from the 'Multcomp' package (Hothorn *et al.,* 2008) in R, were used to generate multiple comparisons amongst sites, averaged across all significant main effects in the model (excluding covariates and interaction terms). Significance between pairs of sites were estimated using the Studentized range distribution with p-values adjusted for simultaneous inference (Wright, 1992).

Relative abundance estimates

Indices of relative abundance at each trapping session were calculated for each site using catch per unit effort (CPUE), which corresponds to the number of different water voles captured per 100 trap-nights of effort. Sprung traps (false triggers) were corrected for by considering them to represent ½ of a night of trap effort (Nelson & Clarke, 1973).

The effects of habitat type (HT = linear and non-linear) and PAR on the relative abundance of water voles were tested for significance using generalized linear models (GLM) with either habitat type or PAR (these two factors were confounded), season and year as main effects. To test whether the relative abundance of water voles was more variable between seasons in linear habitat types, the significance of an interaction between HT and season was tested in the model. Differences in the relative abundance between trapping sites, season and year were assessed using a separate GLM.

Population structure and breeding

The operational (breeding) and non-breeding YOTY sex ratios were calculated as the proportion of females per male within each site per habitat type (linear

vs. non-linear) and were compared to a binomially distributed 50:50 ratio for each trapping session using a binomial test. An index of age structure was calculated as the number of YOTY (breeding and non-breeding) per overwintered adult captured within each site per trapping session. A GLM with binomial error structure and logit link was used to model the differences in the age structure and sex ratios between habitat types in order to identify whether these vary between seasons and years.

Recruitment into the breeding population by young of the year was calculated for individuals initially captured as YOTY that were either breeding at the time of first capture or were recaptured in subsequent trapping sessions as a breeding adult within their natal site. Only YOTY captured between spring 2012 and spring 2013 were used as there was no further opportunity to recapture any YOTY that became sexually mature after the September 2013 trapping session. The influence of habitat type on recruitment into the breeding populations were analysed by logistic regression: Recruitment (0 = not recruited, 1 = recruited) was the dependent variable with body weight (grams), sex (male = 0, female = 1), cohort (spring and autumn), site (N =1 - 8) and habitat type (linear, non-linear) as independent variables. Body weight and the cohort in which the YOTY were first captured were included in the model to investigate whether early or late born individuals were more likely to be recruited into the breeding population. Differences in weight and sex effects on the probability of being recruited, between sites and between habitat types, were tested for using two way interactions between weight and site; weight and habitat type; sex and site and sex and habitat type.

To determine if the relative abundance of YOTY captured in the autumn cohorts was a function of the relative abundance of adult females that overwintered in the population in spring, and whether the relationship was different between habitat types, data was analysed using a GLM. Relative abundance of YOTY in autumn was the dependent variable and spring abundance of breeding females, habitat type and year were considered as main effects with an interaction between number of breeding females in spring and habitat type.

It was hypothesised that territorial females in linear habitats are a limiting factor that negatively influence breeding opportunities for female YOTY, due to resource competition. The weight at which female YOTY water voles became sexually mature was used to test this hypothesis. If competitive exclusion amongst territory holding females and female YOTY is present, one would expect there to be an increase in the weight at which YOTY females become sexually mature relative to non-linear wetlands, which were predicted to be more tolerant of YOTY females establishing breeding territories. For the analyses, all YOTY females captured in autumn cohorts (avoiding bias of sites where early born young were present in spring) were scored either a 1 if they were in breeding condition and 0 if they showed no signs of breeding, creating a binary response variable of reproductive status. A GLM with binomial errors and logit link was then used with reproductive status as the dependent variable and body weight, the relative abundance of adult breeding females (per 100 trap nights in autumn) at each site, habitat type and year as indicator variables. To test for differences in the influence of breeding females on YOTY weight at sexual maturation between habitat types, a three-way interaction between weight, breeding females and habitat types was included. The interpretation of significant interactions was facilitated by visualising interaction plots, using the 'Effects' package (Fox & Hong, 2009) in R. Weight was centred by the mean weight to improve interpretation of the coefficient estimates and interaction terms (Aiken & West, 1991).

4.2.4 Mark-recapture modelling

Water vole survival rates, population sizes and population growth rates were estimated for each site separately using maximum likelihood Robust Design models (Pollock, 2000; Kendall *et al.,* 1997; Pradel, 1996; Kendall & Nichols, 1995) implemented in the programme MARK (White & Burnham, 1999). The robust design incorporates closed population and open population models based on sampling data obtained across two temporal scales comprising of a) short-term secondary sessions in which the population is assumed to be biologically and geographically closed (i.e. no births, deaths, immigration and emigration) and from which the probability of capture and population size is estimated and b) longer-term primary periods which separate secondary

sessions when the population is assumed to be open to births/deaths immigration and emigration and from which estimates of apparent survival, movement, population growth and recruitment can all be obtained (White & Burnham, 1999).

The programme MARK provides flexibility in the modelling approach under the robust design framework by incorporating different models for the estimation of open and closed demographic parameters depending on the data type, biology of the study species and the overall objective of the study (White *et al.,* 2001). For the purpose of estimating population size at each trapping session in this study a full likelihood closed capture model was used which is based on closed population models described by Otis *et al. (*1978). This model allows for variation in the probability of encountering both new (initial capture probability = p) and previously marked individuals (recapture probability = c) across the sampling period and provide means to estimate behavioural responses to trapping, all of which are common sources of bias in the estimation of population size (Chao *et al.,* 1992). Because of small sample sizes obtained from some of the study sites, individual heterogeneity was not included in the closed population models due to the risk of models becoming too complex and over-parameterised (Tattersall *et al.,* 2004).

For the estimation of open population parameters for the breeding and overwinter intervals between secondary trapping sessions, two modelling approaches were initially investigated. The first was the standard Robust Design model described by Kendall *et al. (*1997) & Kendall & Nichols (1995) which provides estimates for a) the probability of survival of previously marked individuals between trapping periods (S), based on the probability of detection (p*) at least once within a secondary trapping session, given that the individual is present in the study area, b) the probability of temporary emigration from the study area, given that the animal was previously present in the population (γ'') and c) the probability of staying away from the study area, given that the animal had not previously been observed in the trapping area (γ', where 1-γ' corresponds to the return rate of temporary, marked emigrants). Initial examination of this model using data obtained from each population, however,

showed this model to be too complex for sites where a low number of individuals were trapped in any one session and resulted in a number of models in the candidate set failing to converge. The second modelling approach was Pradel's extension of the Jolly-Seber model (Jolly, 1965, Seber, 1965) within a Robust Design framework, which proved to be more robust given the data available from each site. This model, referred to herein as the Pradel Lambda model, permits the estimation of the finite rate of population change (λ) between trapping sessions, where $\lambda = N_{(t+1)} / N_{(t)}$ (where t = time), by incorporating estimates of the probability of survival, recapture and the probability that an animal in the population at time t was also in the population at time t-1 (γ), all of which explain changes in a population from one time period to another.

Given that survival (Ø) requires data on the previous state of marked individuals within the population extending beyond the previous trapping session, only sites where three or more trapping sessions were conducted could be used in this Robust Design analyses. For example, to enable estimation of survival rates (Ø) of marked individuals between trapping sessions, three or more sessions are required to be able to identify if the failure to capture a marked individual during session $t + 1$, is because they have died (or permanently emigrated) over period $t \rightarrow t + 1$, or because the individual was not re-encountered, but could have been subsequently recaptured at $t + 2$. This discounted populations NL-LW and L-RD respectively, where only two and one trapping occasions were conducted. For site NL-LW the number of captures was sufficient to obtain estimates of population size for each trapping session, using full-likelihood closed capture design. This is analogous to the procedure used to estimate population sizes for each trapping session in the Robust Design models and, as such, the same *a priori* models for capture and recapture probabilities, as detailed above, were used in the analyses. Survival at this site was substituted for the persistence of marked individuals within the trapped area. Persistence was calculated as the proportion of marked individuals that persisted between trapping session t and t + 1. For site L-RD where the number of captures was insufficient to provide estimates of population size the minimum number known to be alive (MNKA), was used. In all instances, the procedure used to estimate population size and survival is annotated within the results.

Modelling procedure

Sampling data consisted of capture histories (where 1 = encountered, 0 = not encountered) for each individual permanently marked in each population and comprised of a maximum of 24 encounter histories (i.e. six encounters for secondary trapping occasions and (four encounters for primary periods). Capture histories were inputted into the programme MARK and the time intervals between secondary trapping sessions were adjusted to account for unequal intervals between seasonal capture sessions.

Although it is biologically relevant to assume that survival is dependent on sex and age, it was not possible to include these group covariates across all sites used in the analyses due to there being an insufficient number of observations from each of these groups at some of the study sites. As such, no group covariates were included in the capture history input file.

Modelling followed a two phase *a priori* approach. Firstly, the capture and recapture probabilities used to derive closed population size estimates for each secondary trapping session were modelled to investigate what factors best explained the variation observed in the capture probabilities. As the trapping effort remained constant across trapping sessions, time variation in capture probabilities (c & p) was not considered to vary between trapping sessions. Instead, time variation within each secondary trapping session (t) and between morning and evening capture occasions (AM:PM) was considered to account for observed differences in the trappability of animals within a trapping session. The alternative models for capture probabilities were that they remained constant (denoted by '.') within each capture session. Behavioural effects on capture probabilities were tested for by comparing models where the probability of capture and recapture were set to be the same (p = c) or to be different (c, p). Accounting for differences in the probability of capture and recapture allows for more precise estimates of population size during each trapping session to be obtained (Otis *et al.,* 1978). Using the best fitting models from the first phase, open population parameters Ø and λ were then estimated to be either time variant (t), remain constant over the study period (.) or to vary with season (SEAS).

Because the robust design has no goodness-of-fit test available, extra binomial variation (over-dispersion) was tested for using the median c-hat (model deviance/df) approach by collapsing secondary occasions in the context of the live encounter Cormack-Jolly-Seber model (Seber, 1970; Cormack, 1964). The median c-hat approach provides an estimate of over-dispersion and simulates data with a range of c values (bounded between 1 and 3 for each site), obtaining a deviance c-hat = deviance/df for each of the simulated datasets. Logistic regression was then used to estimate the value of c when it falls mid-way between the simulated deviance/df values (White & Burnham, 1999). The number of replicate simulations to generate each c value was set to five and 100 simulations were used for all sites. Over-dispersion was identified in one site (NL-AW) where c-hat = $1 \leq 3$, and the estimated c-hat value was adjusted to correct for extra-binomial variation in the data (Burnham & Anderson, 2002).

Model Selection

A maximum of 11 models were generated for each population (some models failed to converge for sites with a low number of captures and were removed from the model set during the modelling phase) representing *a priori* hypotheses on the influence of time and season on the probability of capture, survival and population growth. Seasonal differences were confounded by time in populations with only three secondary trapping occasions, which reduced the model set for populations L-EM, L-CC and NL-PV.

Model selection was based on Akaike Information Criteria and corrected for small sample size (AICc). AICc weights were used to assess the relative support of each of the candidate models. For over-dispersed data, the Quasi-AICc values and weights were used. Models with the lowest AICc values were most supported by the data and where the difference between AICc values were less than 2.0, it was assumed that they had approximately equal support from the data (Cooch & White, 2010). Model averaged estimates of Ø, λ and N were then obtained to account for model selection uncertainty (Burnham & Anderson, 2002).

Overall Recruitment

Overall recruitment rates (number of new animals entering the population per existing member) during breeding and non-breeding seasons for each population were derived using Pradel's parameterization of recruitment rate (f) where f = λ - Ø. Estimates of λ and Ø were taken from model averaged estimates from the capture-recapture analyses in MARK.

Statistical analyses for site and geometric effects

To determine whether differences in survival, population rate of change and overall recruitment between sites could be explained by either habitat type or by PAR of the sampled patch, separate GLMs were used with the parameter estimate as the dependent variable and either HT or PAR, season and year as the main effects. Interactions between season and HT or PAR were tested to investigate whether seasonal variation can be explained by the two measure of habitat geometry.

Analyses of the effects of habitat type and PAR of the patch on survival, population size and population growth could not be conducted from within programme MARK due to there being unequal time intervals between sites.

4.3 Results

4.3.1 Overview of captures

Between spring 2012 and autumn 2013 a total of 896 water voles, consisting of 392 individuals, were captured during 6866 trap nights across the eight study sites. Across all sites and years, the mean number of individuals captured in any one trapping occasion was 6 (SD = 6.5) and ranged from no observations (occurring 12% of the time) up to 34 individuals being captured on one occasion. The highest proportion of occupied traps on any one occasion was 76% and the mean maximum occupation across all trap nights and sites was 22% (SD = 19%), indicating that none of the sampled populations reached trap saturation during the study (Gurnell & Flowerdew, 1990).

Between spring 2012 and spring 2013 a total of 221 water voles were permanently marked with Trovan PIT tags for future identification. A high proportion of individuals were only trapped in one primary session (74.3%), 16% were trapped across two sessions and only 1% (3 individuals) were observed on three of the four trapping sessions. By season, the recapture of marked individuals between trapping sessions was highest in spring 2013 when 13-32% and 9-33% of individuals from non-linear and linear sites respectively, were recaptures from the previous year. The proportion of autumn cohorts that were recaptures from previous sessions were higher in populations from linear habitats in 2012 (range: linear = 20-21%; non-linear = 0-9%) but were similar between populations from both habitat types in 2013 (range: linear = 0-21%; non-linear = 0-18%).

Details of the number of voles captured per site, season and year are shown in Table 4.2.

Site	2012							2013							
	Spring			Autumn				Spring				Autumn			
	N CP	N IND	% YOTY	N CP	N IND	N RC	% YOTY	N CP	N IND	N RC	% YOTY	N CP	N IND	N RC	% YOTY
NL-AW	80	31_{30}	13	66	33	3	67	73	28	9	11	142	67	7	47
NL-SM	12	7	14	13	7	0	71	22	8	1	0	7	3	0	67
NL-PV	7	4	50	18	11	0	50	-	-	-	-	29	16	2	44
NL-LW	-	-	-	18	9	na	67	-	-	-	-	23	11	2	55
TOTAL	**99**	**42_{41}**	**17**	**115**	**60**	**3**	**65**	**95**	**36**	**10**	**8**	**201**	**97**	**11**	**47**
L-HB	31	15_{14}	0	28	14_{10}	3	50	50	21	2	14	54	29	6	49
L-EM	-	-	-	65	32_{22}	na	66	14	6	2	0	72	40	3	47
L-CC	20	13	23	33	20_{19}	4	33	-	-	-	-	12	7	0	43
L-RD	-	-	-	7	4	na	50	-	-	-	-	-	-	-	-
TOTAL	**51**	**28_{27}**	**11**	**133**	**70_{55}**	**7**	**53**	**64**	**27**	**4**	**11**	**138**	**76**	**9**	**47**

Table 4.2 *Number of captures (N_{CP}), number of individual voles captured (N_{IND}) with the number of permanently marked individuals in subscript if different from capture number, number of N_{IND} that had been previously marked (N_{RC}) and percentage of captured individuals classifed as young of the year (YOTY), by site, season and year. Total captures including recaptured individuals (TOT) and the percentage of these that were YOTY are shown for both non-linear (outlined boxes) and linear (shaded boxes) habitat types. Symbol '-' denotes missed trap sessions.*

4.3.2 Relative abundance

The relative abundance of water voles, as determined by the number of new individuals captured per 100 trap nights is shown for each population and trapping season in Figure 4.2. There was no significant effect of PAR or habitat type on the relative abundance of water voles and seasonal variations were not significantly different between habitat types (as tested by the interaction between habitat type and season), nor did the relative abundance significantly differ between years. However, relative abundance was significantly different between sites and was significantly higher in autumn than in spring (Table 4.3). A Tukey's post hoc pairwise comparison test between sites revealed that site NL-AW had significantly higher relative abundance than all sites apart from L-EM, which did not differ significantly from the other sites apart from site NL-SM which also had a significantly lower relative abundance of voles than site L-HB.

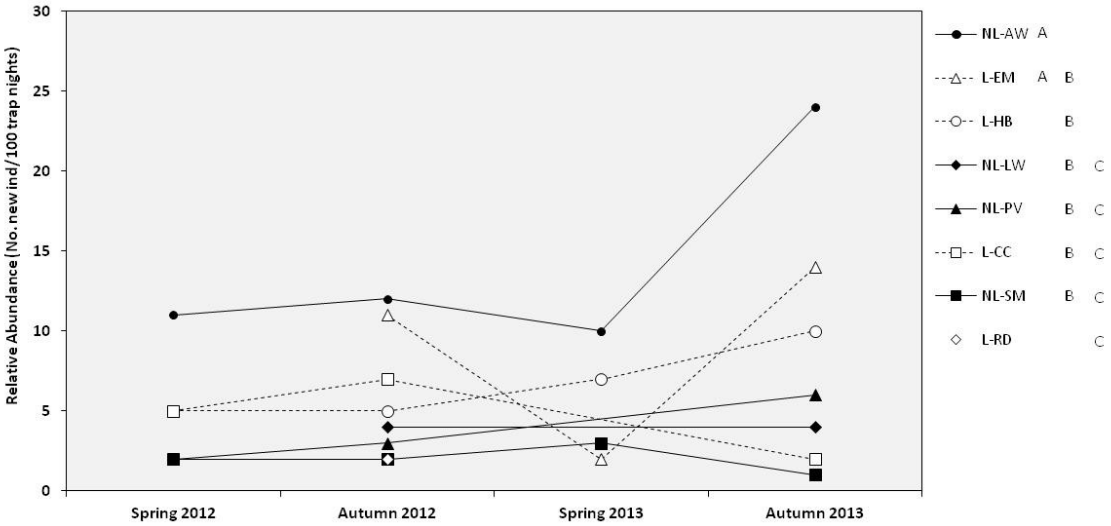


Figure 4.2 *Relative abundance of water voles, calculated as the number of new individuals captured per 100 trap nights at each site per trapping season. Populations from linear habitat types are shown as outlined boxes and hatched lines and populations from non-linear habitat types are shown as solid boxes and lines. Results of Tukey's post hoc pairwise comparisons between sites are shown next to legend where different letters indicate significant differences (i.e. sites denoted with A's are significantly different from B's but are not significantly different if letters are shared between sites).*

Coefficients	Estimate	Df	% Deviance	P-value
Intercept	2.3792			
Site	*	7	73.8	0.000
Season	0.4946	1	9.1	0.005

Table 4.3 *Final model showing significant factors for the relative abundance GLM with binomial errors and logit link (reference coefficients are Site (NL-AW) and Season (Spring). For each term, the deviance explained refers to the change in deviance attributed to the term in question when fitted last, as a proportion of total deviance explained by the main effects in the minimum adequate model. *Coefficient estimates for each site are not shown as differences between sites are described in the main text. Total deviance explained by final model = 82.9%.*

4.3.3 Population structure and breeding

Sex Ratios

The number of sexually mature males and females and the total number of males and females identified as YOTY, per site and trapping season are shown in Table 4.4. The results of binomial tests showed that there was no bias in sex ratios across non-linear wetlands in any capture session. However, in autumn 2013, a female bias sex ratio was observed in the YOTY captured at site L-EM, and when pooled across linear wetlands, females constituted a significantly higher proportion of the breeding and YOTY cohorts.

	2012								**2013**							
	Br Adults				**YOTY**				**Br Adults**				**YOTY**			
Site	Spr		Aut		Spr		Aut		Spr		Aut		Spr		Aut	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
NL-AW	9	18	5	11	2	2	12	10	14	13	23	18	1	2	12	22
NL-SM	2	4	1	1	0	1	2	3	7	1	1	0	0	0	1	1
NL-PV	1	1	2	4	1	1	4	2	-	-	6	3	-	-	4	3
NL-LW	-	-	3	3	-	-	4	2	-	-	3	3	-	-	3	3
Total	12	23	11	19	3	4	22	17	21	14	33	24	1	2	30	29
Mean Ratio	1	1.9	1	1.7	1	1.3	1	0.8	1	0.7	1	0.7	1	2	1	1
L-HB	6	9	4	3	0	0	5	2	14	6	8	13	0	3	5	9
L-EM	-	-	6	12	-	-	9	12	5	1	8	22	0	0	**2**	**17**
L-CC	6	4	4	6	1	2	5	7	-	-	2	3	-	-	0	3
L-RD	-	-	1	1	-	-	2	0	-	-	-	-	-	-	-	-
Total	12	13	15	22	1	2	21	21	19	7	**18**	**38**	0	3	**7**	**29**
Mean Ratio	1	1.1	1	1.5	1	2	1	1	1	0.4	**1**	**2.1**			**1**	**4.1**

Table 4.4 *Total number of sexually mature individuals by sex (including breeding YOTY) and the total number of all individuals classified as YOTY by sex (including those breeding) captured in the 2012 and 2013 spring (Spr) and autumn (Aut) cohorts, by site. Populations from non linear and linear sites are shown in outlined and shaded boxes respectively. Mean Ratios denote number of females per male. Significant departures from equal proportions, as determined by binomial tests, are shown in bold.*

Results of the GLM of proportion of females in the breeding cohort showed that there were no significant differences between sites (X^2 = 8.26,14, p = 0.31). The interaction between habitat type and season, however, was significant, and tests of the simple slopes for season and habitat type coefficients, show that females constituted a significantly higher proportion of the overwinter breeding cohort in non-linear wetland types in spring. In autumn, however, a significantly higher proportion of the breeding cohort were female in linear wetlands (Table 4.5). Year was also a significant factor in the model showing there was a significant reduction in the proportion of sexually mature females to males in 2013 compared with the previous year.

Both habitat type and year were significant factors in the GLM for the proportion of female YOTY indicating that a higher proportion of YOTY were observed in linear than non-linear wetlands and constituted a higher proportion of the YOTY observed amongst study populations in 2013 than 2012. Neither population, nor season were significant factors in the model, indicating that there was no significant skew in YOTY sex ratios between sites or seasons.

a) Breeding Cohort					**b) Young of the Year**				
Coefficients	**Est**	**df**	**% Dev**	**P**	**Coefficients**	**Est**	**df**	**% Dev**	**P**
Intercept	0.621				Intercept	-1.30			
HT	0.536	1	0.6	0.5	HT	0.61	1	12.9	0.003
Season	0.057	1	9.1	0.07	Yr	0.93	1	31.3	0.052
Yr	-0.53	1	15.7	0.02					
HT:Season	-1.09	1	15.6	0.02					

Table 4.5 *Final models showing significant factors for the proportion of females GLM with binomial errors and logit link for* ***a)*** *breeding cohorts and* ***b)*** *YOTY cohorts (including those breeding). Reference coefficients are Habitat Type (HT = non-linear), Season (Spring) and Yr (2012). For each term, the deviance explained refers to the change in deviance attributed to the term in question when fitted last, as a proportion of total deviance explained by the main effects in the minimum adequate model. Total deviance explained for a) = 41% and b) = 44.2%.*

Age structure

The age structure, as determined by the proportion of YOTY per adult, was not significantly different between linear and non-linear habitat types (non-linear HT coefficient = 0.29 ± 0.79, z = 0.362, p = 0.718). Both year and season were significant factors in the model, showing that a higher proportion of YOTY per adult was observed in 2012 than 2013 and the autumn cohorts comprised of significantly more YOTY per adult than in spring (Figure 4.3).

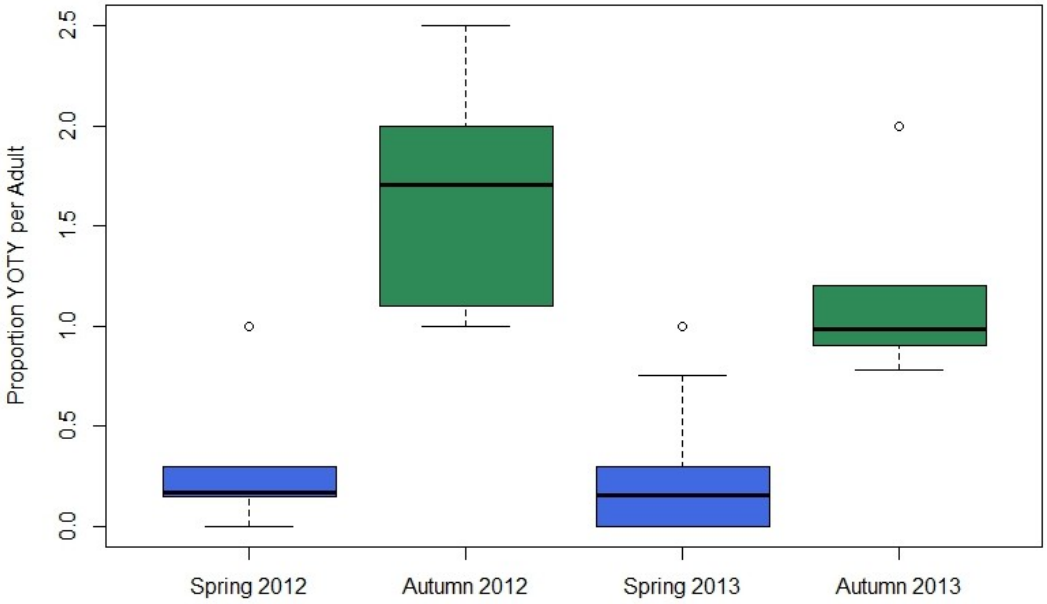


Figure 4.3 *Boxplot of the proportion of young of the year water voles per adult vole captured across all sites in spring and autumn 2012 and 2013. Horizontal lines represent the median, with the box representing the 25th and 75th percentiles and the whiskers representing the 5th and 95th percentiles. Outliers are shown as outlined circles. Significant coefficients for final GLM (reference coefficients of Spring and 2012) for Season (Coefficient = 2.104 ± 0.28 SE, % Model Deviance = 77.8%, p = 0.000) and Yr (Coefficient = -0.451 ± 0.215, % Model Deviance = 4.5%, p=0.035).*

Breeding

The GLM of the relative abundance of YOTY in autumn, showed that the relative abundance of breeding females, year and the first order interaction of breeding females with habitat type and with year to be significant (Table 4.6). Significantly more YOTY were observed in 2012 and testing simple slopes of the coefficients indicates that spring abundance of breeding females positively influences the abundance of YOTY in autumn in non-linear habitat types, whilst in linear wetlands this relationship is negative (Figure 4.4).

Factor	Coefficient	% Deviance	df	p
NULL	-0.0921			
Br. Female abundance	1.5623	52.9	1	0.000
Year (2013)	2.3886	22.0	1	0.020
Habitat Type (Linear)	4.9029	0.05	1	0.731
Br. Female abundance:HT	-2.3605	16.2	1	0.051

Table 4.6 *Final GLM for factors influencing the relative abundance of YOTY in autumn, with significant main effects and first order interactions. Br. Female abundance relates to the relative abundance per 100 trap nights in spring. Total deviance explained by model = 91.2%.*

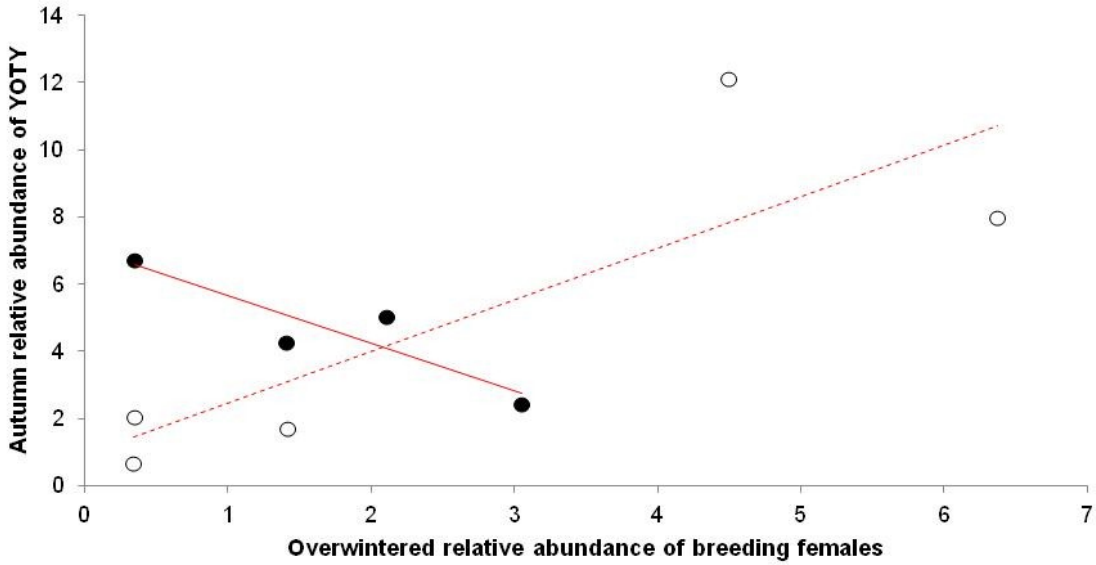


Figure 4.4 *Scatterplot showing the relationship between relative abundance of overwintered breeding females in spring and the relative abundance of YOTY (including those identified as sexually mature) observed in autumn across both study years. Solid marks and trend line relate to linear habitat types, where* R^2 *= 0.82 and outlined marks and dashed trend line relate to non-linear populations, where* R^2 *= 0.71.*

Recruitment of YOTY into breeding population

The probability of YOTY being recruited into the breeding population was positively correlated with weight for both sexes and was significantly different between males and females with the odds of females being recruited being 4.3 times higher than for males (Table 4.7). Habitat type and population were not significant factors in the model.

Factor	Coefficient	% Deviance	df	p
NULL	-3.040846			
Weight	0.022358	10.08	1	0.000
Sex (M)	-1.45149	7.31	1	0.002

Table 4.7 *Final GLM (with binomial errors and logit link) for the number of YOTY recruited into breeding population with significant main effects. Total deviance explained by model = 17.4%.*

Weight at sexual maturity

The mean weight at which female and male water voles were recorded as sexually mature was 206.1 g (range: 95-340 g) and 234.5 g (range: 110-310 g) respectively and was similar between the study sites and sampling years (see summary of breeding weights by site and trapping session provided in Appendix Four).

The probability of female water voles becoming sexually mature was positively correlated to weight and conditional on the number of breeding females and habitat type (Table 4.8).

Factor	Coefficient	df	% Deviance	*p*-value (Chi)
Intercept	-1.2555			
Wgt	0.0468	1	10.60	**0.000**
BF	0.3411	1	0.00	0.945
HT (NL)	0.5114	1	1.65	0.165
Wgt:BF	-0.0055	1	5.32	**0.013**
Wgt:HT	-0.0825	1	0.01	0.904
BF:HT	-0.7722	1	0.80	0.347
Wgt:BF:HT	0.0413	1	3.70	**0.039**

Table 4.8 *Final GLM (with binomial errors and logit link) of significant effects of weight (Wgt), habitat type (HT) and the relative abundance of breeding females (BF) on the probability of sexual maturity for YOTY females. Significant high-order interaction term, lower-order terms and conditional effects are shown with coefficients relative to weight centred to mean (Aiken & West, 1991) and Linear habitat type (HT). Total deviance explained = 23%.*

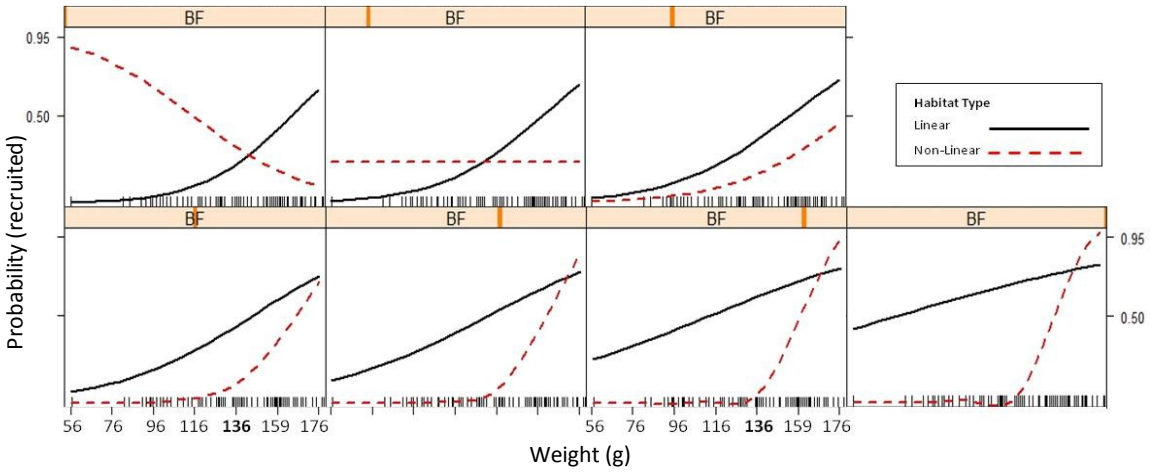


Figure 4.5 *Effects display for three way interaction Wgt:BF:HT, showing the change in probability (y-axis) of females sexually maturing with increasing weight (Wgt) (x-axis, mean shown in bold) and relative abundance of breeding females (BF) (shown across panels with increasing abundance displayed from left to right, top to bottom) by linear and non-linear habitat type (HT).*

Figure 4.5 shows that in linear habitats, the probability of sexual maturity increases with increasing weight, but is relatively unaffected by breeding female abundance, as reflected by the similarity in slopes across panels. Whilst in non-linear wetlands, the slope varies in direction and height relative to linear and indicates that the probability of sexual maturity is high for lighter individuals when abundance of breeding females is low and as abundance increases, the probability increases with increasing weight, but is lower than linear wetlands with increasing abundance of females, as reflected by the effects slope being mostly below that for linear wetlands.

4.3.4 Demographic models and parameter estimates

A total of 221 permanently marked water voles captured between spring 2012 and spring 2013 and 153 individuals captured in the final capture session in autumn 2013 were used in the MARK analyses. Population demographic parameters were obtained from seven of the eight sites and included parameter estimates for apparent survival (Ø), population rate of change (λ) and population size (N), with capture probabilities (p and c) within each secondary trapping session for six sites using Pradel's Lambda model with robust design and population size with capture probability estimates for site NL-LW using closed capture models for the 2012 and 2013 trapping sessions. The results of the top ranking models obtained for each site are shown in Table 4.9.

Over-dispersion was observed in only one site (NL-AW) where the median c-hat value was 1.21 which was adjusted accordingly and the Quasi AICc values were used for selecting the best models amongst the candidate model set. Due to model selection uncertainty, whereby more than one model provided adequate support for the data (Delta AICc $\leq$2.0), parameter estimates, with standard error and 95% confidence intervals, were obtained using the weighted averages of parameter estimates from models with a Delta AICc value of $\leq$2 (White & Burnham, 1999).

Site	Model				AIC_c	Δ_i	ω_i	L	K	Dev
NL-AW	$Ø_{seas}$	λ_t	$p_{AM:PM}$	$c_{AM:PM}$	660.29	0.00	0.29	1	13	633.2
	$Ø_.$	λ_t	$p_{AM:PM}$	$c_{AM:PM}$	660.47	0.18	0.27	0.92	12	635.6
	$Ø_t$	λ_t	$p_{AM:PM}$	$c_{AM:PM}$	661.49	1.20	0.16	0.55	14	632.2
	$Ø_{seas}$	λ_{seas}	$p_{AM:PM}$	$c_{AM:PM}$	661.86	1.56	0.13	0.46	12	636.9
NL-SM	$Ø_{seas}$	λ_{seas}	p_t =	c_t	210.41	0.00	0.24	1	9	188.3
	$Ø_t$	λ_{seas}	p_t =	c_t	210.41	0.00	0.24	1	10	188.3
	$Ø_.$	$\lambda_.$	p_t =	c_t	211.56	1.15	0.13	0.56	8	192.4
	$Ø_t$	λ_t	p_t =	c_t	212.14	1.73	0.10	0.42	16	187
	$Ø_{seas}$	λ_t	p_t =	c_t	212.14	1.73	0.10	0.42	10	187
	$Ø_{seas}$	λ_{seas}	$p_{AM:PM}$ =	$c_{AM:PM}$	212.15	1.74	0.10	0.42	5	200.9
NL-PV	$Ø_t$	λ_t	$p_{AM:PM}$ =	$c_{AM:PM}$	168.95	0.00	0.58	1	9	149.6
	$Ø_t$	$\lambda_.$	$p_{AM:PM}$ =	$c_{AM:PM}$	170.67	1.72	0.25	0.42	8	151.3
NL-LW 12*			p. =	c.	45.54	0.00	0.75	1	2	32.9
NL-LW 13*			$p_{AM:PM}$ =	$c_{AM:PM}$	48.34	0.00	0.81	1	3	29.7
L-HB	$Ø_.$	λ_{seas}	$p_{AM:PM}$	$c_{AM:PM}$	456.76	0.00	0.31	1	11	432.9
	$Ø_.$	λ_t	$p_{AM:PM}$	$c_{AM:PM}$	457.05	0.30	0.27	0.86	12	430.8
	$Ø_{seas}$	$\lambda_.$	$p_{AM:PM}$	$c_{AM:PM}$	457.76	1.01	0.19	0.61	11	433.9
L-EM	$Ø_.$	λ_t	$p_{AM:PM}$	$c_{AM:PM}$	245.90	0.00	0.61	1	10	224.1
	$Ø_t$	λ_t	$p_{AM:PM}$	$c_{AM:PM}$	246.83	0.93	0.39	0.63	11	222.7
L-CC	$Ø_t$	λ_t	p_t =	c_t	220.19	0.00	0.42	1	12	190.1
	$Ø_t$	$\lambda_.$	p_t =	c_t	221.02	0.83	0.28	0.66	11	193.9
	$Ø_.$	$\lambda_.$	p_t =	c_t	222.05	1.86	0.17	0.39	11	194.9

Table 4.9 *Top competing models describing apparent survival rates (Ø), population rates of change (λ), capture (p) and recapture (c) rates of water voles in non-linear (outlined) and linear habitat types (shaded). Subscripts in model parameters are '.' = time constant, 't' = time variant, 'seas' = seasonal variation, 'AM:PM' = varies by morning and evening captures and '=' where capture and recapture probabilities are equal. * Closed capture models obtained only for population size estimates.* Δ_i *= difference between the* AIC_c *value of the model and the* AIC_c *value of the most parsimonious model (Delta AICc);* ω *= Akaike weight in relation to top model; L = model Likelihood; K = number of parameters; Dev = model deviance.*

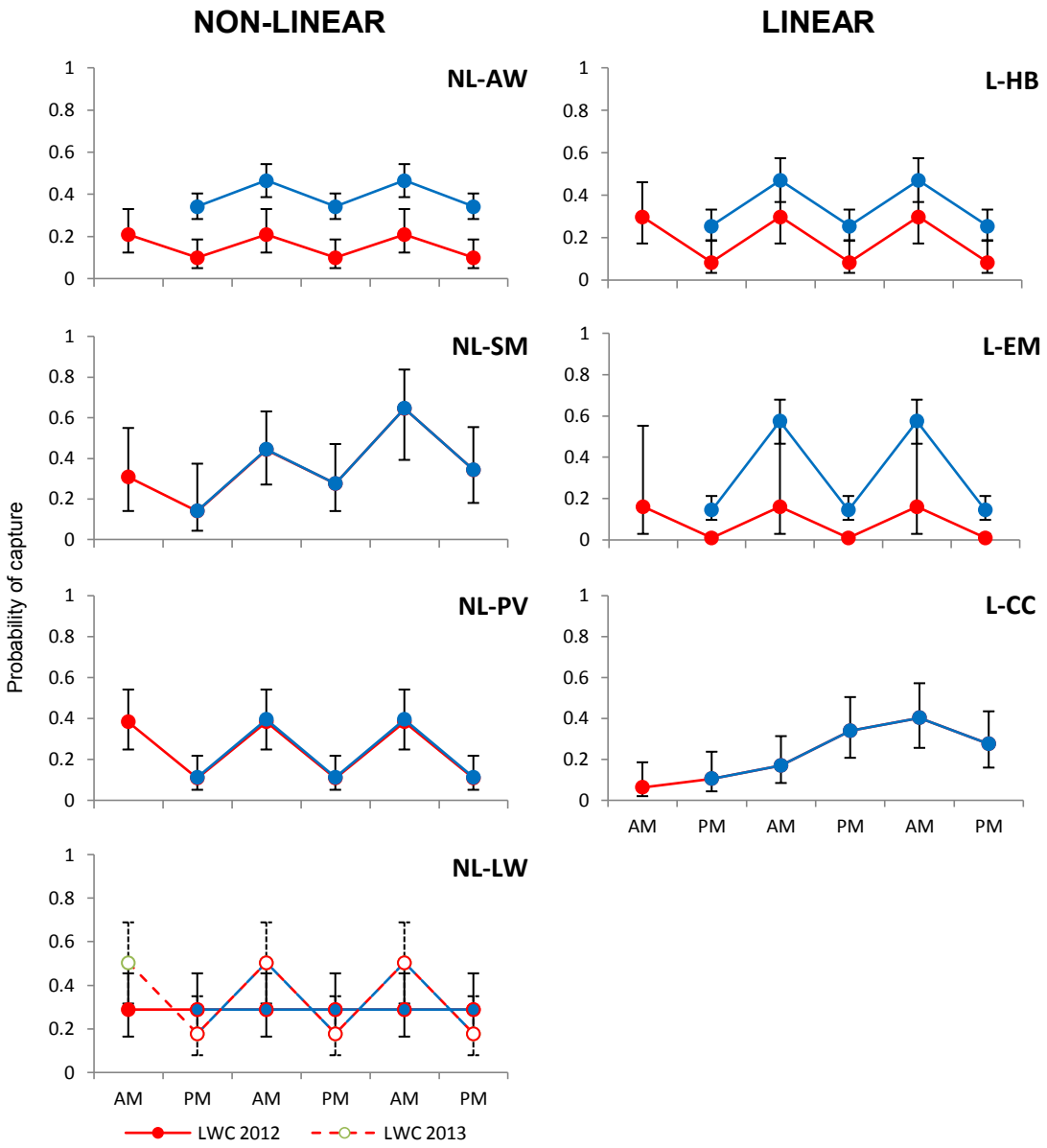


Figure 4.6 *Capture probabilities of water voles in non-linear and linear habitat types. Red and blue lines denote initial capture and recapture probabilities respectively. Error bars represent 95% CI's. Values obtained from model averaged estimates from all sites apart from NL-LW where no other competing models (Δ ≥ 2) were observed.*

Population size and rate of change

The best supported models for water vole population size estimates, across all but one site (NL-LW), included time variant probabilities of capture, whereby the probability of capturing a water vole was not equal across morning and evening release sessions (AM:PM) or varied by trapping occasion (t) (Table 4.9, Figure 4.6). Behavioural responses to trapping were observed within three of the study sites whereby the probability of recapturing an individual was higher than the probability of capturing an unmarked vole, indicating that there was a positive

response of water voles to being trapped. The probability of capture was rarely above 0.5 across all sites and was higher during morning capture occasions.

Abundance estimates were highly variable between sites and ranged from four to 105 individuals across seasons (Figure 4.7). The abundance of voles, post-breeding, increased in 2013 compared with 2012 at five study sites which also showed a positive mean geometric growth over the study period (λ_G = >1). At two sites, NL-SM and L-CC, a reduction in post breeding abundance estimates was observed between 2012 and 2013 and both sites showed a negative mean growth rate during the study period. Large confidence intervals in geometric growth rates were observed at sites that exhibited a higher variation in abundance between seasons.

Seasonal or time variant population rate of change, as estimated by Lambda parameter (λ), were the best supported models across all sites. Seasonal variation (between successive trapping sessions) in the rate of population change for each population is shown in Table 4.10. A positive growth (λ >1) in populations was observed during the breeding season in both years for all sites sampled pre and post breeding, apart from non-linear site NL-SM, where the population growth rate was considerably lower (λ <1) during both breeding seasons. The highest rate of population change was observed in a non-linear site (NL-PV) during the 2012 breeding season and in a linear habitat (site L-EM) during the same period in 2013.

	NON-LINEAR			**LINEAR**		
Season	**NL-AW**	**NL-SM**	**NL-PV**	**L-HB**	**L-EM**	**L-CC**
Summer 2012	1.3 ± 0.38	0.8 ± 0.4	2.4 ± 0.5	1.0 ± 0.3	-	1.2 ± 0.5
Winter 2012/13	0.8 ± 0.17	1.1 ± 0.4	1.2 ± 0.2	1.6 ± 0.4	0.4 ± 0.1	0.7 ± 0.2
Summer 2013	2.2 ± 0.60	0.6 ± 0.3		1.2 ± 0.3	3.3 ± 0.9	

Table 4.10 *Population growth rate estimates ± standard error obtained from model averaged estimates of Pradel Lambda model. Estimates are displayed by site and season seperating each trapping session. For sites NL-PV and L-CC, joint entries are shown for population growth estimates for the period between autumn 2012 and autumn 2013 trapping sessions. Missed trapping sessions are denoted by a dash (-).*

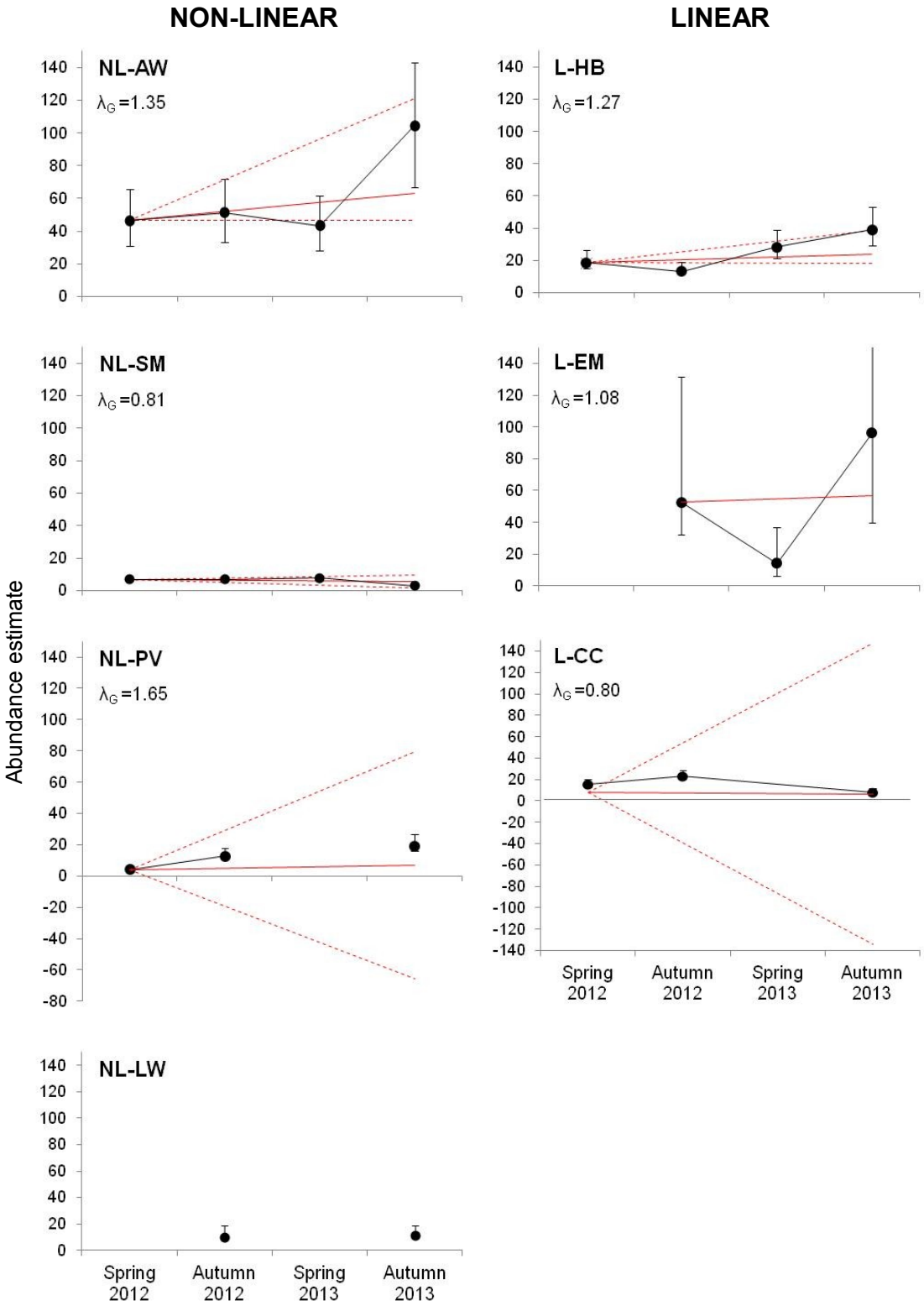


Figure 4.7 *Population size estimates (circle markers) per study site, by habitat type, as estimated by Pradel Lambda with robust design. Lower bound errors relate to the MNKA (included individuals without permenant identification) and upper bound are 95% confidence intervals estimated by MARK. Mean geometric growth rates (solid red line) calculated by each population and 95% confidence intervals (light red lines) as estimated using DA method (Dennis et al., 1991) for complete and missing intervals.*

For sites sampled pre and post breeding in both years, Pradel's population growth estimate (λ) was higher in 2013 than in 2012. Two sites (NL-SM and L-HB) showed a positive growth in population size overwinter and for site L-HB this growth was higher than during the breeding season. The highest seasonal variation in population growth rates was observed at L-EM where overwinter population growth was lower (λ = 0.4 ± 0.1) than the other sites during the same period and when compared to the growth rate estimated during summer 2013 (λ = 3.3 ± 0.9).

Overall survival rates of populations

The apparent survival rate estimated by season, are shown in Figure 4.8. For sites where one trapping session was missed (NL-PV and L-CC), estimates of apparent survival were obtained for comparable time intervals but do not reflect differences in seasonal survival if present. Survival estimates were variable between study sites and seasons and were bounded by large intervals resulting from model averaging procedures. Overwinter estimates of survival were generally lower than during the summer breeding season in populations occupying linear habitat types, whilst in non-linear wetlands, voles had a higher probability of survival during winter than during summer.

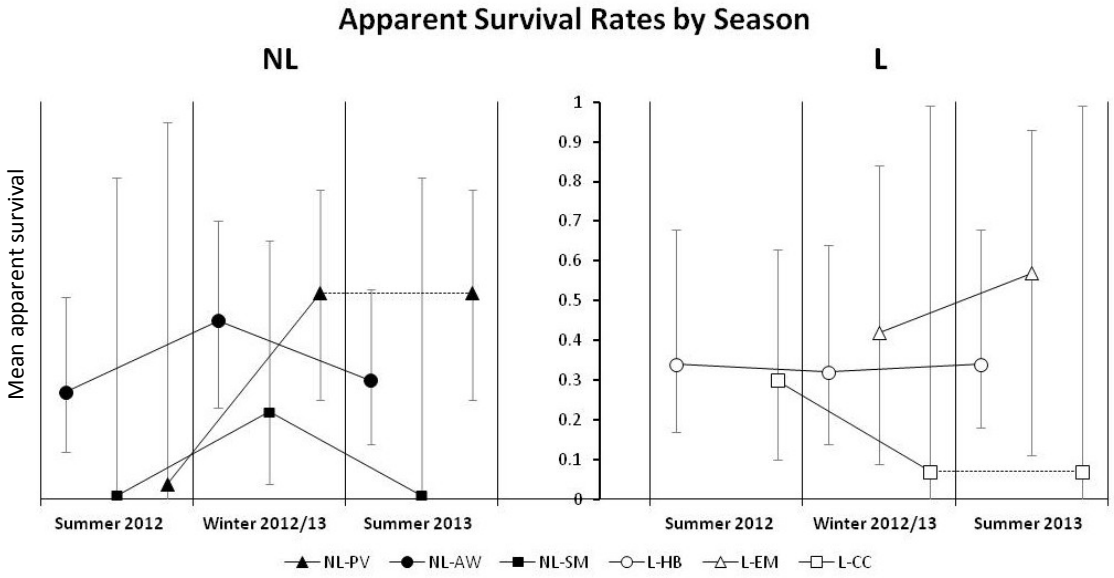


Figure 4.8 *Apparent survival rates for voles occupying non-linear (NL) and linear (L) habitat types, derived by model averaged estimates from Pradel Lambda models. Error bars are 95% CI's. Concurrent estimates for each site are shown by solid lines and dotted lines denote estimates that were obtained for the comparative time interval, but with missing data from autumn 2013.*

Annual survival rates between autumn 2012 and autumn 2013 were higher in the non-linear site (NL-PV, Ø = 0.52, 0.25-0.75, 95% CI's) than in the linear site (L-CC, Ø = 0.07, 0.0 - 0.99, 95% CI's). The persistence rate of water voles during the same annual period for the remaining non-linear site (NL-LW) where survival rates were not obtained was 22%.

Overall recruitment

The overall recruitment of water voles per resident individual between each trapping session, as estimated by f = λ - Ø, for each site is shown in Table 4.11. Estimates of recruitment rates were generally within 0-5 individuals of the number of new recruits observed across sites suggesting the Pradel Lambda model with robust design performed well. The recruitment rate at all but two sites (NL-SM & L-HB) was higher during summer breeding than overwinter and was higher in 2013 than in 2012 for two of the three sites (NL-AW & L-HB) where comparable summer recruitment estimates were obtained. The highest and lowest recruitment rate was observed at site L-EM where nearly three individuals per resident water vole in spring 2013 were recruited into the population by September of the same year and less than 1 individual per ~17 water voles resident in the population in September were recruited overwinter to join the population by spring the following year.

	Summer 2012			**Winter 2012**			**Summer 2012**		
Site	**ḟ**	**r**	**R**	**ḟ**	**r**	**R**	**ḟ**	**r**	**R**
NL-AW	1.05	49	30	0.39	20	19	1.92	83	60
NL-SM	0.81	6	7	0.86	7	7	0.54	5	3
NL-PV	2.02	9	11	-	-	-	0.73	9	14
L-HB	0.60	12	11	1.39	19	19	0.86	25	23
L-EM	-	-	-	0.06	4	4	2.76	39	37
L-CC	0.87	14	16	-	-	-	0.67	16	7

Table 4.11 *Overall recruitment rate per resident water vole (ḟ), for each site per season, derived from model averaged estimates of apparent survival (Ø) and population rate of change (λ). Linear habitat types are shown in shaded boxes. No. of new recruits at time + 1 expected (r) using ḟ rounded up to a whole number and actual number of recruited water voles (R) . '-' denotes seasonal estimates not obtained due to missed trapping occassion.*

4.3.5 The effects of habitat type on parameter estimates

Apparent Survival

The estimates of apparent survival were significantly higher in linear habitat types than in non-linear habitat types (non-linear habitat type coefficient estimate = -0.196 + 0.08, t-value = -2.46, p = 0.032) of which habitat type explained 36% of variation in survival estimates. There was no significant correlation between survival estimates and the perimeter-area ratio of each patch, nor did the survival estimates vary significantly between trapping season, which concurs with the MARK analyses that found no significant differences between the models with and without seasonal variation in survival rates.

Population rate of change

No significant variation in the population rate of change was observed between habitat types, seasons or between the two sampling years, nor was the perimeter area ratio a significant influence on the rate of population change observed across sites.

Overall Recruitment

The perimeter area ratio of the patch did not significantly correlate to the overall recruitment estimates and no significant difference was observed between seasons, years or between habitat types.

4.4 Discussion

Mark-recapture models are a widely used method for estimating demographic parameters such as population size, rate of population change, survival and recruitment in wild populations. This is due to the inherent value that these parameters play in predicting population viability, which is strongly influenced by environmental and individual covariates (Mills, 2007; Franklin, 2001). However, the ability of this method to provide robust estimates of demographic parameters is sensitive to the rate at which marked individuals are re-encountered in a population. If recapture rates are low, parameter estimates lose precision and can become biased (O'Brien *et al.,* 2005). In the context of this study, the majority of recaptures of marked-individuals occurred within trapping sessions, with only 16% and 1% of the 221 permanently marked individuals being recaptured between one or more seasonal trapping sessions respectively. Whilst this data suggests a high turnover of water voles during the summer and overwinter, which is consistent with other studies (Telfer *et al.,* 2003; Aars *et al.,* 2001; Stoddart, 1970), the interval between capture sessions was too long to obtain sufficient recapture rates as the majority of marked individuals had either died or emigrated from the study area. This reduced the capability of programme MARK to provide robust estimates of open population parameters and to detect significant effects of season and year (Franklin, 2001). Furthermore, the number of individuals captured and marked at a number of sites was small, which further reduced the precision of estimates and also prohibited the inclusion of sex and age effects on the estimation of apparent survival.

Study designs are often a trade-off between the effort and cost, and the ability to obtain sufficient samples to generate unbiased, precise estimates of parameters (Bolger *et al.,* 2012). In this study, the decision to not trap more regularly during the summer breeding season was based on both the ethical considerations of trapping pregnant and lactating females and obtaining information from a representative number of populations in order to discern trends that were accountable to the different geometric habitat types. This undoubtedly explains the poor precision of parameter estimates for survival,

population rate of change and recruitment (shown in Figure 4.8 and Table 4.11). For this reason, cautionary conclusions have been drawn when estimates weighted across competing models have been used. These are discussed in context with patterns of population dynamics that characterised the sampled populations during this two year study.

The main aim of this study was to identify if and how habitat geometry influences the demography and dynamics of water vole populations. It was predicted that increased intraspecific competition in linear habitats, particularly amongst breeding females, would result in a higher loss of individuals by mortality or emigration. Contrary to these predictions, apparent survival was significantly higher in linear habitat types than non-linear wetlands, suggesting that individuals in linear habitats are either more philopatric or have increased survivorship when compared to individuals occupying non-linear wetlands. However, a great deal of uncertainty was observed in the estimates of survival obtained from the Pradel lambda models built within programme MARK (Figure 4.8), which are not consistent with the regression model on the influence of habitat type on survival. Thus, it is difficult to conclude whether this difference represents a true difference in survival between linear and non-linear wetlands. Despite this, the monthly survival rate estimates during summer in linear sites, which ranged from 59% to 89% (where monthly survival = $\sqrt[m]{\widehat{\emptyset}}$, and m = number of months seperating trapping sessions) are in concordance with other studies on water voles that have reported survival rates ranging from 63% to 88% in Scottish and lowland watershed systems (Moorhouse *et al.,* 2009; Capreolus Wildlife Consultancy, 2005; Telfer, 2000). In comparison, non-linear sites were characterised by a lower monthly survival rate during summer which ranged between 40% and 78%. Although no comparative data is available on summer survival in non-linear habitat types, the lower survival rate limit is below that reported elsewhere and is thus of concern. These estimates relate to Stodmarsh (NL-SM), which is recognised as a national key site for water voles and where water voles have co-habited with mink for at least 30 years (Carter & Bright, 2003). However, during the period of the study there were numerous accounts of mink within the same areas that fieldwork was being conducted;

making it likely that mink activity is a probable cause for the lower apparent survival at this site. Overwinter survival (spanning ~ 5 months), however, was similar between linear and non-linear wetlands ranging from 32% to 42% and from 22% to 45% respectively and are comparable to estimates obtained from other lowland watershed populations (Moorhouse & Macdonald, 2005). This concurs with other studies that have shown overwinter mortality rates of up to 70% resulting from high predation rates and the death of individuals that had not accumulated sufficient fat reserves before vegetation dies off in the winter (Strachan *et al.,* 2011). Interestingly, patterns in survival were different between habitat types with linear wetland populations exhibiting higher summer and lower overwinter survival, whilst in non-linear wetlands the populations exhibited higher overwinter and lower summer survival. Although these patterns were not significant, given the low precision of these estimates, this could suggest that different factors may contribute to the survivorship and/or emigration of water voles between habitat types.

The overwintered population in linear habitat types was consistently male biased and resulted in a higher proportion of females being observed in the non-linear wetland populations compared with the linear sites in spring (Table 4.5). Two plausible explanations could be invoked to explain this. Firstly, females may suffer a higher mortality rate overwinter compared with males, particularly as the autumn breeding cohorts in linear habitat type consisted of a significantly higher proportion of females compared with non-linear wetland sites (Table 4.5 a). This is shown in the capture data from L-EM where a higher loss of females, compared with males, was observed over the winter of 2012/13 (Table 4.4) and would support the overwinter refuge benefits of non-linear wetland habitats suggested by Carter & Bright (2003). Furthermore, differences in the overwinter survival between male and female water voles has been found in populations occupying wetlands in Russia, where up to two thirds of the autumn female population do not survive to breed by the following spring (Potapov *et al.,* 2004). Secondly, this may reflect the immigration of males into the spring cohorts in linear habitats, as shown by the increased population growth rate (Table 4.10) and number of males observed at site L-HB between autumn 2012 and spring 2013 (Table 4.4). Previous research has shown that

migration in water voles can be male biased with up to 33% of males, compared with 17% of females having dispersed from their natal population by the following spring (Telfer *et al.,* 2003). Furthermore, immigration of water voles has been shown to be influenced by the presence of opposite sex residents but is constrained when populations are at high density (Fisher *et al.,* 2009). Thus, migration by males would be best suited to finding conspecifics in spring, when populations are at their lowest numbers. Unfortunately, it was not possible in this study to estimate differences in male and female survival, due to low capture numbers at a number of sites. However, this data suggests that there may be differences between the survival of different sexes and this warrants further investigation.

Despite differences in the proportion of females overwintered between linear and non-linear habitat types, there was no evidence to suggest that fewer breeding females resulted in a significantly lower population growth rate or overall recruitment in linear sites (Section 4.3.4 and Tables 4.10 and 4.11). However, there was evidence to suggest that the abundance of females that overwintered had a significant influence on the relative abundance of YOTY that were observed in autumn (Table 4.6). The results of the regression model suggest that, in non-linear wetlands, increases in spring female abundance had a predictable positive influence on the autumn abundance of YOTY. However, within the linear sites this relationship was negative, suggesting that increasing female abundance results in a higher loss of YOTY during the summer (Figure 4.4). This supports the prediction that competition in linear habitats results in higher dispersal and/or mortality of YOTY. Higher rates of emigration are often associated with decreasing patch size and the increased proportion of edge influenced habitat due to resource or social competition (Schippers *et al.,* 2009; Kindvall & Petersson, 2000; Gunderson & Andreasson, 1998; Fauske *et al.,* 1997) and can increase an individuals' propensity to emigrate due to behavioural responses to habitat edges (Stamps *et al.,* 1987). Other studies on the dispersal behaviour of water voles have reported high emigration rates in watershed systems, with up to 25% of individuals dispersing from their natal site (Aars *et al.,* 2006; Telfer *et al.,* 2003) and thus the loss of YOTY from linear

sites in this study are in concordance with high dispersal rates in linear systems reported elsewhere.

Despite the potential resource and social limitations on the number of YOTY remaining in linear wetlands during the summer, the probability of being recruited into the breeding population over the duration of study was similar for YOTY captured in both linear and non-linear habitat types and was significantly influenced by weight and sex. Results of the generalised linear model suggest that the probability of recruitment increased with increasing weight and that females were 4.3 times more likely to be recruited than males (Table 4.7). This is in concordance with other studies on water voles (Nazarova, 2013; Strachan *et al.,* 2011; Moorhouse & Macdonald, 2005) and reflects a pattern of female philopatry and male biased dispersal that is particularly characteristic of arvicoline rodents (Le Galliard *et al.,* 2012; Lambin & Krebs, 1991). Interestingly, this pattern appears to be more prevalent in populations occupying linear habitat types, where a significantly higher proportion of the autumn YOTY cohorts were female, when pooled across linear sites and when compared with populations occupying non-linear wetlands (Tables 4.4 and 4.5 b). This may reflect a higher degree of dispersal by YOTY males and/or a higher level of female philopatry in linear sites, which would concur with the higher survival estimates from linear systems (Figure 4.8 and Section 4.3.4). Both are equally plausible and may not be mutually exclusive. For instance, male biased dispersal is considered an inbreeding avoidance strategy which is particularly prevalent in polygynous mating systems (Krebs & Davies, 1993; Gunderson & Andreasson, 1987; Greenwood, 1980), thus increases in female philopatry would increase the rate of dispersal by males. This may be particularly important in linear habitat types given that encounters between related males and females are likely to be higher than in non-linear wetlands, due to constraints in the direction of movement imposed by the linear distribution of resources.

Although this study found no evidence to suggest that female recruitment into the breeding cohort varied between linear and non-linear systems, there was evidence to suggest that the weight at which YOTY became sexually mature in

their year of birth increased with increasing female abundance in non-linear wetlands (Table 4.8). The effects plot presented in Figure 4.5 shows that the weight of sexual maturity for YOTY females in linear patches was relatively unaffected by the relative abundance of breeding females as determined by the consistent pattern in probability slopes of sexual maturity with increasing female abundance. Conversely, in non-linear wetlands, both the direction of the probability slope and its height, relative to linear habitats, suggests that the probability of sexual maturity at most weights decreases with increasing abundance of breeding females when compared with linear sites. This was surprising, as it was predicted that increased competition amongst females in linear wetlands would either suppress sexual maturation or would increase the dispersal of females. One contributing factor may be that there are more opportunities for philopatric females, in linear systems, to become sexually mature in their year of birth, given that the linear sites were comprised of significantly fewer females compared with spring populations occupying non-linear wetlands. It may also be plausible that adult females with established territories along linear margins may be more tolerant of sexually mature YOTY and may incur some inclusive fitness benefit such as cross-lactation or a reduced risk of infanticide by unfamiliar female neighbours (Lambin & Krebs, 1991). This may be more important in linear systems due to the increased competition for resources whereby favouring relatives over strangers may aid in securing saturated resources (Walker *et al.,* 2008). Evidence of territory sharing amongst female kin has been previously reported in water vole populations (Strachan *et al.,* 2011), however, distinguishing between opportunistic sexual maturation and kin selection is beyond the scope of this study and warrants further exploration.

Despite the identification of differing demographic patterns between water vole populations occupying linear and non-linear systems, there was considerable variation in water vole abundance between the study sites (Figures 4.2 and 4.7). These are likely to have accrued due to both extrinsic factors, such as predator occurrence and to demographic stochasticity, which resulted in the variable rates of population change observed during the study period. It is also likely that climatic conditions influenced populations during the study period and

contributed to the significant differences in population structure between years. During 2012, when spring droughts and summer floods were reported across the south east of England, the proportion of YOTY per resident adult was significantly higher than in 2013, suggesting that these conditions resulted in a reduction of breeding adult abundance. Interestingly, a higher proportion of breeding females were also observed during this year, suggesting either higher survival of females or that more female YOTY were able to obtain breeding status due to a reduction in breeding female abundance. The perceptively poor climatic conditions in 2012 however, did not significantly reduce the relative abundance of populations in comparison to 2013, but for the majority of populations, the relative abundance of voles, population growth and overall recruitment were higher in 2013 than in 2012, suggesting that extreme climatic conditions are a limiting factor on the demographic performance of water vole populations.

4.5 Conclusions

This study has shown that water vole populations show a high variation in their demographic rates and are characterised by low spring abundance, high autumn abundance and female biased recruitment. Between habitat types, linear wetlands were characterised by male-biased overwintered populations and female-biased populations in autumn. Increases in the overwintered female abundance in linear wetlands significantly reduced the abundance of young of the year that remained in the population over summer, providing evidence that, as predicted, female territoriality and competition for space in linear wetlands, increases dispersal from linear wetlands. Conversely in non-linear wetlands, overwintered populations were generally female-biased and increased female abundance in spring significantly increased the relative abundance of young of the year in autumn. However, the results suggest that increases in breeding females suppresses maturation of young within their year of birth in non-linear wetlands, which may reflect an inbreeding avoidance mechanism due to the higher proportion of breeding and young of the year males that were in the autumn populations, or to avoid depletion of saturated resources. Different patterns in survival were also observed in linear and non-

linear wetlands, of which the former were characterised by lower overwinter relative to summer survival, whilst non-linear populations showed the opposite, with higher overwinter survival than summer, which may suggest that non-linear wetlands provide refugia from predation during summer.

Within linear wetlands, the small effective population sizes in spring and higher rates of dispersal concur with other studies of water voles in linear systems (Strachan *et al.,* 2011; Aars *et al.,* 2006; Moorhouse, 2004). This highlights the importance of habitat connectivity for populations in linear wetlands that will be reliant on the immigration of individuals to provide both demographic and genetic rescue of local colonies. Increased fragmentation between suitable linear wetland sites could threaten the viability of populations, reducing the chances of successful immigration into sites that could lead to a deterministic reduction in the realised local population growth. This is supported by Rushton *et al. (*2000) in which simulated increases in the fragmentation of habitat patches resulted in population persistence being significantly influenced by reproductive output and survival that would counteract the loss of individuals via dispersal.

It may therefore be of considerable value preserving both linear habitats, that promote the movement of individuals through the processes of resource competition and social exclusion, alongside creating more non-linear wetlands that have the additional benefit of securing more breeding individuals and potentially higher population sizes (in the absence of mink) which will be less influenced by demographic and environmental stochasticity.

Chapter Five: Does habitat type influence sociality in water vole populations?

5.1 Introduction

Social structure exists in most group-living species and is generally associated with securing fitness benefits through competition, co-operation or dominance in the acquisition of biological resources that promote survival and reproductive success (Gardner *et al.,* 2011). Such structure can have a considerable influence on the demographic rates (Johannesen *et al.,* 2000; Lambin & Yoccoz, 1998; Lambin & Krebs, 1991) and genetic structure of populations (Kozakiewicq, 2009; Matocq & Lacey, 2004; Storz, 1999) by affecting the physical arrangement of individuals (Kitchen *et al.,* 2005), limiting the number of reproductive units (Piertney *et al.,* 1999), controlling the extent of dispersal by specific individuals (Liu *et al.,* 2013) and by preventing random mating within populations (Storz *et al.,* 2001; Storz, 1999). Consequently, there is considerable interest in determining the extent of social affiliation within populations to help understand patterns in demography and genetic structure and for identifying mechanisms of adaptive evolutionary change.

Most mammalian systems are characterised by male-biased dispersal and female philopatry which primarily serves to reduce the risk for males breeding with closely related females and allows for females to secure resources required for bearing and rearing young (Le Galliard *et al.,* 2012; Greenwood, 1980). As a result, populations are often structured around females that share recent ancestry and whose spatial arrangement is expected to impact significantly on patterns of social behaviour and population dynamics. For instance, the formation of kin groups that aggregate within a habitat patch is thought to be a prerequisite for kin selection (Chesser, 1991) whereby fitness benefits are accrued from living adjacent to or within the same home range as a close relative (Gardner *et al.,* 2011). Numerous studies of social mammals, including rodents, have documented greater spatial association between female kin than unrelated females (examples within Stockley & Bro-Jørgensen, 2011; Maher, 2009; Kitchen *et al.,* 2005; Lambin & Yoccoz, 1998) suggesting that

there is likely to be some inclusive fitness gained from having kin as close neighbours. Territorial individuals, for example, may react more amicably toward kin than non-kin, which would reduce the costs inherent in home range defence (Kitchen *et al.,* 2005), decrease the frequency and severity of aggressive encounters towards adults and young (Lambin & Krebs, 1991; Ostfield, 1990) and may facilitate co-operative behaviour that could act in reducing the risk of predation with increased attendance at nests (McShea & Maddison, 1984) and increase the growth of offspring through cross lactation (Lambin & Krebs, 1991). As such, kin-affiliation would serve to increase the survival and reproductive success of females and their offspring within the group (Burton & Krebs, 2003; Mappes *et al.,* 1995).

The extent of kin affiliation and social structure however, is expected to vary under different ecological conditions (Chesser *et al.,* 1993). Resource limitations are likely to increase competition amongst relatives and may thus negate the beneficial effects of kin selection (Maher, 2009) resulting in increased rates of natal dispersal that dissolve the formation of temporally stable kin groups (Gunderson & Andreassen, 1998). Conversely, at high population densities, individuals may have increased tolerance of kin, favouring relatives over strangers in securing saturated resources (Walker *et al.,* 2008). Given the high diversity of kinship both within and amongst populations and species, social behaviours and competitive strategies are argued to be 'soft' rather than 'hard' in their selective value (Wade, 1985) allowing the sociality of individuals to adapt in a plastic fashion to different ecological conditions that maximize their survival and reproductive success.

One of the basic predictions of social groupings is that some measure of genetic relatedness among group members exists, and that this reflects the familiarity and behaviour that is observed in spatially aggregated groups. Thus the development of hypervariable molecular markers and theoretical models of gene dynamics can facilitate the study of social behaviour and kin affiliation across a range of taxa (Piertney *et al.,* 1999). Under classical population genetics theory, genetic loci are assumed to be sampled from large, panmictic populations, leading to an expected distribution of alleles and genotypes within

populations: the Hardy-Weinberg principle (Frankham *et al.,* 2011). However, natural populations rarely interbreed randomly and an individual's genetic representation in future generations is generally shaped by socially mediated factors that influence both mate accessibility and mate choice (Kokko *et al.,* 2003). This inevitably prevents complete genetic admixture, resulting in the presence of fine scale genetic structure and patterns in genetic relatedness within natural populations (Balloux *et al.,* 1998). Therefore, molecular data is becoming increasingly valued as a tool for studying social behaviour and demographic processes by measuring both departures from Hardy-Weinberg expectations (Blouin *et al.,* 1996, Queller & Goodnight, 1989) and by using relatedness estimators for determining the probability that two individuals share alleles that are identical by descent.

To date however, there is no consensus on the single best relatedness estimator that outperforms others; rather their performance is largely dependent on the underlying genetic structure of the study population (Anderson & Weir, 2007; Oliehoek *et al.,* 2005; Wang, 2002). Current relatedness estimators are either moment based methods that quantify relatedness in terms of the probability of a pair of alleles being identical by descent (Lynch & Ritland, 1999; Ritland, 1996; Li *et al.,* 1993; Queller & Goodnight, 1989; Lynch, 1988) or utilise a likelihood approach to determine the most likely relationship between a pair of alleles, and hence individuals (Wang, 2007; Millegan, 2003; Goodnight & Queller, 1999). In both instances, when studying natural populations, the estimate of relatedness between two individuals is obtained by using the estimated allele frequency of the sampled population in order to differentiate between alleles that are shared due to identity by descent and those that are shared due to being alike in state (Wang, 2011b; Wang, 2002). For example, when an allele is rare within a population, it is more likely to be shared due to identity by descent rather than simply by chance. As a result, much of the variation that has been observed in the accuracy of relatedness estimators to capture true relatedness has been explained by factors that influence the underlying frequency distribution of alleles within the sampled population (Wang, 2011b; Wang, 2002). These factors include the presence of close inbreeding, differences in social structure and breeding strategies that can

cause fine scale genetic structuring, sample size and sampling error and the degree of polymorphism at a particular molecular marker (Wang, 2011b; Anderson & Weir, 2007; Wagner *et al.,* 2006; Blouin *et al.,* 1996). Despite this, the value of relatedness estimators for determining social behaviours, dispersal and mating strategies is becoming increasingly recognised and applied, particularly for the study of elusive or threatened species for which this information is valuable for their conservation both in the wild and in captivity.

This study uses molecular and demographic data to identify whether there is a relationship between genetic relatedness and the spatial organisation of water vole populations and to identify the extent to which social structure and kin affiliation vary under different ecological conditions. Water voles are semi-aquatic arvicoline rodents that display a wide ecological tolerance within wetland environments, forming discrete colonies along riparian edge habitat and within fen and reedbed habitat (Strachan *et al.,* 2011; Lambin *et al.,* 2004). Like most *arvicolines*, water vole colonies are structured around breeding females that compete for resources by forming territories during the summer breeding season that vary in size and temporal stability depending on habitat quality and population density (Strachan *et al.,* 2011; Moorhouse *et al.,* 2008; Moorhouse & Macdonald, 2008; Moorhouse & Macdonald, 2005). These territories are overlapped by multiple males that are considered to display some degree of social hierarchy, with larger males ranging further and gaining access to a greater number of fertilisable females (Moorhouse & Macdonald, 2005). Both males and females demarcate their ranges with latrines (Stoddart, 1970) and commonly display agonistic behaviour directed towards their conspecifics, which is considered to reflect competitive interactions between individuals, particularly amongst territorial females and female offspring (Forman & Brain, 2006). Whilst it is evident from ecological studies that sociality is present in water vole colonies, where it is likely to play a key role in the demographic and genetic structure of water vole populations, the extent of kin affiliation and the influence of intrinsic and extrinsic factors have not, to date, been investigated.

Understanding the social structure of water vole populations is of particular relevance in the UK, where the species has undergone one of the fastest

declines of any British mammal during the last century (Barretto *et al.,* 1998a; Strachan & Jefferies, 1993). One of the key factors responsible for this decline is the widespread loss of extensive wetland habitat due to drainage and modification of land for commodity production. As a result, water vole populations are now commonly confined to narrow linear remnants of wetland habitat that is patchily distributed and bounded by perceptively hostile land use types (Macdonald & Rushton, 2003; Rushton *et al.,* 2000). The sociality of populations within these habitats is unknown. However, given that non-linear wetlands such as reedbeds and fens are likely to be more representative of the ancestral habitat of water voles, it is expected that both resource competition and edge-mediated dispersal will play a key role in the extent of kin affiliation between individuals and possibly negate behaviour strategies that have evolved to promote survival and reproductive success. Given that a key component for the future conservation of this species in the UK is to establish and enhance wetland habitat to promote population expansion, there is inherent value in understanding how habitat type influences their social structure, which will have important consequences for the dispersal, survival and long-term fitness of populations.

Study aims and objectives

The overall aim of this study was to ascertain how habitat type influences patterns of kinship and population structure. The objectives of this study were to a) determine a suitable estimator for measuring the relatedness between individuals and b) to use these metrics of genetic relatedness to measure the extent of kin tolerance and inbreeding in water vole populations occupying both linear and non-linear wetlands.

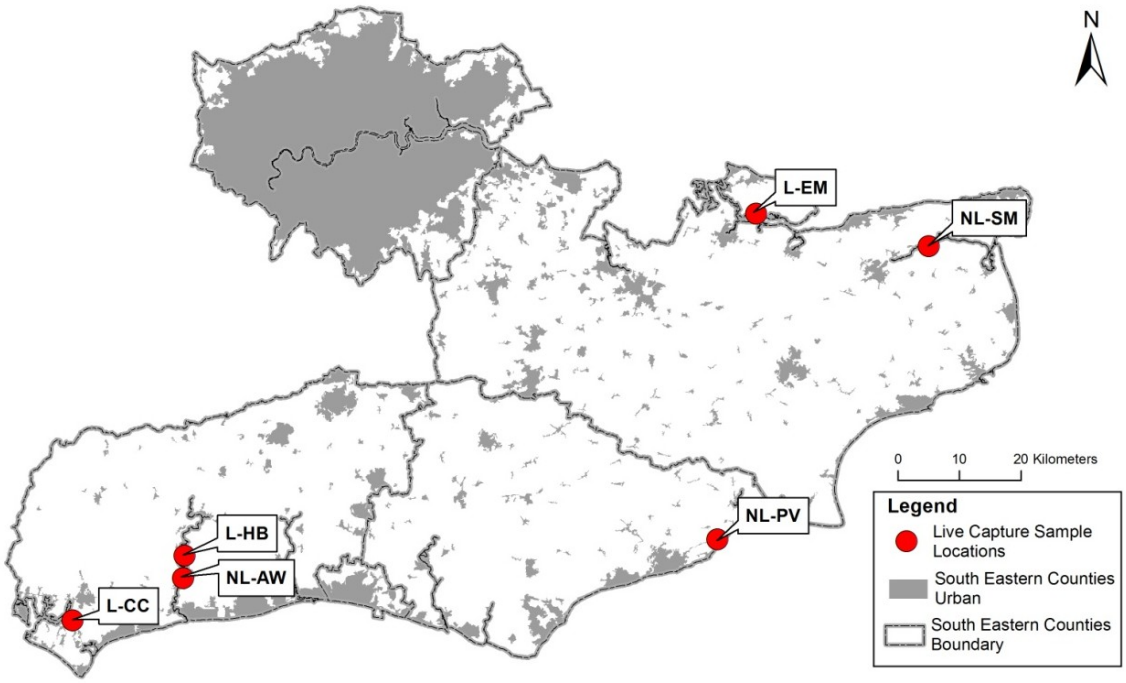
Due to the expected variation in the behavioural response of mammals to edges and resource limitations, two scenarios were investigated. The first tested the hypothesis that increased edge effects and kin competition in linear habitats causes increased rates of dispersal by both male and female water voles resulting in low levels of genetic relatedness and kin tolerance, relative to non-linear habitat types. The second scenario tested the hypothesis that increased edge effects and competition in linear wetlands, causes related individuals to

aggregate within a patch resulting in higher levels of genetic relatedness and spatial affiliation between kin, when compared with non-linear wetlands. Both scenarios were expected to result in differences between mating opportunities and incidence of inbreeding and avoidance of opposite-sex related-pairs was also investigated.

5.2 Methods

5.2.1 Study populations and sampling

This study uses demographic and genetic data collected from six water vole populations occupying wetland habitats in the south eastern counties of England (Figure 5.1). Each sampling site was characterised as either a linear wetland (ditch or canal) or as a non-linear wetland (reedbed, fens with pools and/or channels) depending on the geometric distribution of resources for water voles and included three replicates of the two habitat types. Full descriptions of the study sites are provided in the General Methods, Section 2.2.1.



Contains Ordnance Survey Data
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Figure 5.1 *Location of study populations. Study site codes are provided, where prefix L and NL identify populations occupying linear and non-linear habitat types respectively.*

Water voles were sampled using live capture mark recapture techniques for three consecutive days in spring (March-May) and autumn (September-October) during 2012 and 2013. At each site, a total of 50 baited water vole cage and bedding traps (Greenatyle™, Wildcare) were placed at 20 metre intervals along the bank edges and their location (accurate to 6 metres) was recorded using a Garmin Summit hand held GPS. At two non-linear sites, NL-SM and NL-PV, where banks were not wholly available, traps were additionally placed on floating rafts described in Chapter Two, Section 2.3.2. Traps were checked each morning and evening and each newly captured individual was either injected with a unique PIT tag (Trovan ID162) or was given a semi-permanent fur clip (as illustrated in Gurnell & Flowerdew, 1990) for future identification. The weight, sex, breeding condition and trap location for each individual was recorded and a small hair pluck (>20 hairs with follicles) was taken from the rump of each animal for genetic analyses (described in Section 5.2.2).

Data from a single year of trapping from each population was used (Table 5.1) to investigate the spatial patterns, relatedness and kin tolerance of water voles and, where possible, datasets were selected from autumn trapping sessions, as populations were larger than in spring and comprised of a mixture of breeding females and males, as well as young of the year. These individuals were assumed to represent the resident populations, after the majority of summer dispersal had occurred, and where adult water voles had formed breeding territories and home ranges during the preceding breeding season.

Population	Capture Season	Year
NL-AW	Autumn	2012
NL-SM	Spring and Autumn	2012
NL-PV	Autumn	2013
L-HB	Autumn	2013
L-EM	Autumn	2013
L-CC	Autumn	2012

Table 5.1 *Details of datasets used for each study population in the analyses of spatial and genetic relatedness. Shaded boxes are populations sampled from linear habitat types and non-shaded boxes are populations occupying non-linear habitats.*

5.2.2 DNA extraction and microsatellite analyses

DNA was extracted from each hair pluck samples following digestion (described fully in section 2.4.1), using the QIAGEN DNeasy™ protocol. All samples were genotyped at eight microsatellite loci using pre-designed 5' fluorescently labelled primers and PCR cycling conditions described in Chapter Two, Section 2.4.2. Samples were multiplexed into two groups and separated by capillary electrophoresis on an ABI Prism 3730 Genetic Analyser using a ROX 500 bp size standard (Applied Biosystems). Genotypes were sized using Peak Scanner™ Software v1.0 (Applied Biosystems, 2006) and individuals genotyped for four or more microsatellite loci were used in the analyses.

Genetic data from both spring and autumn sampling occasions within a sampling year were pooled for one of the six sites (NL-SM), where sample sizes from the autumn season were deemed insufficient (n=7) and where the genetic differentiation, measured as F_{ST} (Weir & Cockerham, 1984) between seasons did not differ significantly from zero (data shown in Chapter Six).

5.2.3 Quantifying spatial patterns

To investigate the spatial patterns in genetic relatedness and the extent of kin tolerance, it was first necessary to identify the space use of each live captured individual presumed to be resident within each study population. Where noted, the spatial patterns between breeding adults, males and females were analysed independently due to published differences in the ranging behaviour within each demographic group (Stoddart, 1970). Females were classed as breeding adults and presumed territory holders if they had everted nipples and/or perforated vaginas (Stoddart, 1970). Males were assumed to be breeding residents if they were testes scrotal and/or weighed over 180 g (as described fully in Chapter Two, Section 2.3.1).

Capture locations for individuals were used to determine the spatial arrangement of water voles within each sampling site. Although this method has been found to underestimate or bias the true ranging behaviour of multiple small mammal species (Powell, 2000), its application herein is to determine the spatial pattern and tolerance between individuals by using the location of

capture to indicate areas of activity. Temporary excursions away from home ranges are considered rare for water voles (Stoddart, 1970) and as such their capture position should incorporate at least part of an individual's space use. Where individuals were captured in two or more trap locations, the area in between the furthest two capture points was presumed to be utilised by that individual and was recorded as their minimum home range (MHR). For individuals that were captured once or multiple times in a single location, it was assumed that their capture location was within their normal range of activity. This location was then used in relation to other individuals capture points or boundaries of their MHR to determine the spatial organisation of captured individuals in relation to distance and relatedness. The short duration of trapping prohibited the estimation of MHR for all individuals, who were rarely recaptured in multiple locations, thus comparisons of MHR could not be made across all populations and habitat types.

Geographic distances among all individuals were calculated based on the minimum distance in metres along the bank edge, between the two closest capture locations (Figure 5.2). Where MHR's of captured individuals

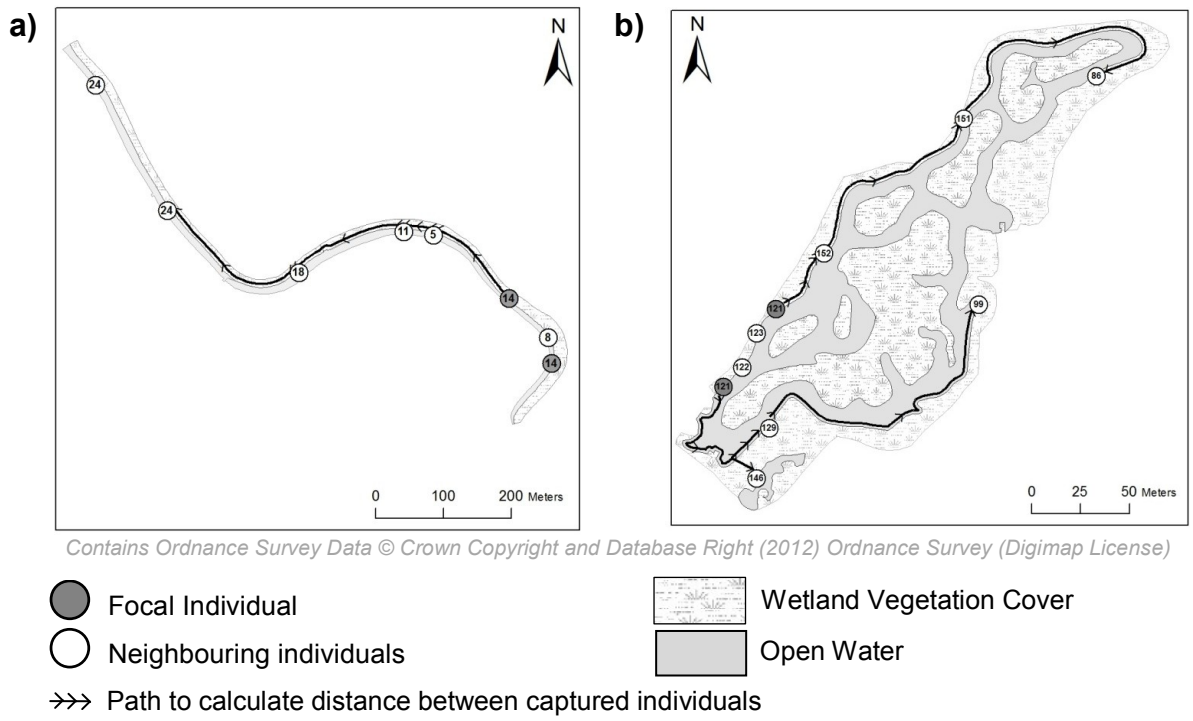

Figure 5.2 *Diagram illustrating the method used to calculate distances between individuals using capture location data in linear (a) and non-linear (b) habitat types.*

overlapped or where individuals were captured within the MHR of another individual, the distance was recorded as '0' metres. Where banks were not present, the minimum Euclidean distance between capture points separated by vegetated wetland habitat were used. Distances across open water were not included in the analyses as, although water forms part of an individual's resource patch, it was considered a secondary resource to vegetation when shaping the spatial pattern of water voles within a habitat. Distances between individuals were calculated from their capture locations using the point distance Spatial Ecology tool for ArcView 10.1 (ESRI Technology).

Nearest neighbours were identified for each individual and for individuals within each demographic group (breeding and non-breeding males and females). Individuals were recorded as neighbours if they were within the closest proximity to each other relative to another individual's capture location within the sampling sites. Neighbours were only considered based on their locations along the bank edge, or trapping line where banks were absent, and were not considered when separated by open water.

Because the distribution of neighbour distances is highly skewed, with a large number of zeroes and a long tail, a generalized linear model with negative binomial error distribution was used to explore whether nearest neighbour distances between breeding adults was influenced by sex, the relative abundance of water voles and the habitat type (linear vs. non-linear). Relative abundance was calculated for each site by determining the number of new breeding individuals, both overall and for males and females separately, captured per 100 trap nights (described fully in Chapter Four, Section 4.1.3). Interactions between sex and relative abundance (all individuals or by sex) and sex and habitat type were included to determine whether the influence of relative abundance and habitat type on spatial behaviour, influenced males and females differently. A global generalized linear model that included all main effects (sex, female = 1, male = 0; habitat type = linear vs. non-linear; relative abundance) and interactions was initially used to derive the dispersion parameter (theta), which captures the skew of the nearest neighbour distance distribution. This estimate was then implemented explicitly in fitting the GLM

models. Due to collinearity between the two relative abundance measures, separate GLMs were used and the significance of each term was assessed by comparing the difference between the deviance values of the model, before and after the term was fitted, to provide the minimum adequate model of significant effects (McCullagh *et al.,* 1998; Crawley, 1993). Estimates of significant coefficients, proportion of the deviance explained by each term and the associated p-value are provided in the results.

5.2.4 Quantifying genetic structure and relatedness

For each population, deviations from Hardy-Weinberg Equilibrium (HWE) were tested using the observed and expected frequencies of genotypes obtained from observed allele frequencies. Significant departures from HWE within each sample were determined from F_{IS} values calculated using a randomization procedure (1000 permutations) implemented in FSTAT version 2.9.3.2 (Goudet, 2001). Positive F_{IS} values showing confidence intervals that excluded zero can be attributed to either inbreeding or unidentified substructure (i.e. the 'Walhund effect', whereby the sample population is comprised of discrete subpopulations that were not interbreeding, resulting in fine-scale substructure) (Wright, 1931).

Genetic relatedness amongst pairs of individuals within each sampled population was used to determine the social structure and kin tolerance of water voles occupying the two different habitat types. Prior to analysing the relatedness between individuals, the performances of different relatedness estimators were examined on individuals of known relatedness from pedigree analysis, to determine the most accurate estimate of relatedness. Two water vole families (mother and two offspring) obtained from a captive breeding site in Devon (Derek Gow Consultancy Ltd) provided individuals with known pedigree. Both families were selected based on their Kent County bloodline, which was the closest available bloodline to the six sampled populations used in this study. For each individual, DNA extraction and microsatellite genotyping was undertaken as described in Section 5.2.2. The pedigree genotypes were pooled with a dataset of 197 water vole genotypes that had been created from hair pluck and tissue samples obtained by the author from eight water vole populations (including the six populations used in this study) since 2010. Full

details of these populations are provided in Chapter Six and allele frequencies for the pedigree inclusive dataset and for each study population are provided in Appendix Five. This dataset was then used to generate allele frequencies in the program Coancestry v1.0.1.2 (Wang, 2011a), from which the coefficient of relatedness and bootstrapped 95% confidence intervals between each pair of individuals of known pedigree (n = 30 pairwise comparisons) was calculated using six of the most widely applied relatedness estimators; TrioML (Wang, 2007), Wang (Wang 2002), LynchLi (Li *et al.,* 1993; Lynch, 1988), LR (Lynch & Ritland, 1999), Q&G (Queller & Goodnight, 1989) and DyadML (Milligan, 2003). The estimator which provided the most accurate (r = 0.5 for parent-offspring and full-siblings) and precise (the smallest confidence intervals) estimate for the known pedigree families was selected for use in estimating relatedness within each of the study populations. Results are presented in Section 5.3.2.

For each population, pairwise estimates of relatedness and 95% confidence intervals were obtained according to Wang's moment-based estimator (Wang, 2002), using allele frequencies that were estimated from the focal population being examined. Relatedness coefficients range from between -1 and 1. Positive values indicate that two individuals are more closely related than by chance, i.e. they share alleles that are identical by descent more frequently than alleles drawn from two individuals randomly drawn from the same population. Negative values indicate that pairs of animals are less related than expected by chance (Kitchen *et al.,* 2005). To determine whether the observed mean relatedness between pairs of water voles within each population was significantly different from populations comprised of unrelated individuals (i.e. having mean r = 0), pair-wise relatedness estimates for each population were resampled with replacement 1000 times, using a resampling macro implemented in Microsoft Excel (Wood, 2003), to determine the 95% confidence intervals around the mean.

Dispersal and natal philopatry

As dispersal is expected to dissolve the degree of relatedness between pairs of individual water voles, differences between breeding male and breeding female relatedness were used to investigate sex biased dispersal and natal philopatry.

Young of the year (YOTY) were not considered in this analysis as it was assumed that these individuals were of pre-dispersal age and thus their philopatry to their natal site would not represent true site fidelity. To test whether there were significant differences between the average pairwise relatedness of breeding male and breeding female groups 1000 bootstrap samples were generated from each group separately. The average relatedness and differences between groups were then calculated from these bootstrap samples to obtain a sample distribution of the difference in the average relatedness between groups. The significance of the differences between groups was determined by comparing the observed difference with the bootstrapped distribution. All analyses were undertaken using the Coancestry package V1.0.1.2 (Wang, 2011a).

The degree of natal philopatry was further investigated by examining the proportion of each population that was estimated to be first or second order relatives. These comprised of pairs of individuals that were assigned relatedness estimates (r) greater than 0.45 for first order kin and between 0.22 and 0.45 for second order kin. Dyadic relatedness estimates can have a high variance, particularly for more distant categories such as third order relatives (Olihoek *et al.,* 2006; Blouin *et al.,* 1996), thus only first and second order relatives (referred to herein as close relatives) were considered in the analyses and a 10% variation around the relatedness coefficients for first order kin (r = 0.5) and second order kin (r = 0.25) were applied (Piertney *et al.,* 1999). To determine whether breeding males or breeding females were more or less philopatric to their natal site, a binomial test of expected equal proportions was used to compare the proportion of closely related dyads that were breeding males and those that were breeding females.

To investigate whether the proportion of closely related adults and of females (males were not considered as individuals estimated to be close relatives were not observed across all sites) that reached sexual maturity within their natal site was different between habitat types and whether this was influenced by resource competition, GLMs fitted with binomial error were used. The proportion of closely related adults or breeding females were included as a

dependent variable and explanatory variables included habitat type (linear vs. non-linear), and the relative abundance of voles. An interaction between abundance and habitat type was included to explore whether the influence of abundance on adult philopatry varied between habitat types.

5.2.5 Quantifying spatial patterns in genetic relatedness

To investigate the spatial genetic structure of water voles within each population, matrices of the geographic distance between each pair of water voles, based on their MHR boundaries or capture location (if only captured in one trap) and their degree of relatedness, were generated and tested for significant correlation using a nonparametric Mantel test (Mantel & Valand, 1970). This test accounts for the unavoidable lack of independence between dyadic data (i.e. many dyads shared one individual) and generates a correlation coefficient R_0 for the original matrices. The rows and columns within the relatedness matrix were then subjected to 999 random permutations to generate a distribution of randomised correlation coefficients. If the original matrices were correlated, the disruption caused by the permutations should reduce the correlation coefficient and thus if fewer than 5% of the permuted coefficients exceeded the original R_0, the spatial patterns in genetic relatedness were deemed significant. Mantel tests were carried out on all pairs of individuals within Microsoft Excel 2007 using PopTools version 3.2.5 (Hood, 2011).

Spatial patterns in genetic relatedness were further investigated by calculating the mean relatedness between neighbouring and non-neighbouring pairs of breeding individuals of the same sex. Sampling variance around the means was calculated by resampling, with replacement 1000 times, to provide 95% confidence intervals. To test the null hypothesis that there is no difference in the degree of relatedness between neighbouring and non-neighbouring pairs a probability distribution of the mean difference in relatedness between the simulated values was compared with the observed difference using a resampling for differences between groups macro implemented in Microsoft Excel (Wood, 2003).

Kin tolerance and inbreeding avoidance

Kin tolerance was first investigated by calculating the proportion of neighbours that were estimated to be close relatives (where $r \geq 0.22$) for same sex and opposite sex pairs. Differences in the tolerance of sexually mature males and females and their same sex neighbouring kin was investigated by comparing between sexes using a Chi-square test. A GLM with a binomial error distribution was used to investigate whether tolerance of neighbouring kin of sexual maturity was influenced by relative abundance and/or by habitat type. Analyses was conducted for breeding females only, as these were most frequently observed, whereby the proportion of sexually mature neighbours that were kin was the dependent variable and habitat type, relative abundance (of voles and of breeding females), and an interaction between habitat type and relative abundance were explanatory variables.

To determine whether sexually mature opposite sex pairs of close relatives avoided each other, spatially, to prevent inbreeding, linear regressions between geographic distance and breeding pair relatedness were first performed for each population using R. The relative distance between 1st and 2nd order kin and unrelated pairs of individuals was also calculated by dividing the distance between pairs by the furthest distance observed between two individuals within each study site. Significant differences between the spatial proximity of kin and unrelated individuals were compared within each site by resampling with replacement 1000 times to provide 95% confidence intervals around the mean. Significant differences were determined by the observation of non-overlapping intervals.

5.3 Results

5.3.1 Social composition and spatial patterns

A total of 152 individual water voles were captured across the six study sites, of which 89 were sampled from linear habitat types and 63 were sampled from non-linear habitat types. The relative abundance of water voles was variable amongst the study sites and within each habitat type, ranging from three to 11 and three to 12 individuals per 100 trap nights in linear and non-linear sites respectively. Breeding adults constituted between 48% and 70% of the trapped populations (Table 5.2).

	NL-AW	NL-SM	NL-PV	L-HB	L-EM	L-CC
Total Captured	33 (32)	14	16	29	40 (34)	20
Relative Abundance	12	3	4	7	11	3
Prop Br. Females	0.30	0.36	0.19	0.41	0.50	0.30
Prop Br. Males	0.18	0.21	0.37	0.21	0.20	0.25
Prop YOTY Females	0.18	0.29	0.19	0.10	0.25	0.25
Prop YOTY Males	0.33	0.14	0.25	0.28	0.05	0.20

Table 5.2 *Demographics summary of study populations with total captured, relative abundance per 100 trap nights of effort, proportion (Prop) of breeding (Br) females and males, proportion of non-breeding young of the year (YOTY) by sex. Populations from linear habitat types are shaded grey.*

On average the distances between neighbouring breeding water voles amongst the six study sites were highly variable ranging from between 4.4 (± 9.7 SD) and 13.2 (± 17.3 SD) metres with the majority of nearest neighbours being located either within the range of another individual or were captured within the same trap, as determined by the zero median values when considering all individuals (Table 5.3).

Nearest neighbour distances between breeding adults were significantly influenced by sex, with males being separated by significantly larger distances than females ($GLM_{108.48, 88}$, breeding male coefficient = 0.900, $p<0.001$), which explained 6% of the total model deviance. The spacing between males and

		NL-AW	NL-SM	NL-PV	L-HB	L-EM	L-CC
All	Mean	4.4 (± 9.7)	11.3 (± 20.9)	5 (± 11)	13.2 (± 17.3)	11.9 (± 15.1)	9.5 (± 20.9)
	Median	0	0	0	0	0	0
	Range	0 - 32.2	0 - 57	0 - 35	0 - 64.1	0 - 58.3	0 - 79.6
Br. F	Mean	24.7 (± 34.8)	49.4 (± 85.8)	95.1 (± 138.4)	48.8 (± 60.9)	27.4 (± 29.5)	23.8 (± 26.3)
	Median	15.3	22.8	15.2	24.4	19.0	18.4
	Range	0 - 91.4	0 - 201.5	15.2 - 255	0 - 161.7	0 - 119.2	0 - 69.5
Br. M	Mean	67 (± 35.6)		25.3 (± 69.5)	126.6 (± 83.2)	83.5 (± 40.5)	59 (± 57.6)
	Median	65.9		36.3	99.4	89.4	79.6
	Range	35 - 132.6		0 - 180.7	66.5 - 287	28.1 - 143.5	0 - 131.5

Table 5.3 *Details of nearest neighbour distances in metres along bank edge for each captured individual and between breeding females and breeding males. (± = mean standard deviation) for each population.*

females was not significantly influenced by habitat type or relative abundance, as determined by the non-significance of interactions between sex and habitat type and sex and relative abundance (both overall and by sex).

5.3.2 Microsatellite DNA analyses

The eight microsatellite loci were highly polymorphic, with between three and 11 alleles detected per microsatellite locus across the study populations (Table 5.4). None of the loci showed significant deviation from Hardy-Weinberg equilibrium, as determined by fixation index (F_{IS}) and after correcting for multiple tests using a Bonferroni correction procedure. Populations L-HB and L-EM showed significant heterozygote deficiencies across all loci when considered together, suggesting that the admixture of individuals within these populations was not random, due to either the Walhund effect, (i.e. the 'site' actually comprised of multiple populations), or that individuals were breeding amongst close relatives.

Locus	NL-AW					NL-SM					NL-PV				
	n	K	H_E	H_O	F_{IS}	n	K	H_E	H_O	F_{IS}	n	K	H_E	H_O	F_{IS}
AV7	32	6	0.82	0.88	-0.07	12	3	0.65	0.50	0.24	16	3	0.62	0.25	0.60
AV8	32	9	0.78	0.63	0.21	11	5	0.77	0.73	0.06	11	7	0.82	0.46	0.46
AV9	32	8	0.80	0.81	-0.01	13	4	0.64	0.69	-0.09	15	5	0.69	0.47	0.33
AV11	32	9	0.84	0.97	-0.16	14	11	0.90	0.86	0.05	14	4	0.61	0.86	-0.42
AV12	30	8	0.81	0.60	0.27	14	7	0.84	0.79	0.06	15	3	0.35	0.13	0.63
AV13	29	6	0.80	0.79	0.01	14	6	0.76	0.71	0.06	15	5	0.78	0.87	-0.12
AV14	30	6	0.64	0.57	0.13	14	4	0.47	0.57	-0.23	15	5	0.75	0.73	0.02
AV15	31	5	0.69	0.58	0.17	13	3	0.38	0.31	0.21	14	5	0.80	0.86	-0.08
Mean	32	7	0.78	0.73	0.06	14	5	0.68	0.64	0.05	16	5	0.68	0.58	0.15
Locus	**L-HB**					**L-EM**					**L-CC**				
	n	K	H_E	H_O	F_{IS}	n	K	H_E	H_O	F_{IS}	n	K	H_E	H_O	F_{IS}
AV7	25	7	0.65	0.76	-0.17	33	7	0.72	0.52	0.29	20	5	0.62	0.65	-0.04
AV8	28	6	0.58	0.50	0.14	29	8	0.83	0.83	0.01	20	3	0.38	0.35	0.07
AV9	28	6	0.64	0.61	0.05	32	8	0.79	0.84	-0.06	20	8	0.78	0.60	0.24
AV11	20	9	0.80	0.75	0.07	29	7	0.59	0.52	0.13	16	6	0.80	0.69	0.15
AV12	22	9	0.80	0.59	0.27	33	7	0.85	0.70	0.18	19	7	0.77	0.79	-0.02
AV13	28	5	0.67	0.57	0.14	34	5	0.65	0.71	-0.09	17	4	0.51	0.47	0.08
AV14	29	6	0.67	0.52	0.23	33	6	0.76	0.61	0.20	19	7	0.68	0.68	-0.08
AV15	29	4	0.54	0.34	0.37	34	5	0.68	0.44	0.36	19	9	0.85	0.74	0.14
Mean	29	6	**0.67**	**0.58**	**0.14**	34	7	**0.64**	**0.74**	**0.13**	20	6	0.65	0.62	0.07

Table 5.4 *Characteristics of the eight microsatellite markers, including number of individuals scored per locus (n), number of alleles (K), expected heterozygosity (H_E), observed heterozygosity (H_o) and fixation index (F_{IS}) per locus and overall. Bold text denotes where F_{IS} showed significant departures from zero, calculated by bootstrapping for 95% confidence intervals.*

5.3.3 Relatedness estimates for pedigree data

A comparison of the accuracy of eight relatedness estimators at determining known kin relationships in two water vole families is shown in Figure 5.3. There was a high level of uncertainty across all eight estimators, in identifying the maximum likelihood value of parent offspring and full sibling relationships (r = 0.5) within both families with known relatedness. The estimator of Lynch and Ritland (LR) was the least accurate at determining the known relationships

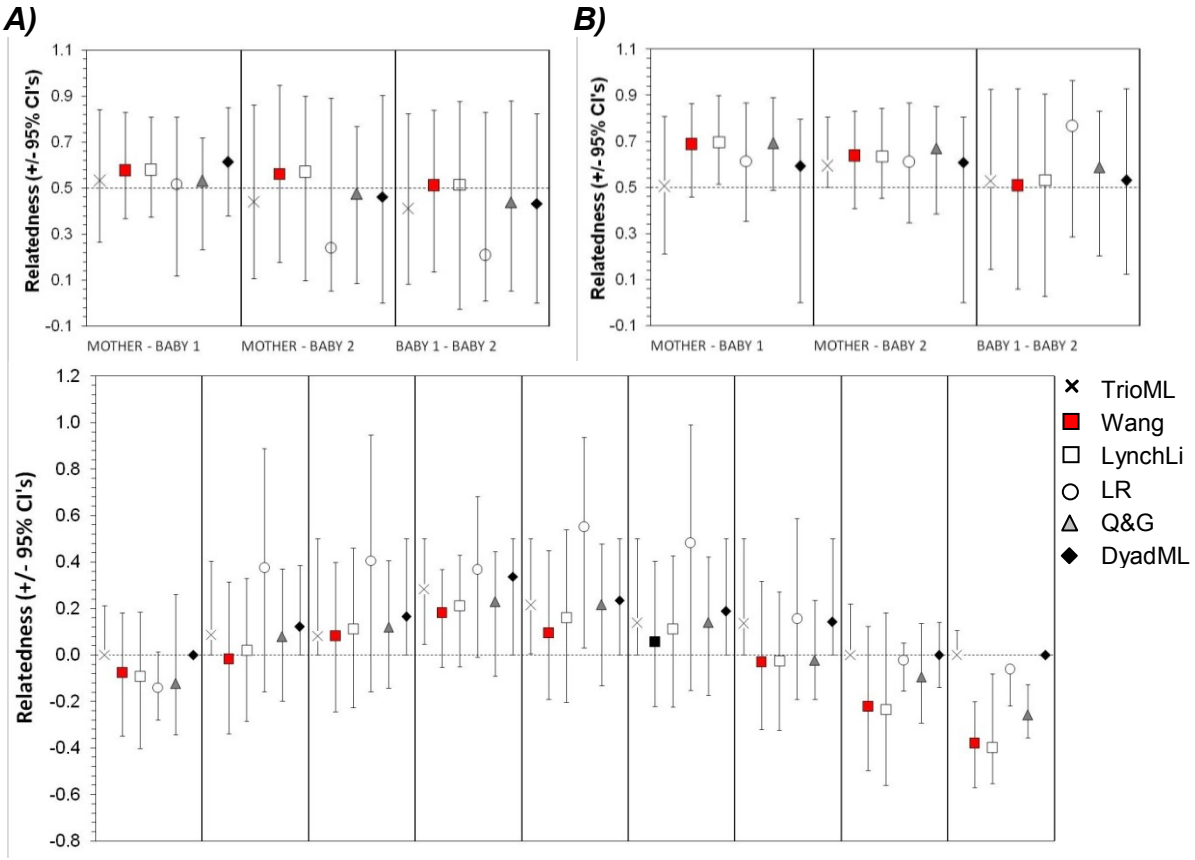

Figure 5.3 *Plot of eight relatedness coefficients obtained for two families of known pedigree (A & B). The respective coefficients for known unrelated pairs between families is shown in the bottom figure. Error bars represent 95% confidence intervals as determined by bootstrapping over loci (1000 times).*

between related and unrelated individuals, whilst both Wang (2002) and LynchLi (Li *et al.,* 1993 & Lynch, 1988) estimators performed best, deriving relatedness coefficients that were relatively accurate for both within and between families. Wang's moment-based estimator showed the greatest precision (as derived from the 95% confidence intervals) and the least bias when estimating relatedness between unrelated pairs.

5.3.4 Relatedness and philopatry

The mean pairwise relatedness estimates for all pairs and for breeding male and breeding female cohorts, considered separately within each study population, is shown in Figure 5.4. Overall, all sites showed low levels of relatedness with mean pairwise estimates ranging from -0.13 and -0.05 in populations occupying linear habitat types and between -0.14 and -0.08 in populations occupying non-linear habitat types. The mean pairwise relatedness

estimates for all dyads and for all pairs of breeding females and breeding males were significantly lower than zero, as determined by resampling 1000 times with replacement.

The mean relatedness between breeding female pairs was higher than the mean relatedness between sexually receptive males in four of the six populations. This difference was significant, as determined by bootstrapping between groups, at site L-HB where the pairwise relatedness between breeding females (r = -0.1) exceeded that observed between breeding males (r = -0.33) and breeding male and female pairs (r = -0.335) (Table 5.5). Breeding male relatedness (range: -0.331 to -0.071) was comparable to the relatedness observed between breeding male and female pairs (range: -0.335 to -0.060) at most sites, apart from non-linear sites NL-AW and NL-PV where breeding male relatedness was lower than that observed between breeding female and male pairs. These differences, however, were not significant at the 95% confidence level. Breeding males at non-linear site NL-AW, however, were significantly less related (r = -0.215) than male and female breeding pairs (r = 0.060).

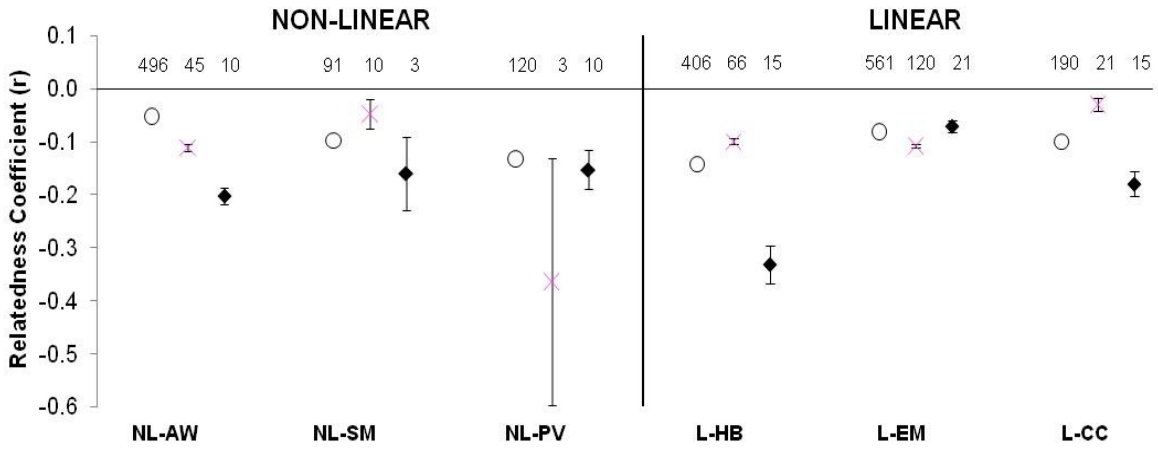

Figure 5.4 *Mean relatedness between all dyadic pairs (outlined circles) and for breeding females (pink cross) and breeding male (black diamond) dyads per population within linear and non-linear habitat types. Values above error bars refer to the number of dyads per group. Error bars are standard error of the mean calculated by resampling 1000 times with replacement.*

Site	$N_{(1)}$	Mean $r_{(1)}$	$N_{(2)}$	Mean $r_{(2)}$	Difference between mean r	95% CI's Lower	95% CI's Upper
$BF_{(1)}$:$BM_{(2)}$							
NL-AW	45	-0.111	8	-0.215	0.104	-0.217	0.186
NL-SM	10	-0.047	3	-0.160	0.112	-0.560	0.369
NL-PV	3	-0.364	15	-0.152	-0.212	-0.457	0.546
L-HB	**66**	**-0.099**	**15**	**-0.331**	**0.232**	**-0.221**	**0.222**
L-EM	120	-0.108	21	-0.071	-0.037	-0.153	0.129
L-CC	21	-0.030	15	-0.180	0.150	-0.207	0.210
$BF_{(1)}$:$BM/F_{(2)}$							
NL-AW	45	-0.111	50	-0.060	-0.050	-0.098	0.096
NL-SM	10	-0.047	15	-0.157	-0.099	-0.216	0.229
NL-PV	3	-0.364	18	-0.216	-0.148	-0.512	0.752
L-HB	**66**	**-0.099**	**72**	**-0.335**	**0.235**	**-0.143**	**0.154**
L-EM	120	-0.108	112	-0.082	-0.026	-0.080	0.080
L-CC	21	-0.030	42	-0.079	0.049	-0.151	0.151
$BM_{(1)}$:$BM/F_{(2)}$							
NL-AW	**8**	**-0.215**	**49**	**-0.060**	**-0.155**	**-0.154**	**0.157**
NL-SM	3	-0.160	15	-0.157	-0.013	-0.297	0.243
NL-PV	15	-0.152	17	-0.216	0.064	-0.321	0.300
L-HB	15	-0.331	72	-0.335	0.003	-0.280	0.269
L-EM	21	-0.071	112	-0.082	0.011	-0.132	0.131
L-CC	15	-0.180	40	-0.079	-0.101	-0.173	0.199

Table 5.5 *Number (N) and mean relatedness (r) between breeding female (BF), breeding male (BM) and breeding male and female (BM/F) dyads, difference between mean relatedness between groups and 95% confidence intervals based on 1000 bootstraps. Subscripts (1) and (2) relates the sample size (N) and mean r estimates to the different breeding cohorts shown in column one. Significant differences are shown in bold for mean r differences that fall outside of the 95% CI's.*

Between 12.1% and 20% of individuals amongst the study populations were estimated as 1st (r = 0.22-0.449) or 2nd (r = $\geq$0.45) order relatives. Of these, non-mating relatives (including full and half sibs, parent-offspring) occurred most frequently in all populations, as determined by relatedness estimates (Figure 5.5). A significantly higher proportion of breeding adults estimated to be close relatives were observed in linear habitat types ($GLM_{2.6632, 4}$, non-linear habitat type coefficient = -1.1705, p = 0.000) than within populations occupying

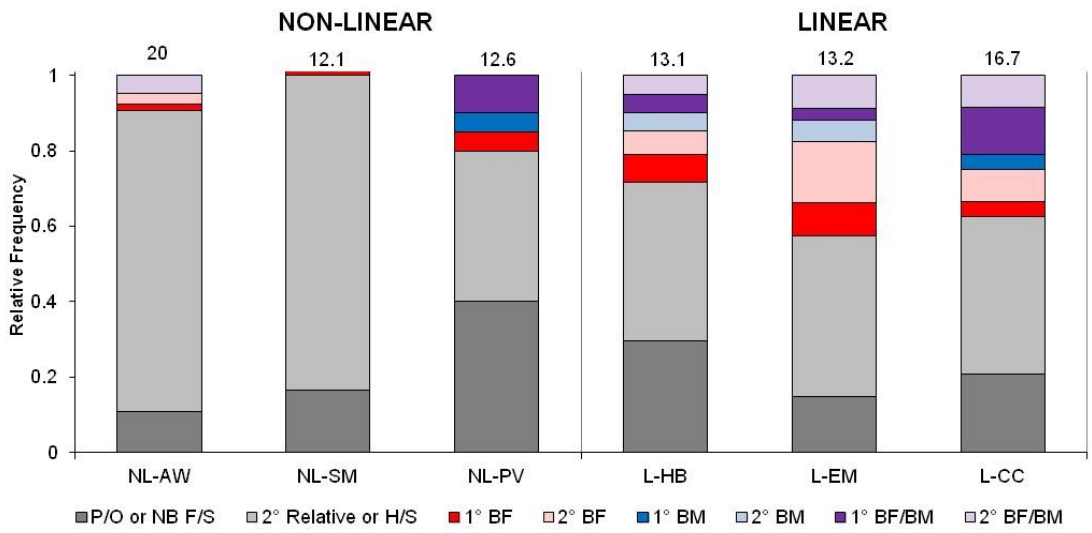

Figure 5.5 *Proportion of 1st (1°) and 2nd (2°) order relatives, by breeding cohorts: BF = Breeding Female, BM = Breeding Male, BF/BM = Breeding Male and Female pairs and by non-breeding cohorts: P/O = Parent-Offspring, F/S = Full Siblings, H/S – Half Siblings. Total proportion of 1st and 2nd order relationships between all dyads in population is shown above each bar.*

non-linear habitat types, explaining 86% of the model deviance. The proportion of breeding females inferred as close relatives were also significantly higher in linear habitat types ($GLM_{3.0454,\ 4}$, non-linear habitat type coefficient = -1.1683, p = 0.003), where habitat type explained 74% of the model deviance. The proportion of adults and of breeding females inferred as close relatives were not influenced by relative abundance (p = 0.493, p = 0.78 respectively), nor was there evidence that the influence of relative abundance varied by habitat type.

When compared to breeding males, a higher proportion of breeding female close relatives (1st & 2nd order kin) were estimated in linear sites and was significantly higher than closely related breeding males at site L-EM (binomial test ratio = 0.5, p = 0.007). Estimates of parent-offspring and full-sib breeding relationships between breeding males were the least observed, occurring in just two of the six study populations. Mating pairs of male and female 1st or 2nd order relatives were estimated in all but one site (NL-SM) and constituted between eight and 16 dyads in linear habitat types and between three and four dyads in populations occupying non-linear habitat types.

5.3.5 Spatial patterns and kin tolerance

Geographic distance and relatedness

The results of the Mantel tests for correlations between genetic relatedness and geographic distance are shown in Table 5.6. There was a negative, but non-significant correlation between genetic relatedness between all pairs of individuals and increasing geographic distance between the locations in which they were trapped in both linear (R_0 range: -0.41 to -0.11) and non-linear sites (R_0 range: -0.99 to -0.07). Similarly, the genetic relatedness between both female pairs and male pairs showed decreasing trends with increasing distance for all individuals, however, none of the observed relationships between genetic relatedness and geographic distance were significant (see Appendix Six for scatterplots of genetic relatedness and geographic distance for all sites and cohorts).

	NL-AW			**NL-SM**			**NL-PV**		
	N	***R_0***	***P***	***N***	***R_0***	***P***	***N***	***R_0***	***P***
All	496	-0.28	0.99	91	-0.14	0.82	120	-0.49	0.99
F	120	-0.35	0.99	36	-0.13	0.70	15	-0.83	0.96
BF	45	-0.07	0.65	10	-0.81	0.68	3	-	-
M	120	-0.40	0.99	10	-0.41	0.85	45	-0.05	0.59
BM	10	-0.99	0.99	3	-	-	10	-0.16	0.66
	L-HB			**L-EM**			**L-CC**		
	N	***R_0***	***P***	***N***	***R_0***	***P***	***N***	***R_0***	***P***
All	406	-0.28	0.99	561	-0.06	0.88	190	-0.31	0.99
F	105	-0.11	0.77	300	-0.07	0.86	55	-0.36	0.97
BF	66	-0.25	0.89	120	-0.11	0.85	21	-0.36	0.76
M	91	-0.41	0.99	36	-0.10	0.66	36	-0.43	0.93
BM	15	-0.33	0.68	21	-0.08	0.59	15	-0.83	0.90

Table 5.6 *Spatial correlation between relatedness coefficients and geographic distance between dyads for All = all pairs, F = all females, BF = breeding females, M = all males, BF = breeding males, showing N = sample size, R_0 = Mantel's correlation coefficient (999 permutations) and P = proportion of permuted R_0 values that exceeded Mantels correlation coefficient where $p < 0.05$ indicates a significant correlation. Symbol '-' denotes groups where sample size was too small (<5) to carry out a significance test.*

The mean estimated relatedness between breeding female neighbours and between breeding females and non-breeding female neighbours was higher than between non-neighbouring pairs across all study sites (Figure 5.6). These differences were not significant, as determined by resampling between the neighbour and non-neighbouring pairs. The estimated relatedness between breeding male neighbours was generally lower than between breeding female neighbours, and when compared with non-neighbouring breeding males was higher, but not significantly different, in four of the study sites. The mean relatedness between breeding males and non-breeding male neighbours was lower than non-neighbours at two sites; however, no significant differences between neighbours and non-neighbour relatedness were observed at any site.

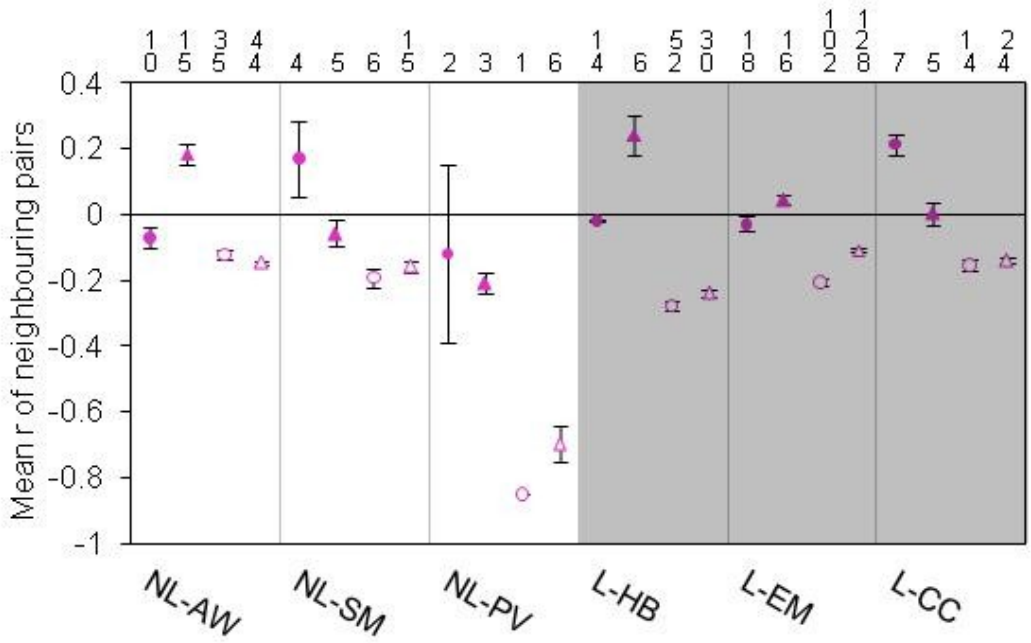

Figure 5.6 *Mean relatedness estimates (r) for female neighbours (solid markers) and non-neighbours (outlined markers). Mean relatedness for breeding female pairs are shown as circles and mean relatedness for breeding, non-breeding female pairs are shown as triangles. Sample sizes (number of dyadic pairs) for each relationship are shown at the top of figure for each site and linear habitats are shown in grey.*

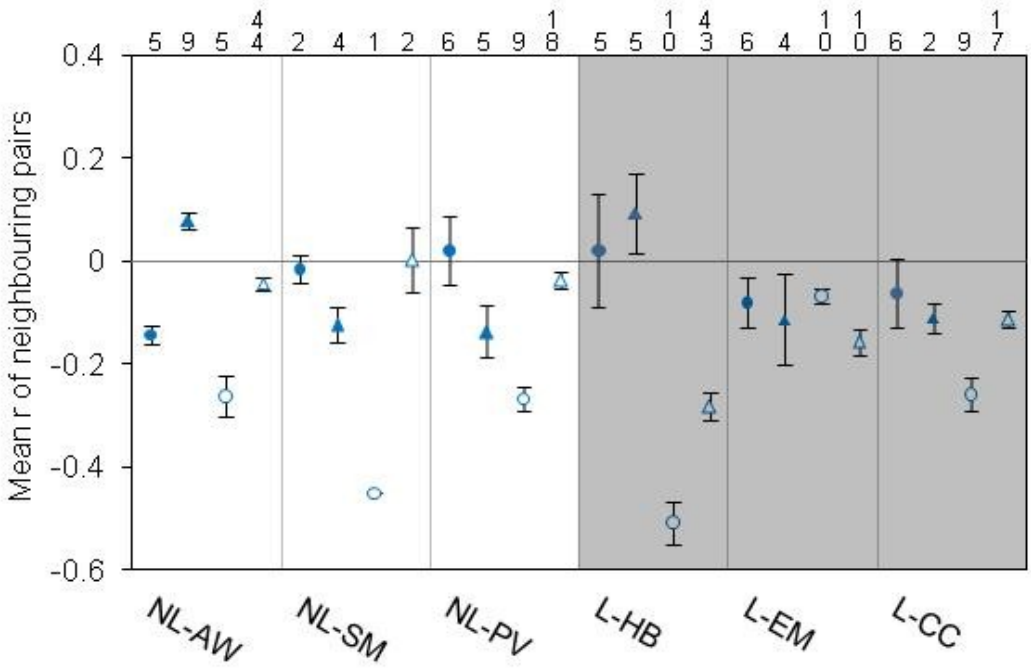

Figure 5.7 *Mean relatedness estimates (r) for male neighbours (solid markers) and non-neighbours (outlined markers). Mean relatedness for breeding male pairs are shown as circles and mean relatedness for breeding, non-breeding male pairs are shown as triangles. Sample sizes (number of dyadic pairs) for each relationship are shown at the top of figure for each site and linear habitats are shown in grey.*

Kin tolerance

Kin tolerance of breeding water voles was first investigated by determining the proportion of individuals that neighboured a sexually mature close relative of the same sex (Figure 5.8). Between 10% and 50% of breeding female neighbours across the six study sites were estimated to be close relatives. In comparison, neighbouring pairs of breeding males that were estimated as close relatives were only observed in linear habitat types where they constituted between 16% and 60% of adult male neighbours. The differences between the proportion of neighbours estimated to be close relatives was only significant at site L-HB, where a higher proportion of closely related breeding male neighbours were observed (χ^2_1, n=19 = 3.9706, p = 0.046).

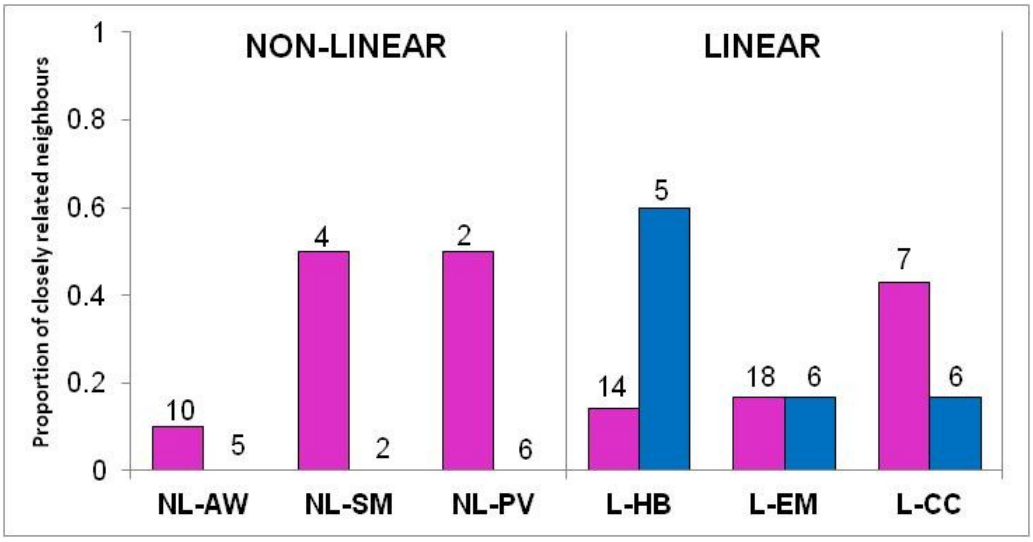

Figure 5.8 *Proportion of breeding water vole neighbours that were estimated to be close relatives (1st or 2nd order kin) for male (blue bars) and females (pink bars) separately. Numbers above bars denote the total number of genotyped neighbouring pairs.*

The relative abundance of water voles significantly reduced the proportion of breeding female neighbours that were closely related ($GLM_{1.36, 4}$, coefficient = -0.198, p = 0.045), explaining 76% of the model deviance (Figure 5.9). The interaction between relative abundance and habitat type was not significant (p = 0.622), suggesting that the effect of relative abundance on the tolerance of neighbouring kin is consistent between habitat types. The relative abundance of breeding females, however, did not influence the tolerance of neighbouring female kin.

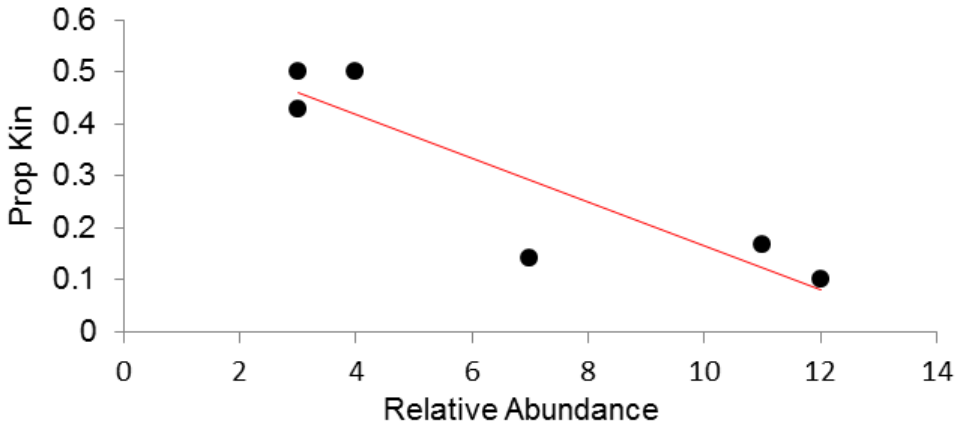

Figure 5.9 *Plot showing relationship between the proportion of breeding female neighbours estimated to be close relatives against the relative abundance of water voles per 100 trap nights. Red line shows linear relationship with $R^2 = 0.8115$.*

5.3.6 Inbreeding avoidance

Closely related male and female pairs that were in breeding condition were observed in five of the six study sites where they constituted between 6% and 22% of all opposite-sex adult pair relationships. Between 20% and 67% and between 50% and 75% of breeding females observed in non-linear and linear sites respectively, were estimated to be closely related to one or more breeding male within the same population. Out of all neighbouring opposite sex adults, estimated close relatives were observed in two linear sites and one non-linear site where they comprised of between 18% and 50% of neighbours (Figure 5.10). As male ranges overlap multiple females, patterns in genetic relatedness and distance were investigated using a linear regression.

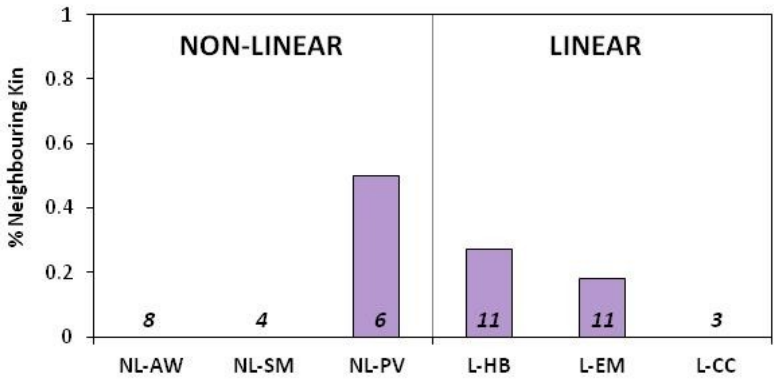

Figure 5.10 *Proportion of adult male female neighbours estimated to be closely related (1° & 2° kin). Numbers within bars are number of genotyped neighbouring pairs.*

Significant negative correlations between sexually mature male and female pairwise relatedness and geographic distance were observed at one non-linear study site NL-PV ($F(1,16) = 7.881$, $p = 0.026$) and one linear site L-HB ($F(1,70) = 10.38$, $p = 0.002$) (Figure 5.11). Comparisons between the relative distance between adult opposite sex pairs estimated as close relatives and non-relatives revealed that at all linear sites close relatives were in closer proximity than those estimated as non-relatives, however, this difference was not significant, as determined from resampling procedures (Figure 5.12).

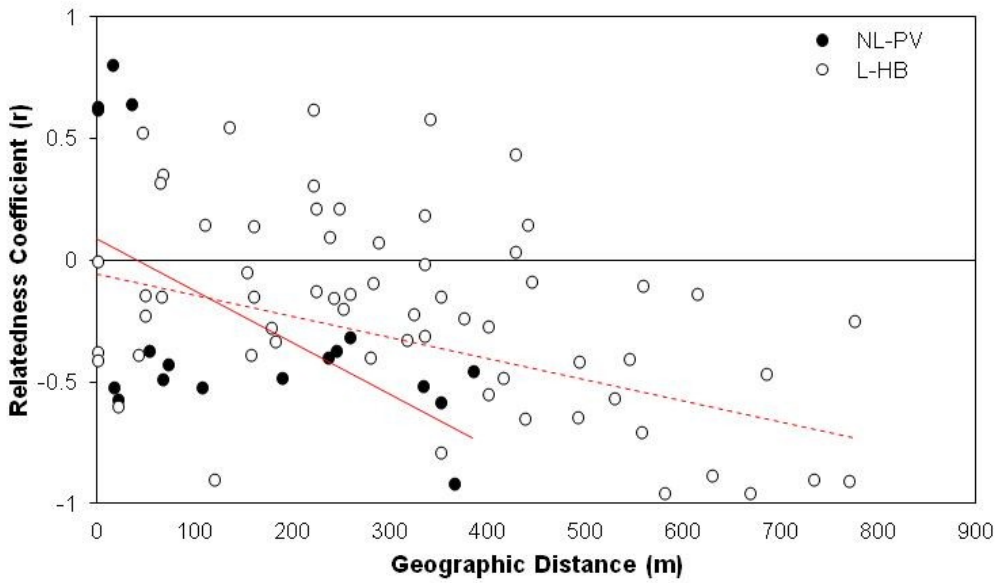

Figure 5.11 *Scatterplot of breeding male and female pairwise relatedness and geographic distance for sites NL-PV and L-HB. Red lines show linear regression for NL-PV (solid), R^2 = 0.34 and L-HB (dashed), R^2 = 0.13.*

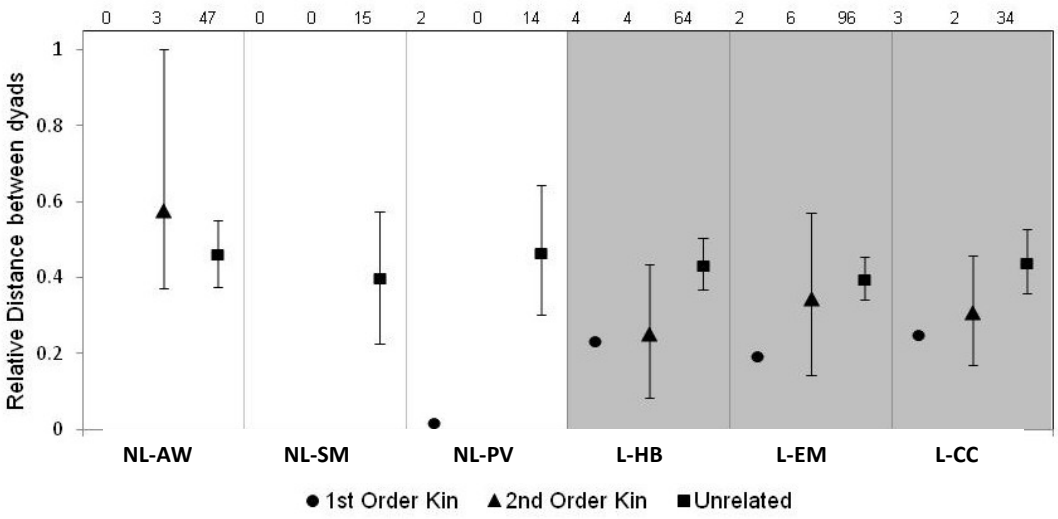

Figure 5.12 *Relative distance between 1st and 2nd order relatives and unrelated breeding male and female pairs. Standard error bars, determined by resampling with replacement 1000 times between 2 or more pairs, are shown. Sample sizes (number of dyadic pairs) for each relationship are shown at the top and linear habitats are shown in grey.*

5.4 Discussion

This is the first study to demonstrate the extent of relatedness and sociality in water vole populations using molecular and demographic information obtained from wild populations in southeastern England. The use of pedigree data, for discriminating between commonly used relatedness estimators, proved an essential step for deriving the most reliable estimates for use in this study, given the high variability that was observed between the eight estimators considered (Figure 5.3). Even with a sample size of nearly 200 voles and their genetic profiles, derived from eight microsatellite markers, all eight estimators were bounded by large confidence intervals for estimating the known pedigree of two water vole families. High variance in relatedness coefficients is commonly encountered when true allele frequencies have to be estimated from sample data (Wang, 2002; Blouin *et al.,* 1996), and is an inevitable constraint in any study of wild populations employing the use of relatedness estimators. The estimator of Wang (2002) provided the most reliable estimates of genetic relatedness between related and unrelated voles. This is likely due to population substructure, due to the pooling of individual's genotypes across multiple populations, as this particular estimator is robust to the effects of substructure within populations (Wang, 2002). This was considered an important attribute for estimating relatedness in wild populations of water voles as the territoriality of females would prevent complete genetic admixture resulting in substructure and possible clusters of related individuals within populations.

The overall aim of this study was to identify patterns of genetic relatedness and the extent of kin tolerance and inbreeding in wild populations of water voles occupying linear and non-linear wetlands, where differences in the proportion of edge influenced habitat and the extent of resources were predicted to influence patterns of water vole sociality. It was hypothesised that the kin tolerance and relatedness would either increase in linear systems, due to related individuals becoming aggregated within a patch or would decrease, due to high dispersal dissolving kin clusters and increased competition amongst kin, when compared with non-linear wetlands. The results of this study are at least partly in

concordance with the former. Although estimates of genetic relatedness between all pairs of individuals were similar in populations occupying linear and non-linear sites (Figure 5.4), a significantly higher proportion of breeding adults and breeding females estimated as close relatives were observed in linear wetlands when compared with non-linear wetlands (Section 5.3.4 and Figure 5.5). This suggests that natal site philopatry is higher in linear systems, particularly amongst adult females where between 43% and 81% had a close relative within the population. Differences in intraspecific philopatry between populations can occur when ecological factors constrain opportunities for dispersal (Matocq & Lacey, 2003). Thus the decision to remain at the natal site can be influenced by the risks of dispersal, the probability of establishing a suitable territory and the probability of finding a mate (Solomon, 2003). The higher proportion of related individuals recruited to breed in linear sites may thus be a result of individuals being less inclined to disperse due to the hostility of the surrounding land use and/or may reflect the ability of early born young to establish a breeding territory due to lower abundances of adults in linear habitat types in spring. Both are equally plausible and are related to the increased edge effects associated with linear systems. Firstly, overwinter predation of water voles has been positively associated with edge habitats (MacPherson & Bright, 2010; Carter & Bright, 2003), and would result in lower spring abundance compared to non-linear wetlands and an inevitable increase in the probability of early-born young being able to establish a breeding territory within their natal site. This is further supported by Stoddart (1970) who reported that the recruitment into the breeding population of water voles occupying riverine habitat was restricted to voles born before July, and would explain the higher proportion of adult males and females that were estimated as close relatives observed in linear habitats (Figures 5.5 & 5.10). Secondly, Stamps *et al. (*1987) suggest that emigration from a patch is positively influenced by the permeability of the patch boundary, thus individuals occupying non-linear sites in this study were less likely to encounter a hostile edge and thus could disperse from the study area to establish a territory without the costs of dispersing through hostile land. Reluctance to cross habitat boundaries has been termed the "fence effect" and has been observed in numerous small mammal species (Lindenmayer & Fischer, 2006) resulting in higher levels of relatedness between

individuals and increased genetic structuring between populations (Stephens *et al.,* 2013; Banks *et al.,* 2007; Banks *et al.,* 2005; Gerlach & Musolf, 2000).

Natal philopatry is a prerequisite for the formation of kin clusters, which are shown through models of social behaviour to be an initial step in developing sociality (Maher, 2009). Whilst female philopatry was significantly higher in linear than in non-linear wetlands in this study, higher relatedness between breeding females compared with males was observed amongst all the populations (Figure 5.4, Table 5.5) suggesting female water voles are more philopatric than males. This behaviour is common amongst most mammal species and is reported to result in female kin groups across numerous taxa, including microtine rodents, carnivores and primates (Le Galliard *et al.,* 2012; Lucia *et al.,* 2008; Matocq & Lacey, 2003; Lambin & Yoccoz, 1998). This study, however, found considerable variation in the spatial distribution of females estimated to be close relatives, suggesting that female philopatry does not consistently result in the formation of kin groups of female water voles. For instance, the relatedness between neighbouring females was generally higher than non-neighbours in both linear and non-linear habitat types, though the difference between relatedness was not significant (Figure 5.6). A strong association amongst female kin is also predicted to result in significant negative relationship between female relatedness and geographic distance, which was not observed here (Table 5.6). Despite this, some kin clustering was observed between breeding females in both linear and non-linear sites where between 10% and 50% of neighbouring adults were estimated to be either 1st or 2nd order relatives (Figure 5.8). Evidence of territory sharing amongst female kin has previously been reported in water vole populations (Strachan *et al.,* 2011) and is common in microtine rodents (Le Galliard *et al.,* 2007; Collins & Barret, 1997). Sharing space amongst female kin may also explain why the distance between neighbouring females in this study was similar between habitat types, despite territories of both water voles and other vole species increasing with decreased forage abundance (Moorhouse & Macdonald, 2008; Jonsson *et al.,* 2002; Fortier & Tamarin, 1998).

The variation in kin affiliation was at least partly explained by abundance, whereby increases in the abundance of voles significantly reduced the proportion of breeding female neighbours that had been estimated as close relatives (Figure 5.9). This suggests that adult female water voles respond to increasing competition for resources by reducing their tolerance of kin as neighbours and concurs with observational studies in which adult, female water voles most frequently engaged in competitive agonistic interactions with their female progeny (Forman & Brain, 2006). Similar patterns of kin tolerance have been observed in other vole species (Gunderson & Andreasson, 1998; Lambin, 1994) and in a review by Anderson (1980) on vole behavioural systems, a consensus was reached that female voles will tolerate mature kin when resources are abundant but will be forced to either disperse or be reproductively suppressed when resources are sparse at high vole density. This is not unusual amongst female mammals where any fitness benefits accrued from having kin as neighbours is negated when resources become limited (Maher, 2009).

These observations are based on the assumption that a spatial association between related individuals reflects tolerance and thus some form of kin recognition exists between individuals. Evidence of kin recognition has been observed in water voles in laboratory conditions, whereby olfactory cues, most notably odour, are used to determine kin from non-kin (Evsikov *et al.,* 1997). This is not surprising, given that water voles use both latrines and flank glandular secretion to demarcate ranges, which must serve to express details of sexual condition and group affiliation (Stoddart, 1972). Olfactory dialect would not only allow for philopatric females to adjust their behaviour accordingly and accrue any fitness benefits, such as reduced costs of home range defence, when neighbouring a close relative at low density, but would also allow for intersex relatives to avoid breeding, when both males and females have been recruited into their natal site. In this study, between 20% and 75% of breeding females were estimated to be related to one or more sexually mature males in five of the six study sites, suggesting that natal dispersal by males may not always be the rule in water vole populations and thus there is an inherent risk that inbreeding could occur. Adult male water voles in this study were

separated by significantly larger distances than females (Section 5.3.1), thus their ranges would incorporate multiple female territories, a pattern that is consistent with a polygynous mating system (Clutton-Brock, 1989) and with other studies on the ranging and breeding behaviour of water voles (Moorhouse & Macdonald, 2008 & 2005; Sharhul, 1998; Stoddart, 1970). Therefore, if male and female water voles are able to distinguish between related and non-related mates, then it would be expected that some degree of spatial avoidance by one of the sexes would occur. There was, however, no strong evidence obtained in this study to suggest that adult males spatially avoid sexually receptive females that were estimated to be a close relative. For instance, neighbouring pairs of close relatives were observed in both linear and non-linear habitat types (Figure 5.10) and significant negative correlations between distance and pairwise relatedness of opposite sex adults were observed in one non-linear site (NL-PV) and one linear site (L-HB) (Figure 5.11). Furthermore, the relative distance between opposite sex adult pairs estimated as close relatives, particularly in linear wetland sites were observed in closer proximity than unrelated pairs, however, this difference was not significant (Figure 5.12). These findings contradict the prediction that inbreeding avoidance will result in opposite-sex relatives, that share home ranges, dispersing before reaching sexual maturity (Greenwood, 1980) but are in concordance with other studies that have observed father-daughter matings in meadow voles, white-footed mice, prairie voles and Townsend's voles (detailed in Lambin, 1994), resulting from female philopatry.

Although the number of potential matings between close kin could not be estimated in this study due to limitations on estimating the home range extent of males, significant inbreeding coefficients were estimated in two linear sites L-HB and L-EM where 50% and 70% of females had a close, sexually mature, male relative within the population. It is therefore evident that breeding between close relatives is not avoided, at least in linear wetlands where individuals may choose to stay and risk breeding with relatives rather than risk dispersal into a hostile matrix.

5.5 Conclusions

This study has identified, through genetic analyses, that sociality is present in water vole populations where between 44% and 87% of adults were estimated to have one or more close relatives of sexual maturity within the same population. When considering all possible pairs of individuals, the overall relatedness was significantly lower than a population comprised of unrelated individuals ($r = 0$), thus populations were established from largely unrelated individuals in spring. Sociality was found to increase in linear wetland systems compared to non-linear habitats due to higher levels of female philopatry, however, tolerance between relatives was reduced at higher abundance, undoubtedly due to increasing competition amongst kin for limited resources. Although the exact causes for different patterns of sociality across the two habitat types can only be inferred, it seems logical that edge effects and resource extent are important factors that dictate the extent of sociality in water vole populations.

Water voles have historically colonised both linear and non-linear wetland systems, however, the modification of landscapes across much of the UK has resulted in linear habitats becoming narrow remnants that are bounded by hostile land use types. The costs of dispersal and predation risk are inherently much higher in modern linear wetlands, resulting in higher levels of natal philopatry, which is a prerequisite for kin selection. However, if non-linear wetlands are more akin to ancestral riparian zones that once extended across lowland areas, kin associations between adult water voles may actually be a modern phenomenon that results from decreased dispersal that is otherwise characteristically high in populations of water voles occupying natural wetland systems. Competition for biological resources can either select for kin associations or kin avoidance depending on the fitness benefits that can be accrued. Further investigations using longitudinal data on relatedness and demographic trends from the six study populations are being undertaken to discern whether kin selection exerts any fitness benefits in water vole survival and reproduction

Chapter Six: Spatial and temporal patterns in the genetic structure of water vole populations in modern landscapes

6.1 Introduction

A major priority for the management of threatened species that live within contemporary landscapes is maintaining or restoring the genetic variation of populations to promote their evolutionary flexibility and secure their long-term viability (McNeely *et al.,* 1990). This, however, is challenging, as landscapes are often fragmented and perceptively hostile to the movement of individuals, which can result in populations becoming small and isolated (Gauffre *et al.,* 2008; MacDonald & Rushton, 2003). This can risk the erosion of both neutral and adaptive genetic diversity through random genetic drift, the fixation of deleterious alleles, and a decline in short-term population fitness caused by inbreeding depression (Frankham *et al.,* 2011). The dynamics of individuals within populations have predictable effects on the patterns of genetic variation seen in populations (Bohonak, 1999). Social structure leads to discernible patterns of relatedness within groups (see Chapter Five on relatedness) and barriers to dispersal and gene flow can result in inbreeding, leading to excesses in homozygosity either at the level of the individual (F_{IS}) or between subpopulations (F_{ST}). In addition, changes in genetic diversity over time can be indicative of migration (Frankham *et al.,* 2011; Bohonak, 1999). As such, a study of the patterns of genetic diversity can offer insights in to the dynamics of the population. One of the main influences of breeding and dispersal activity is resource availability, in turn influenced by landscape. It is the purpose of this study to identify how known variables relating to landscape impact population dynamics through analysis of genetic variation.

Both theoretical and empirical studies have made important contributions to understanding the effects of landscape features on genetic variation in plants (Aquilar *et al.,* 2008), mammals (Le Galliard *et al.,* 2012; Macdonald & Rushton, 2003; Gerlach & Musolf, 2000; Peacock & Smith, 1997), birds (Zuckerberg *et al.,* 2014; Vangestel *et al.,* 2011), amphibians (Allentoft & O'Brien, 2010) and insects (Keller & Largiadèr, 2003). Such studies have identified that factors

including patch size and shape, land-use type and the degree of isolation, can exert a strong influence on genetic variation both within and between populations. For instance, landscape features such as habitat type and roads have been found to restrict gene flow between populations of carnivores (McRae *et al.,* 2005), salamanders (Wang *et al.,* 2009) and toads (Dixo *et al.,* 2009) and increasing distance between resource patches is frequently cited to reduce dispersal success (Le Galliard *et al.,* 2012; Bender *et al.,* 1998). These scenarios result in greater divergence between populations and increase their vulnerability to the loss of genetic diversity that is associated with reductions in population size and inbreeding (Allendorf & Luikart, 2006). Several other studies have also shown that the size and shape of the resource patch can influence genetic variation by altering the rate of migration (Kindvall & Petersson, 2000; Stamps *et al.,* 1987), recruitment (Bennett & Saunders, 2010; Fahrig, 2003) and mortality (Fahrig, 1997) and by causing changes to the social behaviour of individuals (Banks *et al.,* 2007) which shape both the demographic stability and effective size of populations and determine the extent of admixture both within and between demes (Ezard & Travis, 2006).

In natural populations, the level of genome-wide variation is generally estimated using a selection of evolutionary neutral loci from which measures of diversity such as heterozygosity and the number of alleles across loci are obtained. These diversity measures tend to decline due to random genetic drift when populations are reduced in size as a result of environmental change (Leblois *et al.,* 2006; Selkoe & Toonen, 2006; Lacy, 1987). For instance, simulation studies and coalescent theory predict that alleles, particularly rare ones, are lost rapidly (< 10 generations) in response to population size reduction (Hoban *et al.,* 2014; Frankham *et al.,* 2011; Young & Clark, 2000; Petit *et al.,* 1998) and as new variation is only introduced into isolated populations via mutation, which occurs relatively slowly (approximately 1×10^{-4} / locus per generation for microsatellite markers) (Frankham *et al.,* 2011), low allelic richness is useful for identifying historic population bottlenecks (Hoban *et al.,* 2014). Heterozygosity loss, however, is far less pronounced in bottlenecked populations *per se* (Frankham *et al.,* 2011; Allendorf, 1986), but is impacted whenever populations have become small and isolated enough, for long enough, for the effects of

random genetic drift to dominate, increasing the proportion of the population that has alleles identical by descent due to random breeding between relatives (Lacy, 1987).

Although the direct association between genetic diversity and extinction risk remains a subject of debate (Leblois *et al.,* 2006), both heterozygosity and allelic richness can provide indications of a population's response to landscape modifications and are considered particularly important when considering short term demographic fitness and long-term adaptability (Tracy *et al.,* 2011; Leblois *et al.,* 2006) for the purpose of in-situ management programmes (Hoban *et al.,* 2014). In this context, measures of genetic variation are often extended to include multiple populations so that patterns in genetic variation amongst populations can be used to investigate potential causes of genetic erosion (Lowe *et al.,* 2007). Allelic variation between populations, for example, will generally increase with decreasing migration between patches (Frankham *et al.,* 2011) and may align with geographic distance (isolation by distance effect) (Wright, 1946, 1943) or be shaped by more cryptic environmental factors such as land use types or geographic features (Gauffre *et al.,* 2008; Leblois *et al.,* 2006; Manel *et al.,* 2003). Furthermore, this information can help to delineate the scale of association between populations so that the most appropriate geographical unit for the management of genetic diversity can be defined (Moritz, 1994). Given the breadth of understanding that can be gained from studies that incorporate genetic variation there is, unsurprisingly, a wide application of such studies for the purpose of species conservation (Allendorf *et al.,* 2010; DeSalle & Amato, 2004).

One of the limitations in managing the genetic diversity of species within human modified landscapes, is that quantifying the negative effects of habitat loss and fragmentation can be challenging, as they depend upon the life history and behavioural strategies of the species affected, which in turn, determine how they respond to landscape change (Fischer & Lindenmayer, 2007; Ewers & Didham, 2006). For small vertebrates, such as the water vole, that exhibit reduced spatial capabilities and that are short-lived (Berthier *et al.,* 2005), the

maintenance of genetic diversity within populations is likely to be threatened by the loss and fragmentation of natural habitat.

Water voles have already undergone a widespread decline across much of the UK due to the extensive drainage and modification of wetland habitat and by the predation of populations by feral American mink (Strachan *et al.,* 2011). As a consequence their current distribution, particularly in lowland England, comprises of patchily distributed extant or reintroduced populations that inhabit small, primarily linear remnants of suitable wetland habitat within agricultural systems (Macdonald & Rushton, 2003). These patches are likely to support fewer individuals given that water vole abundance, like most small mammals, is largely determined by the quality and quantity of available resources (Moorhouse *et al.,* 2009) and that resources within linear systems are often reduced and temporally unstable due to the increased proportion of edge influenced habitat (Gauffre *et al.,* 2008; Gustafson, 1998). This makes discrete populations in linear systems particularly vulnerable to demographic stochasticity, inbreeding and the loss of genetic diversity, particularly if dispersal is negated by hostile land use types (Macdonald & Rushton, 2003).

Study aims

This study investigates spatial and temporal patterns of genetic variation at microsatellite loci within and among populations of water voles in southeastern England. Populations within this area have declined by up to 99% and the reintroduction of populations and enhancement of wetland habitats are amongst a suite of actions that have been implemented to re-establish and secure water voles throughout their historical range (Sussex Otters & Rivers Partnership, 2007; Kent Biodiversity Action Plan Steering Group, 1997). This study had two primary aims concerned with the genetic variation of water vole populations. These were at the patch and landscape levels and were considered to have important implications for the local and national conservation of water voles. The first investigates whether genetic diversity in linear wetlands is comparable to non-linear wetlands, such as reedbeds, where discernible differences in resource extent and predation risk are expected to influence the demographic stability and, thus, the genetic diversity of populations. It is predicted that

populations occupying linear habitats would exhibit higher levels of genetic differentiation across years and reduced levels of genetic diversity due to lower effective population sizes, and increased inbreeding when compared to populations inhabiting non-linear wetland sites. The second aim was to investigate how the genetic variance of populations is spatially structured across river catchments in order to identify suitable management units for conserving genetic diversity. Previous research on microsatellite and mitochondrial DNA variation have suggested that water voles may be structured by river catchments and that gene flow may be restricted by land use type and elevation (Aars *et al.,* 2006; Piertney *et al.,* 2005), thus spatial structure was considered in terms of both geographical features and landscape type. The influence of reintroductions, historical bottlenecks and environmental perturbations that occurred during the study period, on the patterns of genetic variation was also explored.

6.2 Methods

6.2.1 Study populations and sample collection

This study was carried out across eight sites located in Sussex and in Kent and Greater London (referred to herein as KGL). The study sites were distributed amongst five river catchments and were further differentiated by the north and south downland and urban environments (Figure 6.1 and Table 6.1). Each sampling site was characterised as either linear or non-linear depending on the geometric distribution of resources for water voles. Populations from four replicates of each geometric habitat type were studied and are differentiated herein by the prefix L or NL for linear and non-linear sites respectively.

Two of the study sites, NL-AW and NL-LW, consist of historically (2005 and 2001 respectively) reintroduced populations of water voles. Details on the demographic and genetic stock (if known) of reintroduced individuals are presented in Chapter Two Section 2.2.1. No other sites had confirmed on-site reintroductions.

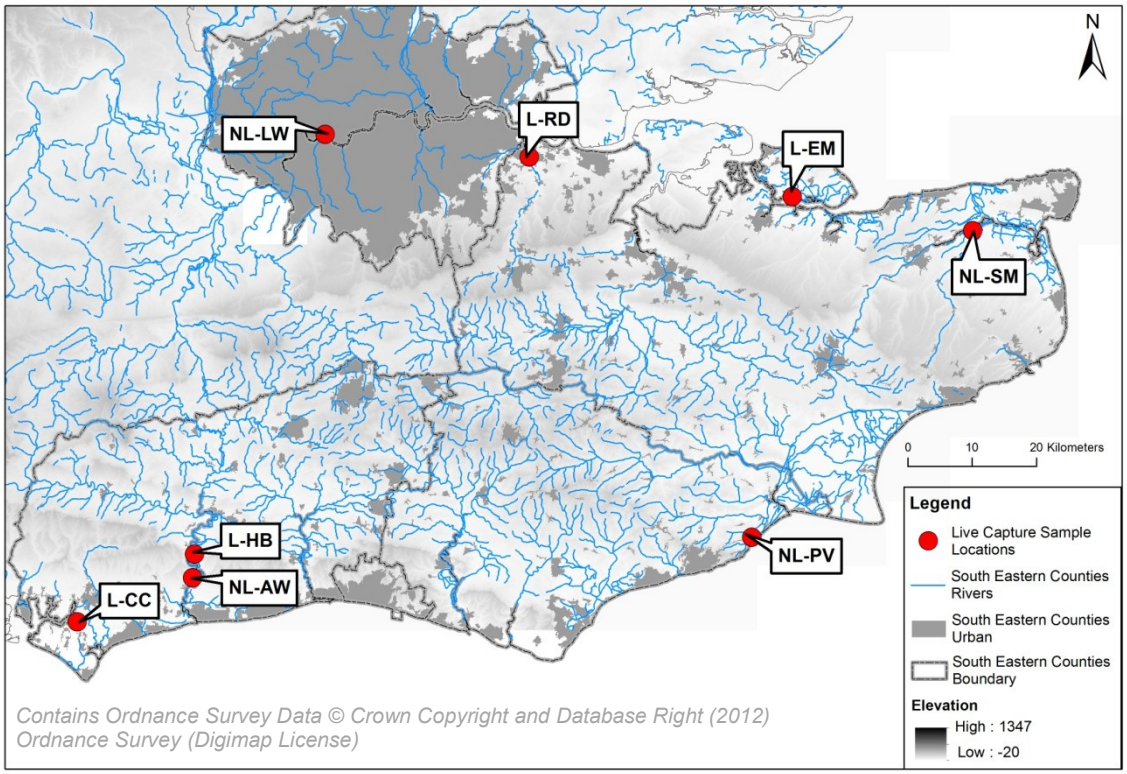

Figure 6.1 *Location of study sites used to investigate the genetic structure of water vole populations. Study sites are labelled and prefixes of 'L' and 'NL' denote linear or non-linear habitat types respectively.*

Site Code	River Catchment	River Sub-catchment	Predominant land-use
NL-AW	Arun and Rother	Lower Arun	Natural and Grazing Marsh
L-HB	Arun and Rother	Lower Arun	Grazing Marsh and Agriculture
L-CC	Arun and Rother	Western Streams	Agriculture
NL-PV	Cuckmere and Pevensey Levels	Cuckmere and Pevensey Levels	Grazing Marsh and Agriculture
NL-SM	North Kent	Wingham & Little Stour	Natural
L-EM	North Kent	Lower Medway & The Swale	Natural and Grazing Marsh
L-RD	London	Darent & Cray	Urban
NL-LW	London	Thames	Urban

Table 6.1 *Details of the river catchments and landscapes differentiating study sites. Predominant land use relates to immediate habitat type(s) surrounding each study site.*

Genetic samples were obtained from water voles using live capture techniques carried out for three consecutive days in spring (March-May) and autumn (September-October) during 2012 and 2013. At each site, a maximum of 50 baited cage and bedding traps were placed at 20 m intervals along the bank edges or among emergent vegetation. Traps were checked each morning and evening and each newly captured individual was injected with a unique PIT tag (Trovan ID162), weighed, sexed and had their breeding condition determined. In addition, a small pull of hair (with follicles) was taken from the rump of each animal for genetic analyses and placed in a 1.5 ml microcentrifuge tube before storing at -20°C until DNA extraction (detailed in Section 6.2.2). Full details of live capture methods are provided in Chapter Two, Section 2.3.1. Additional hair samples obtained during live capture studies conducted by the author during June 2010 and July 2011 at sites L-HB and NL-AW are included in this study to investigate patterns and longitudinal changes in genetic variation. Genetic samples were obtained as per the field methods described above.

6.2.2 DNA extraction and microsatellite genotyping

DNA was extracted from each hair pluck sample after lyses (as described by Pfeiffer *et al.,* 2004) using Qiagen DNeasy™ Blood and Tissue Kit. All samples were genotyped at eight microsatellite loci (Berthier *et al.,* 2006; Stewart *et al.,* 1999a) using pre-designed 5' fluorescently labelled primers and PCR cycling conditions described in Chapter Two, Section 2.4.2. Samples were multiplexed into two groups and separated by capillary electrophoresis on an ABI Prism 3730 Genetic Analyser using a ROX 500 bp size standard (Applied Biosystems). Genotypes were then sized using Peak Scanner™ Software v1.0 (Applied Biosystems, 2006). Any ambiguous genotypes, in terms of stutter and peak-height ratio's and samples with rare and/or private alleles were genotyped twice. Only individuals successfully genotyped for four or more microsatellite loci were used in the analyses.

6.2.3 Microsatellite analyses

Genotyped individuals from each population were grouped into annual samples as water vole populations can undergo changes in genetic composition across years due to fluctuations in population size resulting from high over-winter

mortality (Aars *et al.,* 2006). Each annual group is referred to herein as the population location which are differentiated within populations by the suffix 1,2,3... (Table 6.2). For populations L-CC, NL-PV, NL-LW and NL-SM, samples include individuals captured in spring and autumn of the same year, which were pooled due to the sample size within each sampling period being insufficient for accurate estimates of genetic composition to be obtained (based on simulated data from 50 individual water voles genotyped at eight microsatellite loci. Refer to Chapter Three; Section 3.2.3). Furthermore, no significant deviation in the genetic structure, as determined by F_{ST} (described in detail below), between the spring and autumn populations was observed, thus pooling the seasons was considered appropriate.

	Pop ID	Season	Year	Sample Year Code	N	N_F	N_M
SUSSEX	NL-AW	Summer	2010	1	81 (64)	41 (32)	40 (32)
		Summer	2011	2	60 (33)	29 (16)	31 (17)
		Autumn	2012	3	33 (32)	17 (16)	16 (16)
	L-CC	Spring	2012	3	13 (13)	6 (6)	7 (7)
		Autumn	2012		20 (20)	11 (11)	9 (9)
		Autumn	2013	4	7 (7)	5 (5)	2 (2)
	L-HB	Summer	2010	1	43 (33)	19 (16)	24 (17)
		Summer	2011	2	27 (24)	7 (5)	20 (19)
		Autumn	2012	3	14 (11)	5 (3)	9 (8)
		Autumn	2013	4	29 (29)	15 (15)	14 (14)
	NL-PV	Spring	2012	3	5 (5)*	2 (2)	3 (3)
		Autumn	2012		11 (11)	5 (5)	6 (6)
		Autumn	2013	4	16 (16)	6 (6)	10 (10)
KGL	L-EM	Autumn	2013	4	40 (34)	30 (25)	10 (9)
	NL-LW	Autumn	2012	3	9 (9)	3 (3)	6 (6)
		Autumn	2013	4	11 (11)	5 (5)	6 (6)
	NL-SM	Spring	2012	3	7 (7)	5 (5)	2 (2)
		Autumn	2012		7 (7)	4 (4)	3 (3)
		Spring	2013	4	8 (8)	1 (1)	7 (7)
		Autumn	2013		3 (3)	1 (1)	2 (2)
	L-RD	Autumn	2012	3	4 (4)	1 (1)	3 (3)

Table 6.2 *Genotyped populations across eight study sites showing sampling season, sampling year and number of captured and genotyped individuals (including recaptures) listed as total (N), females (N_F) and males (N_M) with number genotyped in parenthesis. Sample year codes are provided as they are referred to in the results. Grey shaded boxes show sites that were pooled across season per sampling year. * includes one tissue sample from a dead water vole found by site manager.*

Genotypes of samples from each population were first analysed using the programme Cervus version 3.0.3 (Kalinowski *et al.,* 2007; Marshall *et al.,* 1998) to estimate allele and null allele frequencies and the programme MICRO-CHECKER (Oosterhout *et al.,* 2004) to identify other potential genotyping problems such as large allelic dropout or typographical errors. Genotype files were then reformatted using the calculated allele frequencies within the programme Convert (Glaublitz, 2004) for use in the different genetic analyses software programmes described herein. Allele frequency data for each study population is provided in Appendix Seven.

Genetic variation

Genetic variation was estimated based on polymorphisms at eight microsatellite loci using GDA version 1.1 (Lewis & Zaykin, 2001), unless otherwise stated. Genetic diversity within each sample across all eight populations was measured as the mean number of alleles across loci (*A*), observed heterozygosity (H_O) and unbiased expected heterozygosity (H_E) and mean allelic richness (A_R). Because the number of alleles is strongly dependent on sample size, a rarefaction procedure was implemented using programme HP Rare v.6 (Kalinowski, 2005) to estimate the expected number of alleles in subsamples of *x* genes (where *x* is the smallest number of genes genotyped for a locus in the sampled populations and where gene represents the number of copies of *i*th alleles in a sample of *y* individuals) (Kalinowski, 2004) to allow for comparative analyses across the study populations and regions. Significant departures of genotype frequencies from Hardy Weinberg proportions, which assume random mating within populations, were determined from F_{IS} values calculated using a randomization procedure (1000 permutations) implemented in FSTAT version 2.9.3.2 (Goudet, 2001). Bonferroni procedures were applied to the unbiased probability estimates that were generated across loci within each sample from each population (Rice, 1989). The frequency of rare alleles and identification of private alleles (A_P) within each population was calculated using GenAlEx version 6.5 (Peakall & Smouse, 2012; 2006).

To examine whether the genetic diversity of each population was influenced by increasing linearization, the perimeter-area ratio (PAR) of each patch was first

quantified as

$$\frac{Perimeter\ (m)}{Area\ (m^2)}$$

to provide a value that increases with increasing perimeter relative to the area of the patch (described fully in Chapter Four, Section 4.2.1). Linear regression analysis of observed heterozygosity vs. PAR and allelic richness vs. PAR was performed using the MASS package (Ripley *et al.,* 2013) in the statistical software R, version 2.15.3 (R Core Team, 2013).

To further examine whether the type of wetland habitat (linear vs, non-linear) influences the genetic diversity within populations and to examine whether the genetic diversity of reintroduced populations was comparable to the natural study populations, an analysis of variance (ANOVA) test was performed using R statistical software. Allelic richness (A_R) and observed heterozygosity (H_O) were used as measures of genetic diversity for each population. Unbiased estimates of expected heterozygosity were not considered as they assume the genotypic proportions are in HWE (Stewart *et al.,*1999b). For each model, sampling year was included as a nested factor due to account for annual variation in allelic frequencies and the eight microsatellite loci were used as repeated measures.

Spatial and temporal genetic structure

To investigate patterns in genetic structure across populations spatially and within populations temporally, Weir & Cockerhams F_{ST} (1984) was used as a measure of genetic divergence, along with 95% confidence intervals obtained by bootstrapping across all loci (1000 permutations) and tested for statistical significance using FSTAT version 2.9.3.2 by permuting the genotypes among samples, which does not assume sampled populations conform to Hardy Weinberg proportions (Goudet, 2001). A sequential Bonferroni correction was applied to each probability estimate.

To characterise the spatial distribution of genetic divergence amongst the sampling sites, annual groups of populations were first tested for an isolation by distance pattern by correlating matrices of pair wise F_{ST} with the Euclidean distances (km) between each sampled site (as measured from point data within

ArcGIS 10.1 (ESRI, 2011)). The significance of the correlation between F_{ST} and geographic distance for both 2012 and 2013 sampled populations, across regions, was determined using a Mantel test (Mantel & Valland, 1970) (1000 permutations) using POPTOOLS version 3.2.5 (Hood, 2011).

Hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.,* 1992) was used to investigate the partitioning of genetic variation (using F_{ST} as the genetic distance measure) among sampled populations. Two broad geographical subdivisions of genetic variation across the study area were examined and included region (Sussex and KGL) and river catchment (Arun & Rother, Cuckmere & Pevensey Levels, North Kent and London) to ascertain which grouping explained the greatest proportion of variance (Figure 6.2 a). This approach was also used to investigate the influence of annual variation within each population (Figure 6.2 b). To visualise patterns in genetic distance (pairwise F_{ST}) between sampled populations a principle coordinate analysis (PCoA) was performed and the variation in F_{ST} explained by the first two coordinates was plotted. Both AMOVA and PCoA were performed using GenAlEx 6.5.

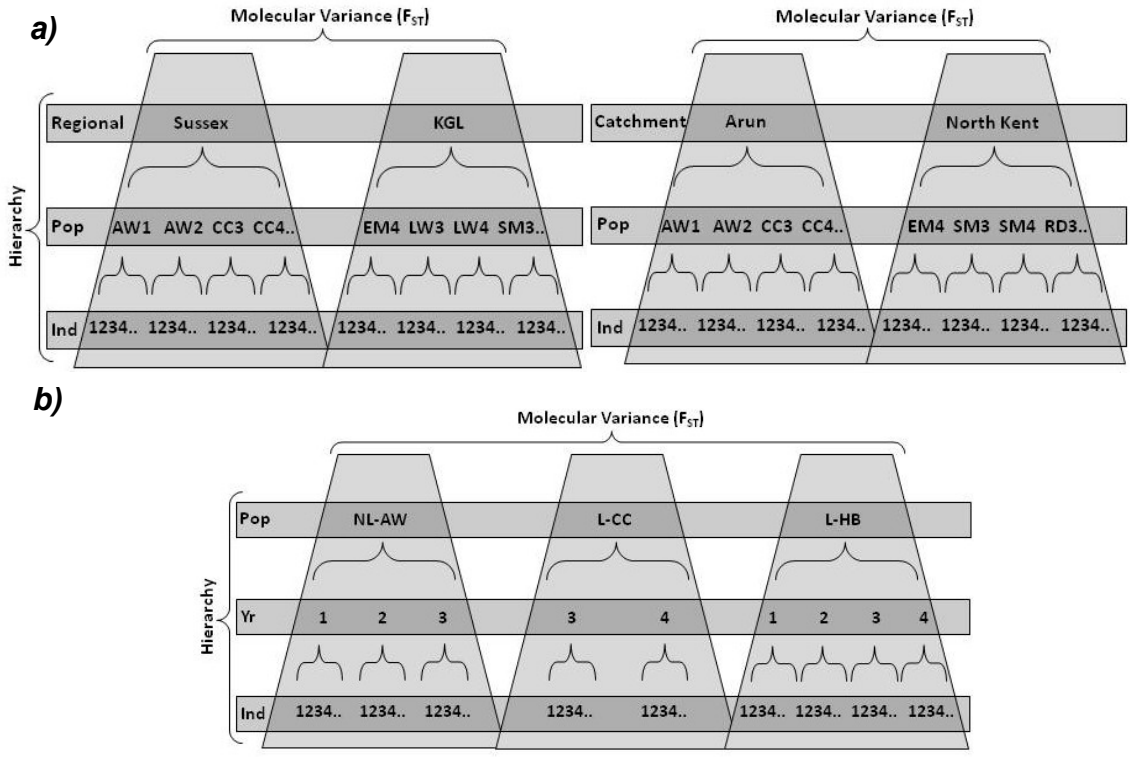

Figure 6.2 *Diagrams showing examples of the hierarchical design of analyses of molecular variance as measured by F_{ST}, accounting for (a) spatial and (b) annual partitioning of variation.*

6.3 Results

6.3.1 Microsatellite markers

Microsatellite amplification success was 91.6% and all eight loci tested in this study were found to be polymorphic in all populations, except locus AV7, which was not variable in the L-RD population, where only four individuals were sampled. A total of 95 alleles were detected for eight microsatellite loci in 372 individuals that were sampled across years from the eight study populations. Six alleles were identified as unique to any one population (Table 6.3). Null alleles were detected across all loci but their presence and estimated frequency were inconsistent across populations and hence did not provide evidence that any one locus would yield consistent genotyping errors.

Locus	n	K	H_E	H_O	N_{AP}	F_{NULL}
AV7	348	10	0.80	0.62	0	+0.1238
AV8	333	12	0.86	0.63	1	+0.1548
AV9	323	12	0.83	0.70	0	+0.0830
AV11	325	17	0.86	0.69	0	+0.1060
AV12	338	12	0.87	0.65	2	+0.1457
AV13	355	8	0.81	0.69	1	+0.0728
AV14	351	10	0.78	0.67	1	+0.0820
AV15	353	14	0.74	0.59	1	+0.1071
ALL	**372**	**95**	**0.82**	**0.65**	**6**	

Table 6.3 *Characteristics of eight microsatellite loci used in study including number of individuals typed per locus (n), number of alleles at each locus (K), expected heterozygosity (H_E) (Nei, 1978), observed heterozygosity (H_O), number of private alleles (N_{AP}) and the estimated null allele frequency (F_{NULL}), computed over all populations and sampling years.*

6.3.2 Genetic variation *within* and *among* populations

The results of genetic diversity measures calculated for annual samples obtained from eight water vole populations are shown in Table 6.4. The mean number of alleles (*A*) observed across loci ranged from 2.5 $\pm$ 1.1 in population L-RD to 7.9 $\pm$2.1 in samples 1 and 2 from population NL-AW.

	Pop Code	n	A	A_R	H_O	H_E	F_{IS}
NON-LINEAR	**NL-AW1**	64	7.9 (2.1)	2.82	0.74	0.75	0.022 NS
	NL-AW2	33	7.9 (2.8)	2.93	0.73	0.78	0.072 NS
	NL-AW3	32	7.1 (1.6)	2.9	0.73	0.78	0.063 NS
	NL-PV3	16	4.6 (1.1)	2.49	0.57	0.65	0.122 NS
	NL-PV4	16	4.6 (1.3)	2.54	0.58	0.68	0.150 NS
	NL-LW3	9	4.4 (1.2)	2.78	0.64	0.75	0.155 NS
	NL-LW4	11	4.8 (1.5)	2.65	0.74	0.72	-0.030 NS
	NL-SM3	14	5.4 (2.7)	2.59	0.64	0.68	0.050 NS
	NL-SM4	11	5.4 (1.8)	2.66	0.70	0.68	0.033 NS
LINEAR	**L-CC3**	29	6.5 (1.9)	2.65	0.62	0.69	0.108 NS
	L-CC4	7	4.6 (1.7)	2.63	0.70	0.70	0.000 NS
	L-HB1	33	5 (1.5)	2.40	0.56	0.64	0.115 *
	L-HB2	24	4.9 (2)	2.53	0.59	0.69	0.142 NS
	L-HB3	11	4.6 (1.6)	2.54	0.70	0.69	-0.023 NS
	L-HB4	29	6.4 (1.6)	2.51	0.58	0.67	0.136 *
	L-EM4	34	6.6 (1.2)	2.75	0.64	0.74	0.125 *
	L-RD3	4	2.5 (1.1)	2.03	0.47	0.47	0.008 NS

Table 6.4 *Genetic characteristics of sampled populations of water voles including, sample size (n), mean number of alleles observed across eight microsatellite loci (A), allelic richness averaged across eight loci and standardised for a sample size of four genes (A_R), averaged observed heterozygosity (H_O), averaged expected heterozygosity (H_E) and multilocus estimates for F_{IS}. Populations with significant heterozygote deficiency are indicated by * according to adjusted (5%) nominal level calculated per annual group. ±StDev is shown in parenthesis. Samples are differentiated by the geometric characteristics of each site (non-linear and linear) and by region with samples obtained from Sussex populations shown as shaded boxes.*

Allelic richness estimates *(A_R),* ranged from 2.03 to 2.93 and were consistent with *A* in identifying populations where the least and most number of alleles were present. Observed and expected heterozygosities were generally high across all samples and ranged from 0.47 to 0.74 and 0.47 to 0.78 respectively. Expected heterozygosity was higher than the observed heterozygosity for the majority of annual samples obtained from each population (F_{IS} = 0.008-0.155,

N=14). Significant heterozygote deficiencies and departure from Hardy Weinberg proportions after Bonferonni correction were observed at linear site L-HB from sampling years 2010 and 2013 and also at linear site L-EM which was sampled in 2013 (Table 6.4).

Of the 95 alleles detected, none of the samples obtained across the eight populations contained all 95. At a regional scale, Sussex populations contained 96% of the total number of observed alleles whereas 79% of alleles were found within individuals sampled from the KGL populations. This reflects the skew in the number of individual water voles sampled across regions (Sussex, N = 292, KGL N = 80) and when accounting for sample size (minimum subsample of 150 genes), A_R is slightly higher in Sussex (*Sussex* = 10.53, *KGL* = 9.37).

Across all populations, the majority of alleles, averaged across all loci, occurred at low frequencies: <0.05 (Figure 6.3) suggesting that none of the populations had suffered from prolonged historical bottlenecks - rare alleles are expected to be lost during bottleneck events. Private alleles were identified from three of the eight study sites, all of which happened to be linear wetlands (Table 6.5).

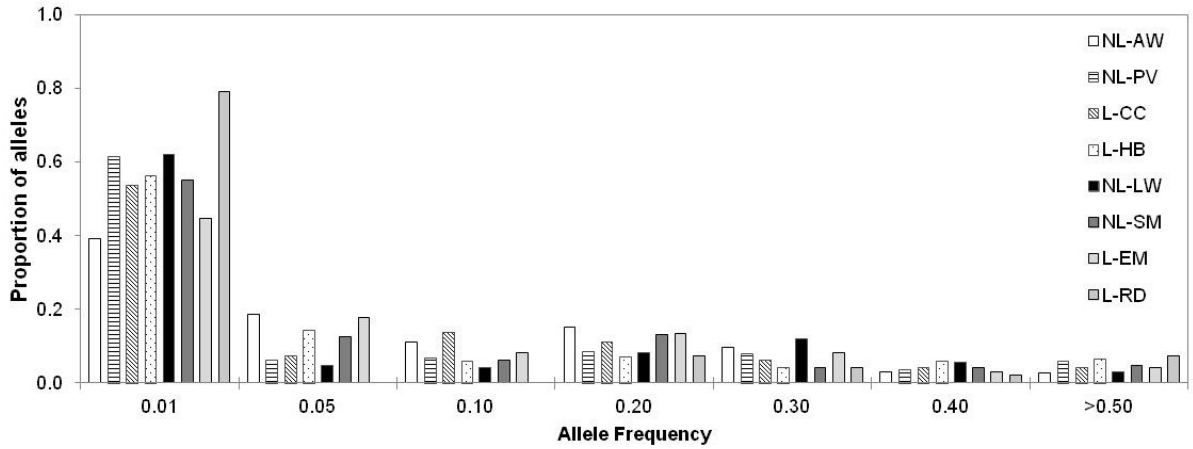

Figure 6.3 *Proportion of alleles across eight microsatellite loci that occur at different frequencies. Solid filled bars indicate sampled populations from KGL; pattern or outlined bars are sampled populations from Sussex. Frequencies are calculated per sampled site and include all annual groups.*

Region	Site Code	N_{PA}
Sussex	L-CC	2
	L-HB	2
KGL.L	L-RD	2
TOTAL		**6**

Table 6.5 *The number of private alleles (N_{PA}) by study population and region.*

The observed heterozygosity and allelic richness showed a slight negative correlation with the perimeter area ratio of each patch; however, this relationship was not significant as determined by linear regression (H_O, r = -0.271, F(1,15) = 2.012, P = 0.176, A_R, r = -0.853, F(1,15) = 2.868, p = 0.111).

Results of the ANOVA analyses investigating the influence of habitat geometry and reintroductions on allelic richness and observed heterozygosity, accounting for sampling year effects (Tables 6.6 a and 6.6 b), indicate that reintroductions were a significant factor in explaining the total variation estimated from both observed heterozygosity and allelic richness. Figure 6.4 shows that reintroduced populations exhibited higher allelic richness (a) and observed heterozygosity (b) compared to the other study populations. Neither habitat geometry nor sampling year was a significant factor in either model.

	(a) Allelic Richness				**(b) Observed heterozygosity**			
Source of Variation	**df**	**SS**	**F**	**P**	**df**	**SS**	**F**	**P**
Geometric type *(L vs.NL)*	1	0.085	0.4628	0.498	1	0.0001	0.0058	0.940
Reintroduction (yes or no)	1	0.998	5.4285	**0.021**	1	0.0243	0.0775	**0.029**
Year (2010-2013)	3	0.358	0.6480	0.586	1	0.0027	0.6677	0.429

Table 6.6 *Results of ANOVA comparing allelic richness (a) and observed heterozygosity (b) amongst water vole populations from linear and non-linear sites and across years (nested factor). Factors in bold indicate significance.*

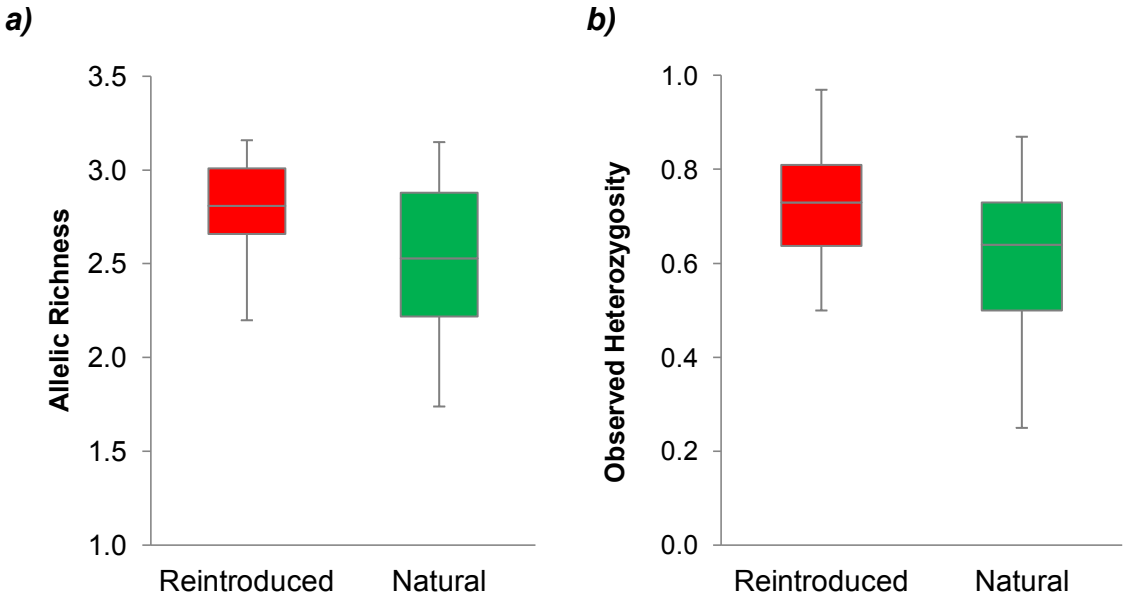

Figure 6.4 *Boxplot of allelic richness (a) and observed heterozygosity (b) across eight microsatellite loci from reintroduced and natural water vole populations. Horizontal lines represent the median, with the box representing the 25th and 75th percentiles and the whiskers representing the 5th and 95th percentiles.*

6.3.3 Spatial genetic structure

Overall, a significant level of genetic differentiation as measured by F_{ST} was identified across all samples (averaged F_{ST} = 0.140, p = 0.001). Of the 136 pairwise tests 80% were significant after Bonferroni corrections were applied. Between pairs of samples from the same region (Table 6.7), none of the pairwise comparisons in Sussex (n = 55, averaged F_{ST} = 0.129) showed significant differentiation, whereas eight of the 15 tests carried out between KGL samples were significant and included samples from three of the four study populations (averaged F_{ST} = 0.170, adjusted p-value = 0.003). Within each sampling year (Table 6.8), pairwise comparisons indicated that there was significant divergence between all sampling sites in 2013 and in 2012, apart from tests comparing sites with L-RD, which proved insignificant but where the number of individuals was unlikely to provide meaningful estimates of F_{ST}. Both populations, L-HB and NL-AW that were sampled in 2010 and 2011 showed significant divergence from each other (F_{ST} = 0.166, 95% CI's 0.11-0.22 and F_{ST} = 0.166, 95% CI's 0.08-0.25 respectively).

	a)	NL-AW			L-CC		L-HB				NL-PV
		1	2	3	3	4	1	2	3	4	3
Sussex	NL-AW2	0.026									
	NL-AW3	0.046	0.018								
	L-CC3	0.164	0.122	0.144							
	L-CC4	0.125	0.112	0.146	0.078						
	L-HB1	0.166	0.135	0.135	0.244	0.234					
	L-HB2	0.100	0.166	0.098	0.194	0.177	0.088				
	L-HB3	0.125	0.079	0.107	0.188	0.186	0.164	0.079			
	L-HB4	0.117	0.093	0.123	0.182	0.173	0.083	0.046	0.106		
	NL-PV3	0.136	0.138	0.108	0.207	0.240	0.240	0.198	0.199	0.214	
	NL-PV4	0.130	0.145	0.118	0.204	0.231	0.237	0.192	0.227	0.206	0.036

	b)	L-EM	NL-LW		NLSM	
		4	3	4	3	4
Kent	NL-LW3	**0.125**				
	NL-LW4	**0.163**	-0.01			
	NL-SM3	**0.145**	**0.170**	**0.181**		
	NL-SM4	**0.158**	**0.160**	**0.170**	0.033	
	L-RD4	0.227	0.232	0.263	0.273	0.257

Table 6.7 *Pairwise population F_{ST} estimates for Sussex (a) and KGL (b) for sampled water voles across eight study sites. Estimates include all annual groups (shown as population codes 1..4) and significant differences are shown in bold after Bonferroni correction.*

2012							2013					
	NL-AW	L-CC	L-HB	NL-LW	NL-PV	NL-SM		L-CC	L-EM	L-HB	NL-LW	NL-PV
L-CC	**0.144**						L-EM	**0.135**				
L-HB	**0.107**	**0.188**					L-HB	**0.173**	**0.147**			
NL-LW	**0.082**	**0.139**	**0.162**				NL-LW	**0.177**	**0.163**	**0.187**		
NL-PV	**0.108**	**0.207**	**0.199**	**0.188**			NL-PV	**0.231**	**0.167**	**0.206**	**0.190**	
NL-SM	**0.147**	**0.214**	**0.139**	**0.170**	**0.232**		NL-SM	**0.212**	**0.158**	**0.187**	**0.170**	**0.209**
L-RD	0.214	0.299	0.227	0.232	0.334	0.273						

Table 6.8 *Pairwise population F_{ST} estimates for 2012 and 2013 for sampled water voles across eight independent study sites. Estimates which showed significant differentiation after Bonferroni correction are shown in bold.*

No evidence was found of a significant isolation by distance pattern between water vole populations sampled within the 2012 and 2013 annual groups across regions (Figures 6.5, a and b). However, when separated by region, the pattern of genetic differentiation and geographical distance suggest that opposing isolation by distance patterns may be occurring within the two regions with Sussex displaying a slight positive correlation across years and KGL showing a negative correlation in 2012 (Figure 6.6).

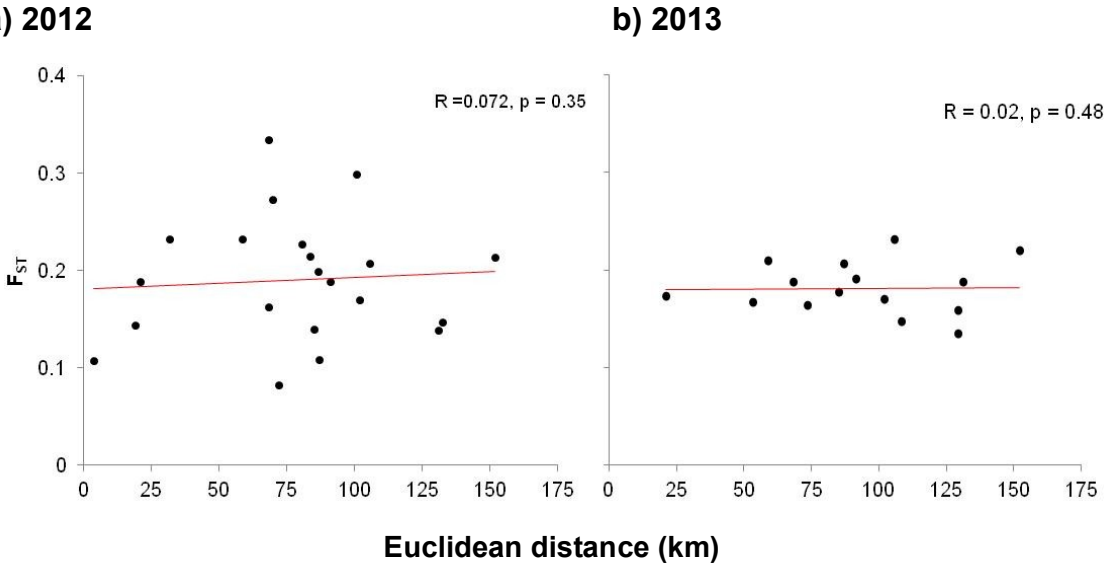

Figure 6.5 *Patterns of genetic isolation by distance for water vole populations sampled in 2012 (a) and 2013 (b) not separated by region. R is displayed as Mantel's correlation coefficient and related p-values for 5% level of significance.*

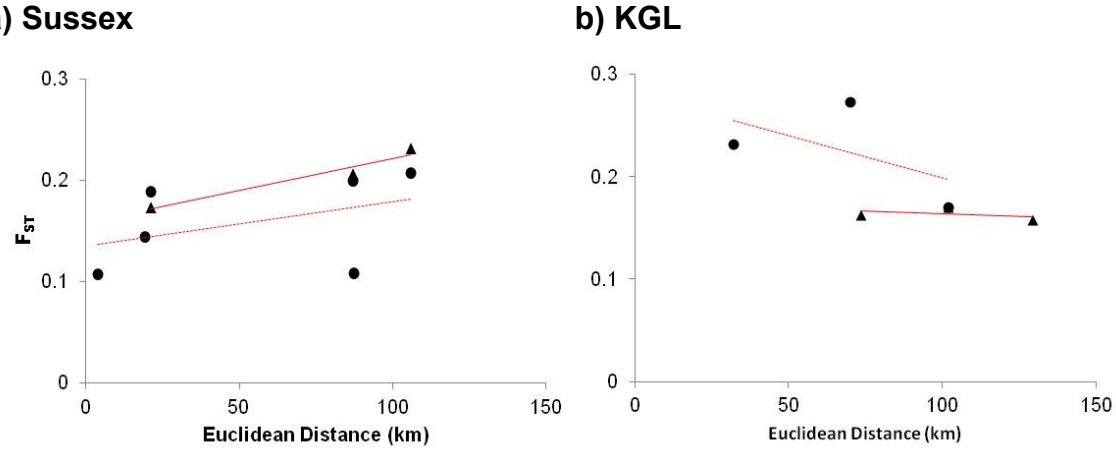

Figure 6.6 *Patterns of genetic isolation by distance for water vole populations sampled in Sussex (a) and KGL (b) in 2012 (circles) and 2013 (diamonds). Lines of best fit are shown as dashed for 2012 and solid for 2013.*

The significance of these relationships was not tested due to an insufficient number of pairwise comparisons within annual groups in both Sussex (2012, n = 6; 2013, n = 3) and KGL (2012, n = 3; 2013, n = 3) (Harmon & Glor, 2010). The R^2 values suggest that the linear relationship between geographical distance and genetic differentiation for pairs of populations explains a large amount of variation for Sussex in 2013 ($R^2 = 0.95$) and 31% of variation for KGL in 2012.

Hierarchical AMOVA results indicated both region and river catchment were significant factors contributing to the genetic variation observed across the study area (Table 6.9 a). Of the two geographical subdivisions considered, river catchment explained more variation (5%) than county (1%), whereas the most variation ($\geq$ 85%) was observed *within* sampled populations. To test whether variation between the eight study populations and divergence between sampling years were significant factors for explaining genetic variance, regional populations were pooled and a second AMOVA was carried out (Table 6.9 b). Variation between populations and between annual groups was highly significant ($p = 0.001$). However, together, they only explained 14% of the variance in the model with the remaining variance being explained by the variation between individuals within the samples.

The relationship between the genetic divergence of all sampled populations across regions and years was represented by principle coordinate analyses (PCoA) using estimates of pairwise F_{ST}. Results are presented in a two dimensional plot (Figure 6.7) showing the partitioning of genetic differentiation across sites and represents 45% of the variation estimated across the 17 samples obtained from the eight study populations. The plot clearly shows clustering of annual groups of samples from the same site and partitioning between the Sussex and KGL populations on the vertical axis, which reflects the significance of region found from the AMOVA results. The two sites where reintroductions are known to have occurred (NL-AW and NL-LW) are clustered centrally and do not appear genetically dissimilar to populations sampled at L-CC and L-EM.

(a,i)	df	Est. Var.	% Tot Var.	P
Among Regions	1	0.047	1	0.001
Among Samples	13	0.421	12	0.001
Within Samples	737	2.947	86	0.001
Total	753	3.415	100	

(a,ii)	df	Est. Var.	% Tot Var.	P
Among Catchments	3	0.158	5	0.001
Among Samples	13	0.346	10	0.001
Within Samples	737	2.956	85	0.001
Total	753	3.460	100	

(b)	df	Est. Var.	% Tot Var.	P
Among Pops	7	0.291	8	0.001
Among Years	9	0.189	6	0.001
Within Samples	737	2.947	86	0.001
Total	753		100	

Table 6.9 *Hierarchical analyses of molecular variance (AMOVA) of genetic differentiation, according to Weir & Cockerham's F_{ST}, among 17 sampled populations accounting for spatial variation (a) by region (i) and river catchment (ii) and for annual variation (b) within populations. P-values are based on 1000 permutations across all samples and loci.*

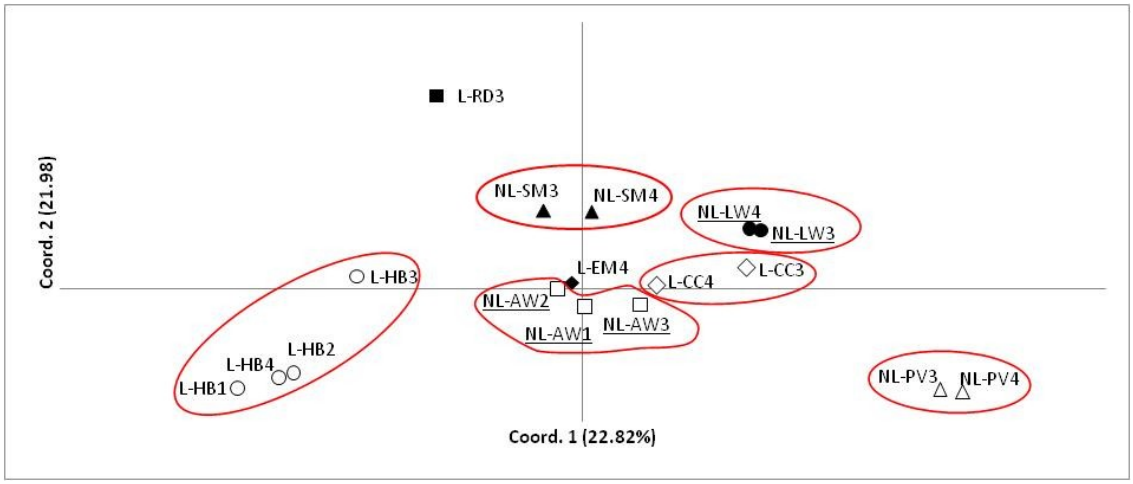

Figure 6.7 *Plot of PCoA showing partitioning of differentiation between all sampled populations across regions. Solid symbols represent Kent populations, outlined symbols represent Sussex populations and ellipses represent annual samples from the same population. Underlined samples are from sites where known reintroductions have occurred.*

6.3.4 Temporal patterns in genetic structure

Temporal variation in the genetic structure of water vole populations, as measured by F_{ST}, was observed between sampling years (Figure 6.8). Tests for significant variation in F_{ST}, after Bonferroni correction for multiple tests, indicated that three of the six populations (NL-AW, L-CC and L-HB) sampled over multiple years showed significant differences in annual genetic structure (Table 6.10). Non-significant temporal variation in F_{ST} between 2012 and 2013 was observed in sampled populations from three of the four non-linear sites (NL-SM, NL-PV & NL-LW) sampled across years.

Region	Population	F_{ST}	Probability of genetic differentiation across sampling years
Sussex	NL-AW	0.032	**
	NL-PV	0.037	NS
	L-CC	0.078	*
	L-HB	0.088	**
Kent	NL-LW	-0.008	NS
	NL-SM	0.033	NS

Table 6.10 *F_{ST} averaged across loci for annual groups within sampled sites. Significance (* = 0.05 and ** = 0.01) of differentiation is after Bonferroni correction for multiple tests across loci.*

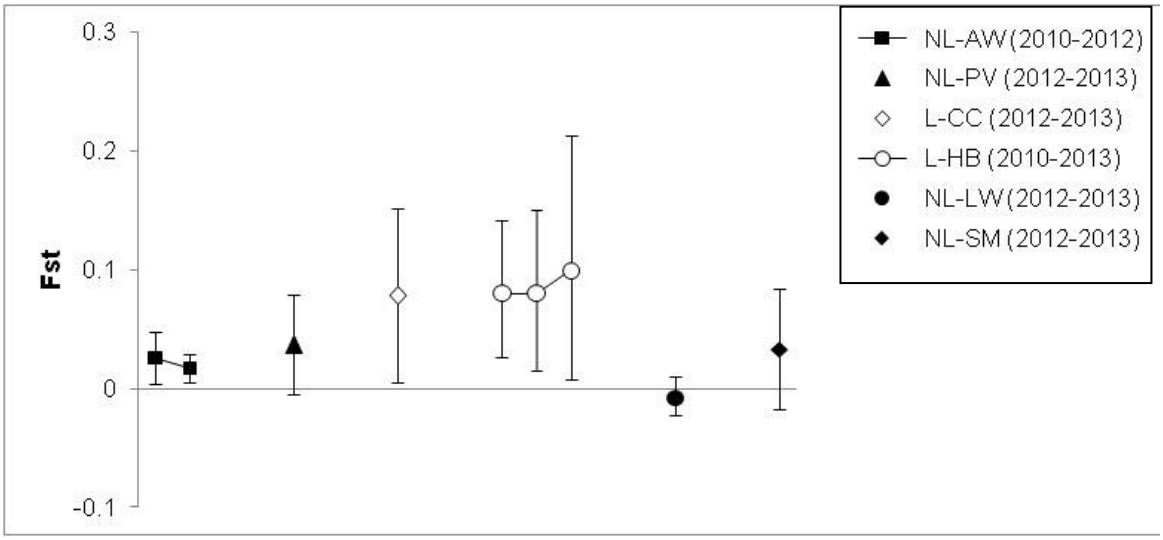

Figure 6.8 *F_{ST} averaged across eight microsatellite loci with 95% confidence intervals estimated by bootstrapping across loci. Estimates are grouped by population, across sequential years. Outlined and solid symbols are samples from populations occupying linear and non-linear habitat type respectively.*

6.4 Discussion

This study examined the spatio-temporal dynamics of genetic loci across eight water vole populations occupying linear and non-linear wetlands and had two primary aims: The first was to determine whether the genetic diversity within water vole populations was significantly lower in those occupying linear wetland systems, where a high turnover of individuals and lower effective population sizes were expected, in comparison to populations occupying non-linear wetland systems. The second was to identify how genetic variation was spatially structured across river catchments and whether factors such as historical bottlenecks and reintroductions have contributed to contemporary patterns of genetic structure in the south east of England.

Intra-population genetic diversity and patterns between wetland habitat types

Contrary to the predictions, this study found no strong evidence to suggest that water vole populations occupying linear wetland systems exhibited reduced levels of neutral genetic diversity compared with non-linear wetland populations. Excluding site L-RD, where only four individuals were sampled, all eight microsatellite loci were highly polymorphic in the study populations. Both the allelic richness and observed heterozygosity averaged across loci, were similar in populations from linear (A_R = 2.4 to 2.75, H_O = 0.56 to 0.70) and non-linear (A_R = 2.49 to 2.93, H_O = 0.57 to 0.74) wetlands and neither habitat type nor the perimeter-area ratio of the occupied patch had a significant influence on the levels of genetic diversity (Section 6.3.2, Tables 6.4 and 6.6).

Compared with other molecular studies on water vole populations that have used the same microsatellite markers, the genetic diversity of the natural populations in this study, in terms of average number of alleles per locus, were similar to those reported by Stewart *et al.,* (1999) from moderately fragmented colonies occupying linear watershed networks of the River Ythan (n = 6 to 107, A = 3.1 to 5.8, averaged across 12 loci). Interestingly, Aars *et al. (*2006) compared allelic richness, standardised by sample size, between the River Ythan colonies with other highly fragmented populations in northern Scotland, which showed that allelic richness on the River Ythan was significantly lower

than in northern Scotland. Whilst these values are not directly comparable, being based on different numbers of loci and samples, this could suggest that water vole populations in southeastern England may have reduced genetic diversity compared with northern Scotland. This may reflect unknown historical events or contemporary ecological conditions such as restricted dispersal, given the agricultural dominance of land use types across the study area. Colonies in northern Scotland, for example, are interspersed by heather dominated moorland (Aars *et al.,* 2006), which may be more permeable for the dispersal of water voles compared with agricultural landscapes in southern England. The widespread loss of historical populations and subsequent fragmentation and isolation of remnant populations resulting from habitat loss and mink predation is another possible contributor to lower allelic diversity. Limitations on dispersal, by distance or by land use type, would reduce the genetic neighbourhood in which genes are sampled and increase the strength of genetic drift, resulting in a loss of allelic richness, compared with populations in northern Scotland. However, this would not explain the loss of allelic richness in the study population L-EM which has been relatively unaffected by habitat loss and mink. It is, however, difficult to generalise the possible causes for any loss of allelic richness in south east England compared with northern Scotland, particularly as only independent colonies and not metapopulations have been sampled, but it does warrant further investigation.

Both allelic richness and observed heterozygosity was found to be significantly higher in the reintroduced populations than the natural colonies (Table 6.6 a and 6.6 b, Figure 6.4 a and b). This is of notable interest as reintroduced populations can often exhibit a loss of heterozygosity and allelic richness due to reduced survival and reproductive capabilities of founders (Kekkenon & Brommer, 2015; Robert, 2009). Comparing genetic diversity (H_O and A_R) between the two reintroduced populations, NL-AW exhibits higher diversity than NL-LW (Table 6.4), despite a similar number of founders having been released (~200) and a comparative amount of suitable habitat being present at both sites (Hutchins, 2007; Strachan & Strachan, 2005). This may be due to unknown differences in microsatellite variation of the founding population, but may also

reflect a higher reproductive success at site NL-AW which can aid in reducing the loss of genetic diversity (Robert, 2009; Leberg, 1993).

Three annual samples from two populations, L-HB and L-EM, showed significant heterozygote deficiencies (Table 6.4) suggesting that samples from these populations were either composed of discrete clusters of parents and their offspring, referred to as a 'temporal Walhund effect' (Rodrigáñez *et al.,* 2008), or that consanguineous mating had occurred, resulting in the presence of inbred individuals. Hardy Weinberg disequilibrium is commonly reported in water vole populations and varies in degree and direction due to different mating patterns and reproductive skew between colonies and will exist when colonies consist of family groups as long as juveniles are present (Aars *et al.,* 2006; Stewart *et al.,* 1999). The significant loss of heterozygosity at both L-HB and L-EM in 2013 corresponds with the recolonisation of these sites after widespread flooding over the winter of 2012/13. Recapture data (presented in Chapter Four), shows that a few (n = 2) water voles survived at both sites to recolonise in spring; thus spring densities in 2013 were likely to have been low compared with other years and study populations. This would allow for higher natal philopatry and the accumulation of related individuals and alleles that are identical by descent and that were subsequently sampled in the autumn. Microtine rodents, such as the water vole, can exhibit high female philopatry which accumulates during the breeding season (Lambin & Krebs, 1991) and is likely to be dependent on resource competition, given that female water voles are also known to disperse before breeding in linear wetland systems (Telfer *et al.,* 2003). It therefore seems plausible that the Hardy Weinberg disequilibrium observed at these two sites in 2013 reflected a 'temporal Walhund effect' and that migration by males prevented the loss of genetic diversity that would be expected when populations are founded by a few individuals (Wade & McCauley, 1988).

Large seasonal fluctuations in local population sizes, such as those observed at sites L-EM and L-HB are commonly observed in water vole colonies occupying linear watershed systems due to high overwinter mortality and high predation rates (Strachan *et al.,* 2011; Aars *et al.,* 2006; Telfer *et al.,* 2003; Stewart *et al.,*

1999). The high turnover of individuals in linear sites is clearly demonstrated by the significant differences in genetic composition, as determined by $F_{ST,}$ between the annual samples obtained from the two temporally sampled colonies L-CC and L-HB (Figure 6.8, Table 6.10). This pattern is congruent with the predicted genetic discontinuity that occurs over time in small populations through drift-migration and when the turnover of individuals within a population is high (Østergaard *et al.,* 2003). Lower F_{ST} values were observed between annual colonies occupying non-linear wetlands, of which one site, NL-AW, also showed significant temporal variation in genetic composition. This was unexpected given that this was the largest colony sampled during the course of this study (Table 6.4 and in Chapter Four). Whilst migration by genetically divergent individuals from neighbouring colonies into this site cannot be wholly disregarded, it seems unlikely given the high abundance ($\widehat{N} =$ 45 - 105 individuals, Chapter Four), which has been shown elsewhere to hinder immigration into water vole colonies via a "social fence effect" (Fisher *et al.,* 2009). Other factors such as high variance in female reproductive success between years, or more cryptic mating patterns could also be invoked; however, it is unclear why such high variance in genetic composition between years is occurring in this population and warrants further investigation. The temporal F_{ST} values for the other non-linear colonies were not significant and may infer a higher survival of individuals and thus the temporal continuity of genetic structure. This would support studies by Carter & Bright (2003) and later Moorhouse *et al. (*2009) that suggest survival of voles is likely to be higher in non-linear, well vegetated habitats, such as the non-linear wetlands sampled in this study. However, this pattern may reflect the low number of samples obtained from these sites (n = 9 - 16), which is below the sample size of 25 individuals, identified in Chapter Three (Figure 3.9) that would maximise the probability of identifying significant differences in genetic composition, given the magnitude of variation across the eight microsatellite markers employed here.

Genetic drift is a powerful evolutionary force that induces a rapid loss in genetic diversity within small populations, and seasonal bottlenecks such as those observed at sites L-EM and L-HB would predictably lead to a rapid loss of rare alleles (Greenbaum *et al.,* 2014). Contrary to these predictions, however, the

majority of alleles, averaged across the eight loci used in this study, occurred at low frequencies in all populations (Figure 6.3) suggesting that seasonal variations in population size did not cause significant genetic bottlenecks and thus the loss of rare alleles. It therefore seems apparent, that migration and/or survival of voles is sufficient across the study populations to prevent any substantial loss of alleles that would be expected to be lost through drift.

Spatial patterns of genetic variation and the influence of historical and contemporary factors.

The genetic variation amongst all samples showed significant differentiation (F_{ST} = 0.140, P = 0.001), and when accounting for sampling year effects all populations, apart from site L-RD where only four individuals were observed, showed significant differentiation from each other (Table 6.8). This was further supported by the hierarchical analysis of genetic differentiation that showed genetic diversity amongst populations accounted for the most variance (8%) after that observed amongst individuals (86%, Table 6.8b). This was expected given that the geographic distance between pairs of populations, ranged from between 6.5 and 130 km, which is well beyond the 1.5 - 2 km dispersal range of water voles (Aars *et al.,* 2006; Telfer *et al.,* 2003, Stewart *et al.,* 1999). Interestingly, however, the geographic subdivisions of region and river catchment, although significant, explained a surprisingly small amount of variation in the AMOVA (1% and 5% respectively, Table 6.8a) and was exceeded by the variability between annual samples from each population. Furthermore, when pooled across regions, no significant isolation by distance pattern in genetic differentiation was observed in either 2012 or 2013 (Figure 6.5). One plausible explanation would be that reintroductions across the study area have lead to the genetic homogenization of some geographically disparate populations. This is evident from the principle coordinate analysis (Figure 6.8) where reintroduced populations NL-LW and NL-AW are clustered with populations from north Kent (L-EM) and the Manhood Peninsular in Sussex (L-CC). The naturally large geographic distance and unconnected river catchments that separate these populations should predictably lead to large genetic differentiations between these sites that are unlikely to have experienced any contemporary exchange of migrants. However, investigations

into the locations of reintroductions and of the founding stocks, revealed that water voles from the Isle of Sheppey had been used in the reintroduction programme at NL-AW (Gow, 2012a) alongside Kent bloodlines which formed part of the genetic stock reintroduced into site NL-LW. This may therefore explain the genetic similarities between these three sites (Strachan *et al.,* 2011; Hutchins, 2007; Strachan & Strachan, 2005). There has also been a reintroduction of unknown source on the Manhood Peninsular, where site L-CC is located, thus the similarities in genetic composition between this population and other reintroduction sites NL-AW and NL-LW, may also reflect human mediated movement.

Despite the contribution of assisted colonisations to the spatial distribution of genetic diversity within the study area, genetic divergence was still observed between regions and river catchments. In Sussex, populations L-HB, NL-PV and L-CC showed particular divergence along the first principle coordinate axis (Figure 6.7) and are supported by the presence of private alleles within both L-HB and L-CC populations (Table 6.5). Of particular local interest is the distinctness of population L-HB, which occurs within the same river valley as the reintroduced population NL-AW. The significant F_{ST} estimates between these sites in each sampling year (Table 6.8), and the partitioning observed in the PCoA analysis, show these two sites to be more differentiated than that which would be expected from populations occupying the same river catchment. This suggests a barrier to dispersal between the reintroduced population (NL-AW), which has expanded from the site into the adjacent floodplain, and L-HB, which occurs 6.5 km upstream, and so warrants further investigation. In Kent, similar divergence between different river catchments was observed with NL-SM, L-RD and L-EM showing differentiation along the y axis of the PCoA. The partitioning in Kent, however, is not as pronounced as in Sussex and may reflect historical rather than contemporary processes. Although declines in water vole populations have occurred in both counties, Sussex has undergone a far greater loss of numbers than Kent, and thus the genetic differentiation between populations may be a relic of historical isolation between connected river catchments (as shown in Figure 6.1) where water voles once exhibited a more continuous distribution.

6.5 Conclusions

This study has explored how both natural and human induced processes have influenced the spatio-temporal patterns in microsatellite loci of water vole populations in southeast England. Comparable levels of genetic diversity were observed between colonies occupying linear and non-linear habitat types and significant temporal variation suggests that the turnover of individuals, particularly in linear habitats, is high and thus migration is effective in maintaining genetic diversity. This is particularly important in linear wetlands, where populations are known to suffer high mortalities due to predation and are vulnerable to environmental perturbations such as flooding, as discussed herein. Significant inbreeding coefficients in two linear sites suggest that, following population declines, related individuals may accumulate within a site in response to low densities, which increases the risk of inbreeding, particularly in the absence of migration. The temporal continuity of genetic structure observed in three of the four non-linear populations, could suggest that voles exhibit higher survivorship at these sites, resulting in annual groups comprising of a greater proportion of shared genes, which would thus contribute to the maintenance of genetic variation. However, these results are not conclusive given the small samples size obtained in three of the four non-linear populations. Although no discernible differences were observed in the genetic diversity between colonies occupying linear and non-linear wetlands, there is tentative evidence to suggest the demographic stability and rate of migration is different between habitat types. It therefore may not be important whether different habitat types influence populations with regard to allelic richness; rather the processes that maintain genetic variation, such as higher survival or migration, may be different between the two wetland systems. Further work to investigate seasonal demography, kin structure and genetic variation will be important to elucidate how different patterns between breeding, survival and kin structure contribute to the maintenance of genetic diversity in water vole populations occupying different habitat types.

This study is the first to monitor the genetic diversity of reintroduced water vole populations, despite the recognised risk of genetic erosion that can arise when the numbers released are limited and when survivorship of captive individuals is reduced (Kekkonen & Brommer, 2015). Despite this however, significantly higher heterozygosity and allelic richness in the reintroduced populations suggest that both reintroductions were successful. Furthermore, this also suggests that releases of approximately 200 water voles into high quality habitat can be sufficient to maintain high genetic diversity and thus secure their long-term fitness and adaptability.

Lastly, this study has identified that genetic variation is partitioned amongst river catchments and may be further differentiated by more cryptic boundaries such as land use types and/or the degree of connectivity amongst river catchments. However, further sampling would be required within different river catchments to discern the scale of management that would preserve genetic integrity, whilst maintaining sufficient gene flow for preserving genetic diversity. This is particularly relevant for mitigation works that can potentially disconnect populations and can lead to populations being translocated outside of catchments. Although this is unlikely to cause adverse effects to the populations in the short term, it may result in the loss of rare alleles that may be useful to the evolutionary potential of the species.

Chapter Seven: The role of phylogenetic relationships in informing conservation management of water voles in southeastern England.

7.1 Introduction

The conservation management of threatened species is primarily focussed on halting the decline and increasing the abundance of remnant populations by implementing remedial management strategies that are underpinned by knowledge of the species' life history and reasons for their original decline (Fischer *et al.,* 2006; Norris, 2004). For many species, particularly those threatened by habitat loss and isolation, management strategies include increasing the habitat availability and functional connectivity and, in more severe cases, the translocation or reintroduction of ex-situ bred individuals to augment already bottlenecked or extirpated populations (IUCN/SSC, 2013; Mills, 2007; Fischer *et al.,* 2006; Norris, 2004). Both strategies aim to provide demographic and genetic rescue amongst threatened populations by facilitating the admixture of new genetic material and demographic groups to ultimately enhance population growth and restore geographical distribution (IUCN/SSC, 2013; Mills, 2007; Leberg, 1993). Despite their theoretical advantages to population viability, it is argued that human-mediated enhancements to the admixture of individuals can result in homogenizing the genetic features of geographically distinct populations, causing a loss to the evolutionary and adaptive flexibility of local populations, thereby increasing the risk of reduced fitness through outbreeding depression (Canu *et al.,* 2013; Wyner *et al.,* 1999; Lacy 1987).

Phylogeographic studies are a popular tool for directing the genetic and demographic management of threatened species (Beheragaray, 2008). Using molecular markers, such as mitochondrial DNA (mtDNA), where mutational differences between distinct haplotypes are assumed to correlate with time since a common ancestor, populations that harbour distinct haplotypes potentially contain significant adaptive variation (Canu *et al.,* 2013; Lowe *et al.,* 2007; Wyner *et al.,* 1999). These units are typically referred to as evolutionary

significant units (ESUs) or conservation management units (MUs) and are characterised by the significant divergence of alleles (Dizon *et al.,* 1992) or diagnosable clusters of shared ancestry (Wyner *et al.,* 1999). Genetic data can therefore provide valuable guidance for captive breeding and translocation programmes allowing, if available, representative genetic founder populations to be maintained and released, reducing the risk of outbreeding depression whilst preserving genetic integrity and diversity (IUCN/SSC, 2013; Leberg, 1993). This can also inform on the spatial scale at which habitat connectivity for the focal species should be restored and of the environmental components that influence historic and contemporary gene flow so that recolonization routes can be predicted, monitored and facilitated by focussed management strategies (Frankham *et al.,* 2011; De Barba *et al.,* 2010).

The European water vole is one of 11 terrestrial mammals in the UK that are regarded as key species for conservation and, as part of the government's commitment to the Convention on Biological Diversity (Rio de Janeiro, 1992), are receiving targeted conservation action through the delivery of a species Biodiversity Action Plan (BAP) (Birks *et al.,* 2012; UK Steering Group, 1994). This plan aims to locally implement remedial actions that will counteract the widespread decline of populations across the UK which have been caused by habitat loss and fragmentation and predation by feral American mink (Strachan *et al.,* 2011). These actions include restoring habitat to improve ecological connectivity between extant populations, sustaining mink control and protecting key source populations to facilitate range expansion and the recovery of water voles within their historical distribution (JNCC, 2010). Given their range contraction over the past century however, the reintroduction and translocation of water voles has also become an increasingly popular tool for re-establishing populations in areas where they are unlikely to recolonize naturally (Moorhouse, 2004); a strategy that has been exacerbated by the need to relocate water voles as part of mitigation work driven by development and other landscape change (Strachan *et al*., 2011; Gow *et al.,* 2004a).

Given the widespread conservation effort to re-establish water vole populations across the UK, it is important that patterns in their historical and contemporary

gene flow are identified, to ensure that the genetic heritage, adaptive flexibility and diversity of water voles are maximised. Previous research by Piertney *et al. (*2005) on the national phylogeographic patterns of water voles, identified that there is a major division between the Scottish and English/Welsh lineages resulting from different post-glacial colonisation events. It was suggested that the two regions be treated as independent ESUs and recommended that admixing individuals may threaten both the viability of populations (Avise, 1995) and their evolutionary heritage (Piertney *et al.,* 2005). Piertney *et al. (*2005) also reported a significant divergence between sampled populations within regions, suggesting that conservation MUs be at least at the river catchment level. This is supported by the fact that water voles, like other small vertebrates, exhibit limited dispersal capabilities (~1.5 km) (Aars *et al.,* 2006; Berthier *et al.,* 2005; Telfer *et al.,* 2003; Stewart *et al.,* 1999b) and given their affiliation to wetland habitat, it is likely that their genetic heritage is also determined by geographical features such as river topology that may influence historical connectivity (Igea *et al.,* 2013; Berthier *et al.,* 2006). However, the phylogeographic structure of water vole populations within regions has not been examined. This information would not only identify historical geographical boundaries to gene flow and hence genetic lineages at a microgeographical scale, it may also prove useful in identifying the scale of population admixture resulting from recent reintroduction or restocking programmes.

Study Aims

This study examines the phylogeographical pattern of water vole populations within the southeastern counties of England, where widespread landscape modification and mink predation have resulted in the loss of up to 99% of historical populations (Smith, 2009). The area is representative of many regions in the UK whereby water vole populations have become increasingly isolated and targets for both human mediated and naturally facilitated recolonization are being implemented. This study aims to; 1) infer the geographical factors and ecological processes that may have influenced the distribution and diversity of genealogical lineages and 2) establish whether the phylogeographic pattern observed can be used to monitor the success and recolonisation of populations that have been reintroduced within the region.

7.2 Methods

7.2.1 Sampling locations and collection method

Samples of water vole hair used for phylogeographic analyses were collected from 13 locations across the south east of England between 2011 and 2014 (Figure 7.1). Samples were obtained using hair pulls (HP) from individuals captured during live capture at eight sites and by non-invasive hair capture tubes (NGS) placed at five sites (Table 7.1) (Full details of methods are provided in Chapter Two, Section 2.3.3). Two of the live capture sites, London Wetland Centre (NL-LW) and Arundel Wetland Centre (NL-AW) were receptor sites in 2001 and 2005 for 45 translocated and 171 captive bred individuals, respectively. Full details of introductions are provided in Chapter Two, Section 2.2.1. All genetic samples obtained were dried and stored at -20°C for a maximum of one month.

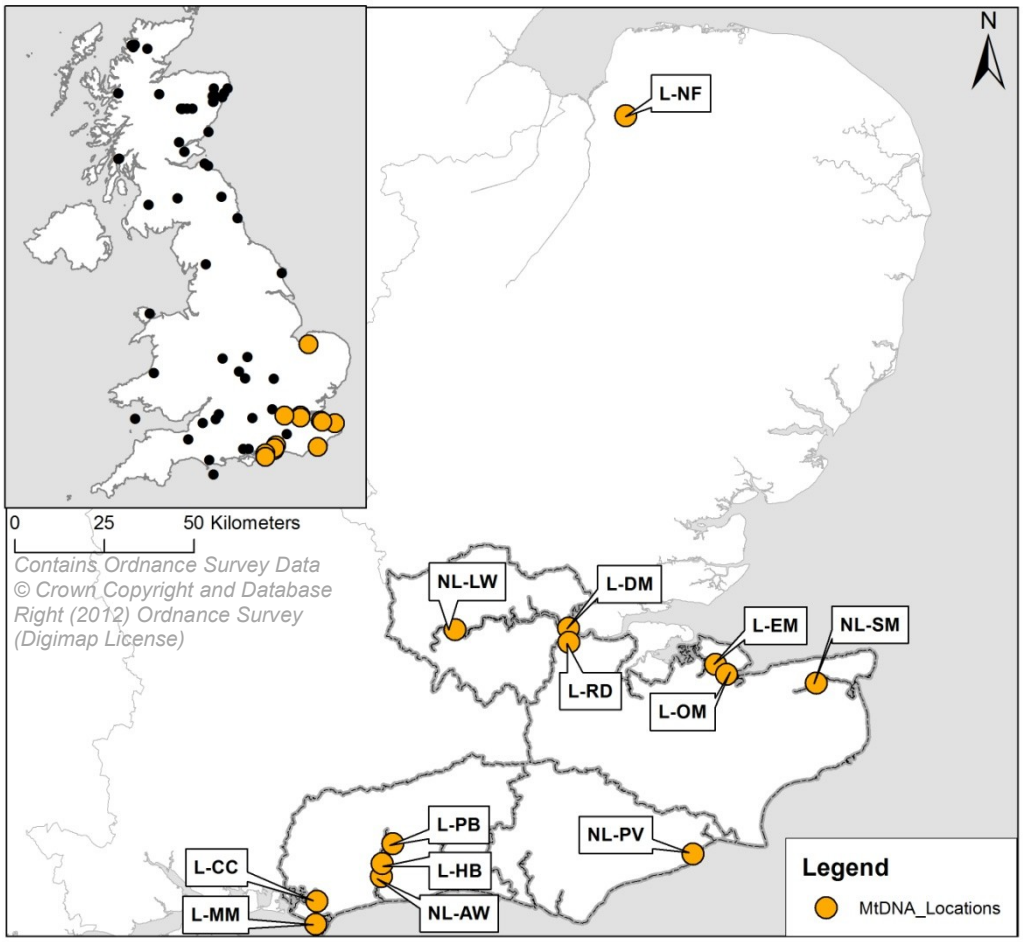

Figure 7.1 *Distribution of mtDNA samples collected from water vole populations in eastern and southeastern counties of England (grey lines). Inset map shows locations (black dots) of supplementary mtDNA sequences resolved by Piertney et al., (2005). Sample site prefix identifies linear = L and non-linear = NL sites.*

Pop No.	County	Sample Area Code	Pop Code	Lat (N)	Long (W)	Yr of sampling	Method
1	W. Sussex	MHP	L-CC	50°80	0°81	2012	LC
2	W. Sussex	MHP	L-MM	50°75	0°81	2013	NGS
3	W. Sussex	AV	NL-AW	50°86	0°55	2012	LC
4	W. Sussex	AV	L-HB	50°86	0°55	2013	LC
5	W. Sussex	AV	L-PB	50°95	0°50	2012	NGS
6	E. Sussex	PL	NL-PV	50°90	0°69	2012	LC
7	Kent	SM	NL-SM	51°32	1°21	2012	LC
8	Kent	NKM	L-EM	51°37	0°81	2012	LC
9	Kent	NKM	L-OM	51°35	0°85	2014	NGS
10	Gr.London	DF	L-RD	51°44	0°22	2012	LC
11	Gr.London	DF	L-DM	51°48	0°22	2012	NGS
12	London	LW	NL-LW	51°48	0°23	2012	LC
13	Norfolk	NF	L-NF	52°76	0°52	2012	NGS

Table 7.1 *Haplotype sampling location, year of data collection and sampling method (LC = Live capture; NGS = Non-invasive hair capture tubes). Population Code prefixes denote the patch geometry of the site where L = linear and NL = non-linear.*

7.2.2 DNA extraction, mitochondrial sequencing and data analyses

Total genomic DNA was isolated from both HP and NGS samples using Qiagen DNeasy™ Blood and Tissue Kit. A 736-base pair region of mitochondrial control region was amplified for between one and seven individuals sampled from each site (Table 7.3) using primers (F15708 5'-TTAATCTACCATCCTCC GTGAAAACC-3' and R92 5'-TKGACACTGGTCTAGGGATATTTGC-3') and polymerase chain reaction (PCR) conditions as described by Piertney *et al.* (2005) (described fully in Chapter Two, Section 2.4.2). Sequencing was performed using both forward and reverse primers for each individual using PeakTrace™ DNA sequence basecaller and run using Applied Biosystems 3730 series DNA Analyzer. Resulting sequences were edited and aligned manually using CLC Sequence Viewer 7.0.1. (CLC Bio, Aarhus, Denmark) (Xue *et al.,* 2013) against one Scottish *A. amphibius (formerly A. terrestris)* sequence retrieved from GenBank nucleotide database under accession no. AY948543. Ambiguous end regions were clipped and a total of six nucleotide insertions were identified resulting in 731 bases being analysed across all individuals. A further 27 *A. amphibius* haplotypes from the UK, France, Spain, Finland and Switzerland that have previously been resolved by Piertney *et al. (*2005) were input manually within CLC Sequence Viewer and used as

supplementary data to establish genealogical relationships with the sampled populations within southeastern England.

The number of haplotypes, haplotype diversity (*h*) (Nei, 1973), nucleotide diversity (π_N) (Tajima, 1983) and the mean number of pairwise differences (π) between all pairs of haplotypes (Tajima, 1983; 1993) was calculated for each sample population and across all samples. The variation in sequences observed amongst sampled populations was tested for selective neutrality using Tajima's (1989) *D* statistic, which compares estimates of the population mutation rate based on different effective population sizes to find departures from a neutral model based on demographic stability (Larsson *et al.,* 2013). The presence of rare variants that may have accumulated as a result of recent population expansions result in Tajima's D becoming negative, whereas the replacement of these with more frequent common variants (alleles at intermediate frequency) suggest a recent population reduction or bottleneck and a positive D value (Schmidt & Pool, 2002). The significance of departure from neutrality was tested using 10,000 permutations.

The phylogeographic structure of the eastern and southeastern haplotypes was investigated using the supplementary sequence data from the UK and Europe. A Bayesian Markov chain Monte Carlo (MCMC) approach implemented in the Programme MrBayes v3.2 (Ronquist *et al.,* 2011) was used to generate a consensus tree based on a standard substitution model where substitution rates were variable for each site and the probability of among site variation followed a gamma distribution and was invariable for a proportion of sites (the software's default settings). These model parameters concurred with the optimal model of *A. amphibius* sequence evolution found by Piertney *et al.* (2005). The Markov chain was run for 400,000 generations and sampled every 100 generations. A consensus was reached once the analyses of multiple trees (n=2) reached convergence, measured by a standard deviation of split frequencies of <0.03. The consensus tree was visualised using Programme FigTree v1.4.2 (Rambaut, 2006-2014). To aid recognition of the relationship between haplotype diversity and geographic location, haplotypes with recent shared ancestry ($\leq$ x mutational differences) were clustered into different haplogroups and their spatial

distribution was investigated in relation to a) geographical features including river topology and elevation; and b) reintroduction and translocation sites.

To investigate the evolutionary relationships between the sampled and supplementary haplotypes, a minimum spanning tree was calculated using Programme Arlequin v.3.11 (Excoffier *et al.,* 2005). The pairwise distance matrix between haplotypes, used to derive the most likely connections between unique haplotypes, was calculated using a modified algorithm of Rohlf (1973) see (Excoffier and Smouse, 1994). The minimum spanning tree was visualised using programme HapStar (Teacher & Griffiths, 2011).

7.3 Results

7.3.1 MtDNA sequence variation

A total of 14 unique and novel haplotypes, defined by 38 polymorphic sites were detected across 58 mtDNA sequences obtained from the 13 sampling locations (Table 7.2). Whole sequences for each haplotype are provided in Appendix Eight. Two haplotypes, WvSE_H02 and WvSE_H08 were most common occurring at frequencies of 34 and 28% respectively. WvSE_H02 was frequently observed in populations from Sussex and Kent, whilst WVSE_H08 haplotype was most common within the Sussex populations (Figure 7.2). A total of six private haplotypes were observed of which four were found in Sussex populations and the remainder were from Norfolk and Greater London. The other six haplotypes were observed at frequencies between 3% and 7%.

Among all samples obtained across east and southeast England, haplotype (*h*) and nucleotide diversity (π_N) was 0.80 (SD = 0.0366) and 0.004258 (SD=0.002) respectively and the mean number of pairwise differences between sequences was 3.108 (SD=1.640). Within populations, where five or more samples were obtained, haplotype and nucleotide diversity ranged from 0 to 0.80 and 0.007, respectively (Table 7.3). Pairwise differences between sequences for these same populations were zero for three sample sites where no polymorphism was found and was highest at the reintroduced site NL-AW where the mean number of pairwise differences between all pairs of haplotypes was 5.14 (SD=2.83).

Conversely, no diversity was observed at site NL-LW where the reintroduced population consisted of both captive bred individuals and individuals translocated from another London site.

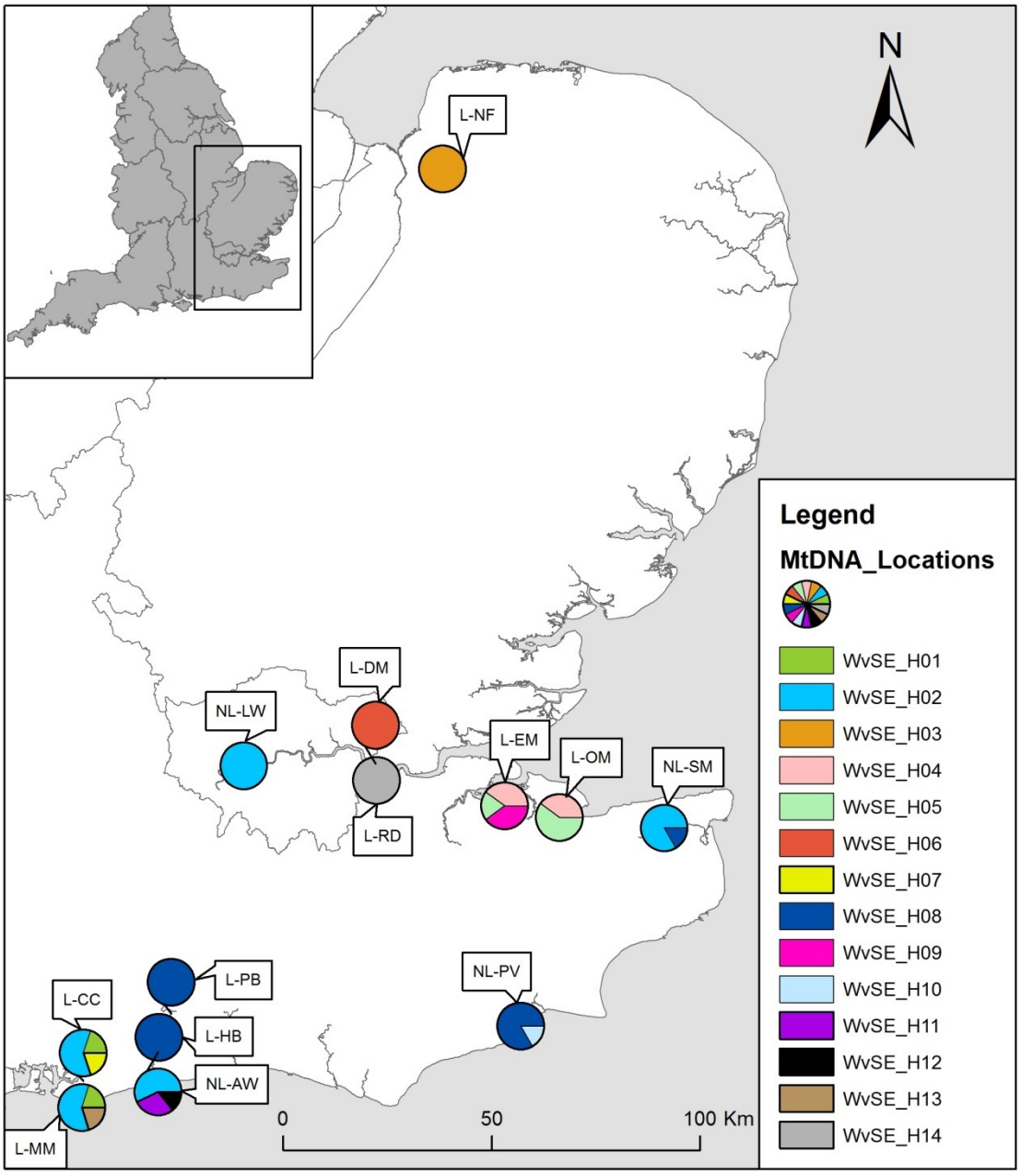

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Figure 7.2 *Map showing distribution of 14 mtDNA haplotypes for water vole (Arvicola amphibius) from data obtained from 13 locations across east and southeastern England.*

Nucleotide positions

Haplotype	11	12	262	270	320	330	335	343	346	361	366	367	369	373	381	391	392	394	407	414	423	436	445	451	458	484	503	517	529	531	548	570	595	607	637	638	658	691
WvSE_H01	T	A	A	C	G	T	T	A	C	C	T	C	A	A	A	A	G	A	T	A	G	-	-	A	T	A	T	T	T	A	C	T	-	T	T	T	T	T
WvSE_H02	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.
WvSE_H03	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	A	G	.	.	.	C	.	.	.	G	C	.	.	.	T	.	.	.	.	-	.	.
WvSE_H04	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.
WvSE_H05	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
WvSE_H06	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
WvSE_H07	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.
WvSE_H08	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.
WvSE_H09	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.
WvSE_H10	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.
WvSE_H11	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	G	.	-	.	C	.	.	.	.	.	.	C	G	.	.	A	.	-	-	.	.
WvSE_H12	.	.	.	.	.	C	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.
WvSE_H13	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
WvSE_H14	C	T	.	T	A	.	C	.	.	.	C	.	G	G	C	G	A	.	C	-	A	.	.	G	C	.	.	C	.	.	.	C	.	C	-	-	A	.

Populations

Haplotype	Sussex						Kent			London			Norfolk	Total
	L_MM	L_CC	NL_AW	L_HB	L_PB	NL_PV	NL_SM	L_EM	L_OM	NL_LW	L_RD	L_DM	L_D5	
WvSE_H01	1	1												**2**
WvSE_H02	3	3	4				5			5				**20**
WvSE_H03													1	**1**
WvSE_H04								2	2					**4**
WvSE_H05								1	3					**4**
WvSE_H06												2		**2**
WvSE_H07		1												**1**
WvSE_H08				5	5	5	1							**16**
WvSE_H09								2						**2**
WvSE_H10						1								**1**
WvSE_H11			2											**2**
WvSE_H12			1											**1**
WvSE_H13	1													**1**
WvSE_H14											1			**1**
Total	**5**	**5**	**7**	**5**	**5**	**6**	**6**	**5**	**5**	**5**	**1**	**2**	**1**	**58**

Table 7.2. *Polymorphic sites of 14 unique haplotypes from 58 samples of water vole mtDNA collected across eastern and south eastern regions of England (top). Nucleotide positions are numbered according to reference sequence WvSE_H01 and nucleotides identical to reference sequence are denoted by dots (.) and deletions are denoted by a dash (-). Number and distribution of haplotypes sampled per study site is shown in the bottom half of table.*

Pop Code	No. Samples	No. Haplotypes	*h*	π	π_N
NL-AW	7	3	0.67	5.14	0.0071
L-CC	5	3	0.70	1.20	0.0016
NL-LW	5	1	0.00	0.00	0.0000
NL-SM	6	2	0.33	0.33	0.0005
L-MM	5	3	0.70	2.20	0.0030
L-NF	1	1	0.00	0.00	0.0000
L-EM	5	3	0.80	1.80	0.0025
L-OM	5	2	0.60	2.40	0.0033
L-DM	2	1	0.00	0.00	0.0000
L-HB	5	1	0.00	0.00	0.0000
L-PB	5	1	0.00	0.00	0.0000
NL-PV	6	2	0.33	0.33	0.0005
L-RD	1	1	0.00	0.00	0.0000

Table 7.3 *MtDNA diversity parameters for 13 sampled water vole populations including sample size, number of haplotypes, haplotype diversity (h), mean number of pairwise differences between haplotypes (π), nucleotide diversity (π_N).*

Amongst the sampled populations within the east and southeast of England, Tajima's D was -2.41 and showed significant departure from selective neutrality (p = 0.000) and thus support that the population has undergone a recent expansion (Schmidt & Pool, 2002).

7.3.2 Phylogeographic structure

The phylogeographic structure of haplotypes sampled from water vole populations in the east and south-eastern counties and their relationship with the UK and European haplotypes previously resolved by Piertney *et al. (*2005) are shown in Figure 7.3. In concordance with Piertney *et al. (*2005) there is a major division between the English/Welsh and Scottish haplotypes, representing a mean number of nucleotide differences of 21.7 between the two major clades. Of notable interest is the well-supported assignment of the London sourced haplotype, WvSE14, to the Scottish clade. The remaining eastern and southeastern haplotypes were clustered within the English clade within which a further two major clades were identified and well supported. This division separated haplotypes originating from reintroduction site NL-AW and clustered the remaining samples with more geographically distinct populations including Birmingham, Oxford and Warwick, in that the haplotypes are essentially indistinguishable. More recent divergence was identified in North

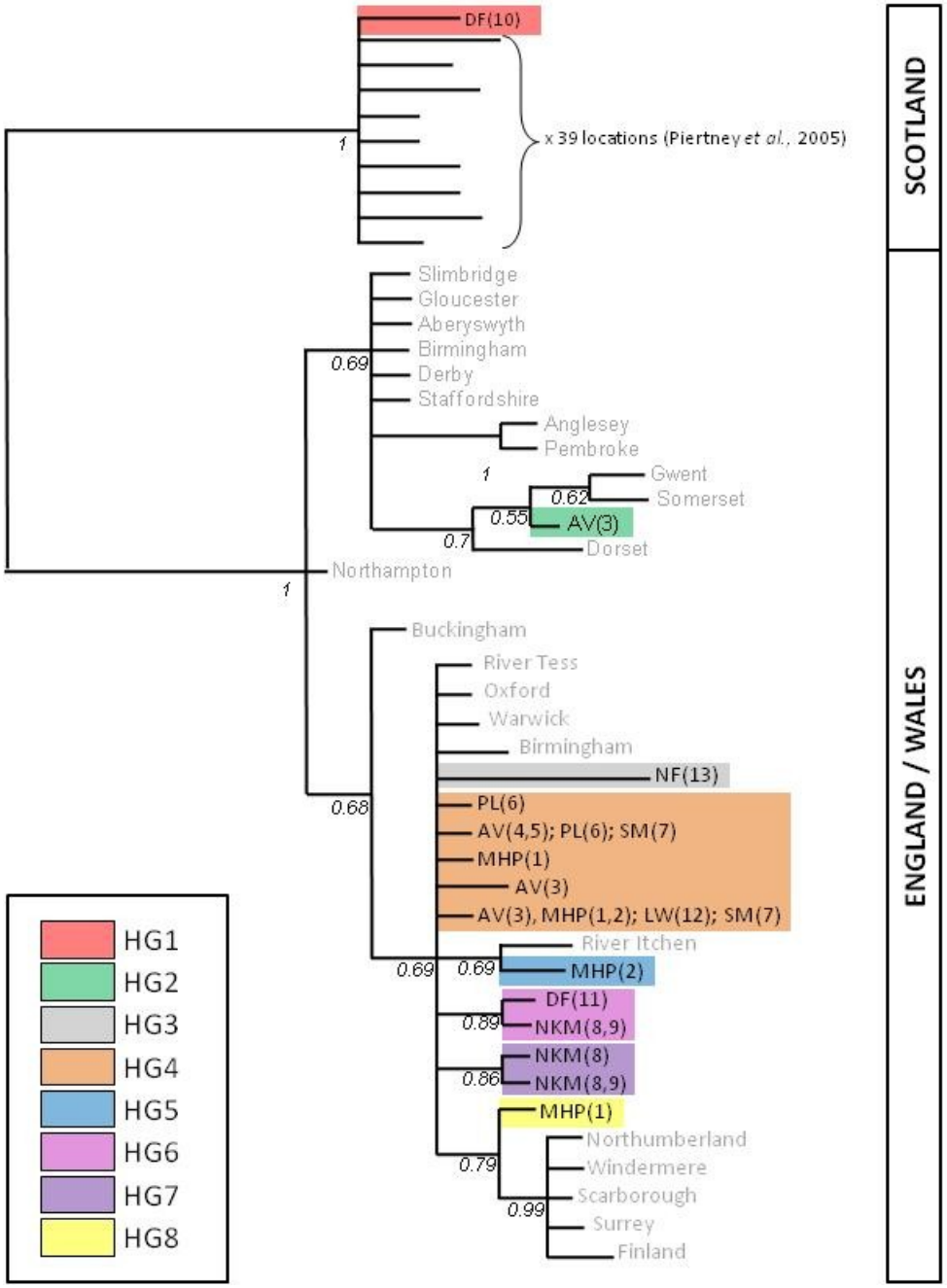

Figure 7.3 *Phylogenetic tree from Bayesian analysis of aligned mtDNA sequences from water voles sampled across 13 study sites and supplementary sequences obtained from Piertney et al., (2005). Bayesian posterior probability values for each branch are shown below nodes. Sampled populations are identified by black labels showing study region code and site number as detailed in Table 7.1. Scottish supplementary mtDNA sequences have been collapsed for simplicity and English/Welsh supplementary data is shown in full to visualise evolutionary relationships with sampled haplotypes from water voles in east and south eastern England. Coloured boxes represent haplogroups of sequences from sampled populations which share most recent ancestry (considering branch length and internal nodes) and are presented in Figure 7.5.*

Kent, where two unique clades were identified amongst the Isle of Sheppey (L-EM), North Kent Marshes (L-OM) and Dartford Marshes (L-DM). Similarly, sites L-CC and L-MM, located on the Manhood Peninsular, West Sussex, also showed divergence and unique ancestry from the shared ancestry exhibited amongst much of Sussex and Kent. The notable branch length of the Norfolk site L-NF also indicates a greater divergence from the shared common ancestor.

The minimum spanning network derived for both sampled and supplementary UK haplotypes is shown in Figure 7.4. The English/Welsh and Scottish clades are clearly visible representing a minimum separation of 16 mutational steps. Haplotype WvSE14 sourced from London site (L-RD) is clearly clustered within the Scottish clade, as per the tree topology in Figure 7.3, and is separated from the east and southeastern samples by a minimum of 30 mutational steps. The closest affiliation to this haplotype is Inverness, which is the common ancestor for a further nine Scottish lineages (see Piertney *et al.,* 2005). Within the English clade, haplotypes WvSE11 and WvSE3 show the furthest affiliation from other known English and Welsh haplotypes and are derived from sites NL-AW and L-NF respectively. The divergence of WvSE3 is in concordance with the geographical separation of site L-NF from the other sampling localities (both sampled and supplementary data) (Figure 7.2), however, haplotype WvSE11 did not originate from a geographically disparate sampling area. The closest known affiliated haplotype to WvSE11 is Somerset (SS), which represents a minimum of six mutational steps. However, when compared to haplotypes WvSE2 and WvSE12, which originate from the same site, this haplotype is further diverged with a minimum of 12 and 14 mutational steps.

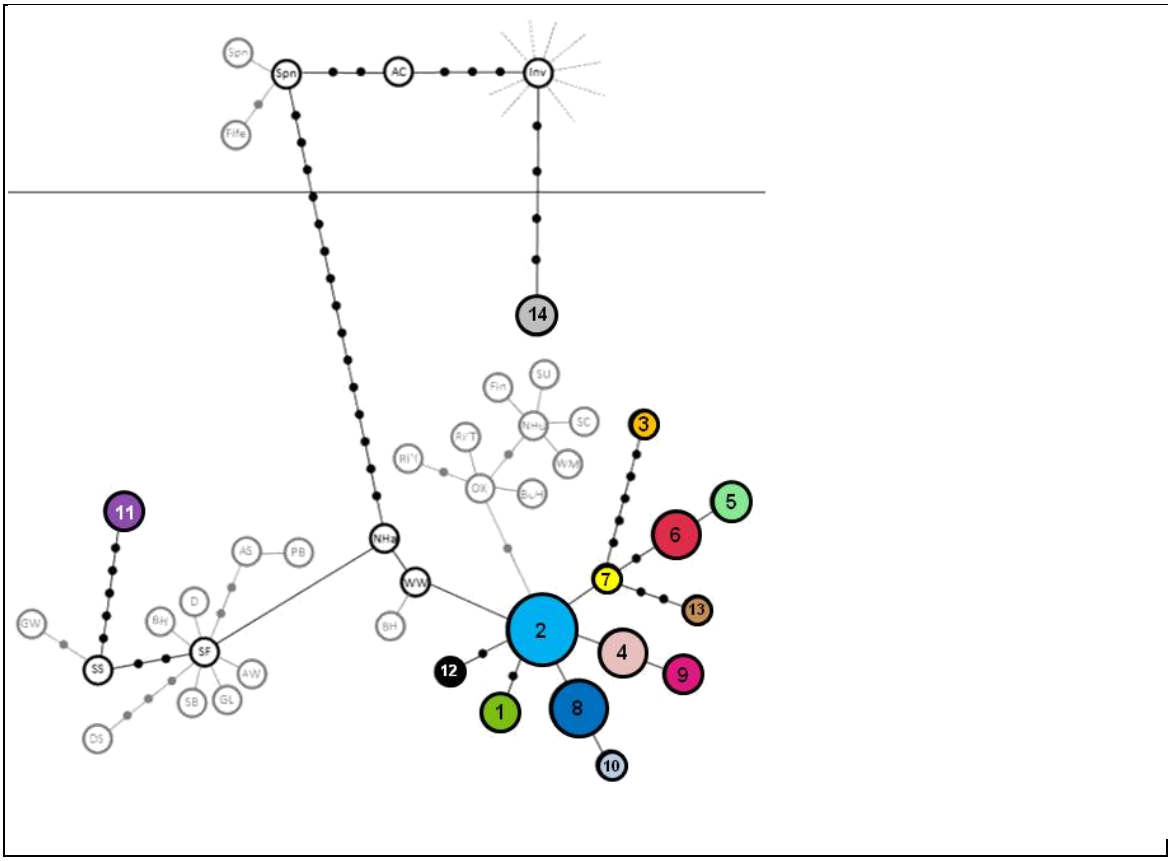

Figure 7.4 *Minimum Spanning Tree constructed from a matrix of pairwise distances between 14 south eastern haplotypes identified across the 13 sampled populations (solid fill) and English/Welsh and Scottish haplotypes previously resolved by Piertney et al. (2005) (outlined). Sampled haplotypes are numbered and correspond to Table 7.2 and coloured to correspond to Figure 7.2. Supplementary haplotypes are labelled by their respective sampling location which have been abbreviated as displayed on right hand side of figure . Genealogical relationships between published (Piertney et al,. 2005) and sampled populations are shown in black and solid dots represent inferred mutational steps between haplotypes.*

7.3.3 Geographical and management influences on phylogeny

The geographical distribution of the unique clusters of haplotypes identified within the southeastern samples is shown alongside major geographical features and known reintroduction sites, in Figure 7.5. This map indicates that there is a large geographical spread of haplogroup 3 (HG3) across Sussex, Kent and London, which include the commonly represented haplotypes WvSE_H02 and WvSE_H08. This spread concurs with the connectivity of river catchments across Sussex and Kent and supports the occurrence of unique groups HG5 and 6, which are clearly isolated in terms of river topology.

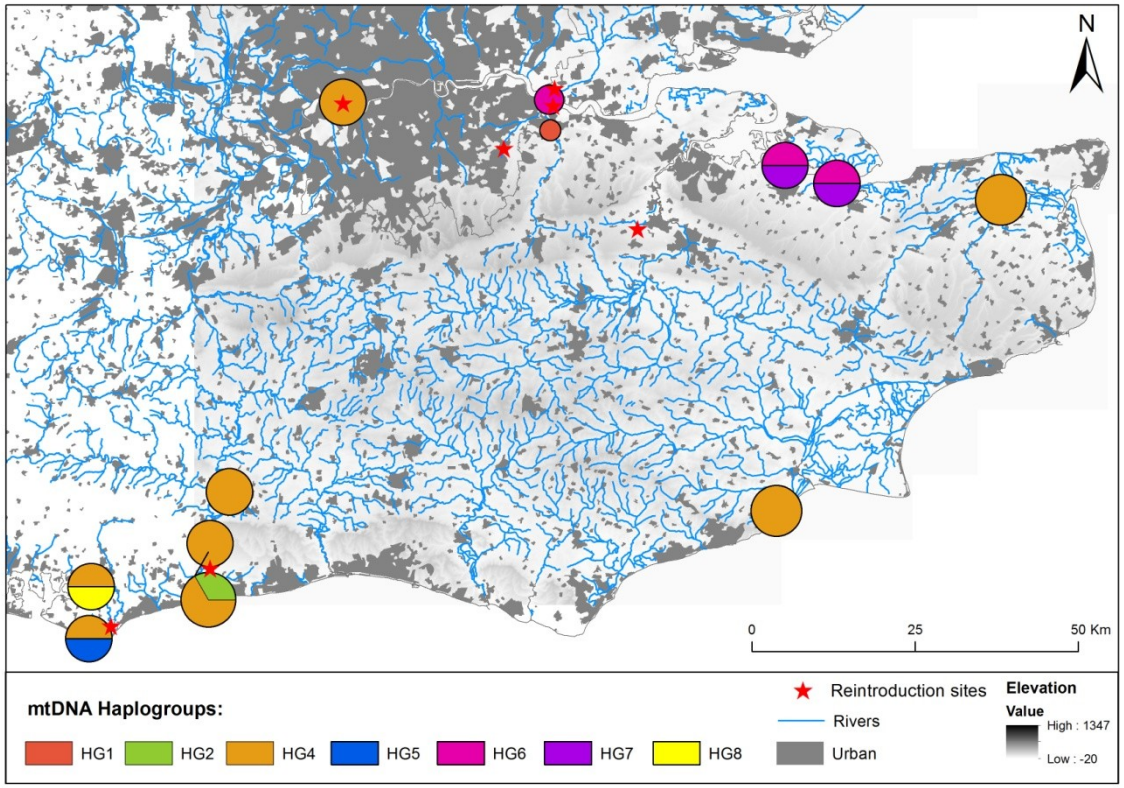

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Figure 7.5 *Geographical features and reintroduction sites in relation to the spatial distribution of haplogroups (HG) identified using tree topology of mtDNA sequences from south eastern water vole populations presented in Figure 7.3. Pie charts are sized according to the number of individuals sampled per site and show proportion of haplogroups observed within each population. Haplogroup HG3 from site L-NF (eastern region) is not shown as it represents genealogical separation due to geographical distance and not geographical features.*

7.4 Discussion

This study has identified fine scale phylogeographic structure in water vole populations occupying modified landscapes in the southeastern region of the UK, facilitating the exploration of both historical and contemporary processes that best explain the distribution and diversity of genealogical lineages. Of notable importance is the genetic signature of reintroduction and restocking programmes that have contributed to both the diversity and divergence that is observed amongst populations. Whilst this certainly has important consequences for the future of human mediated conservation and monitoring strategies, it is highly plausible that the unnatural movement and breeding of genetically distinct individuals has masked the relative contribution of geographical features and demographic history. Despite this, the possible associations between regional genetic structure and both natural and human induced processes and the implications of these for water vole conservation are discussed in detail below.

The overall variability in mtDNA observed in the southeastern region, in terms of nucleotide and haplotype diversity are indicative of the biogeographic history of water voles across Europe which has been proposed and supported by phylogenetic studies by Taberlet *et al. (*1998) and later, Piertney *et al. (*2005). Both studies suggest water vole populations have expanded after postglacial colonisation from European refugia, a scenario that would result in the low nucleotide diversity (π =0.004, SD=0.002) and high haplotype variability (h=0.8, SD=0.03) that is observed here. This is because the further distance from refugia, the greater loss of genetic variation due to the inherent reduction in the effective population size of colonizers (Hewitt, 1996; Nei *et al.,* 1975). However, unlike haplotype diversity, which is derived from new mutations and that occur at a relatively high rate in microtine rodents (0.5-1.0% per Myr) (Piertney *et al.,* 2005), nucleotide diversity takes a much longer time to accumulate due to its dependence on the contribution of moderate to high frequency alleles (Holsinger & Mason-Garner, 1996). These assumptions are further supported given that the observed variability and relationship between both nucleotide and haplotype diversity are consistent with a predictive model of mitochondrial

variation proposed by Goodall-Copestake *et al. (*2012) where $\pi=0.0081h^2$ (based on a meta-analyses of the Cox1 mtDNA variation observed across 23 animal species). As such there is no reason to assume that the variation observed in this study reflect any anomalous biological processes or methodological issues.

Phylogenetic and network analyses revealed that the majority of haplotypes obtained from the southeastern county were clustered within the English clade representing their genealogical association to each other and their geographic origin. The deep phylogenetic structure within this clade and pattern of common, widespread haplotypes connecting to several other population-specific haplotypes within the study region, as shown in the Minimum Spanning Tree (Figure 7.4) is consistent with a relatively recent range expansion of water voles into new habitats following the retreat of the Pleistocene ice sheets and explains the significant negative Tajima's D statistic resulting from the presence of rare alleles that accumulate during the expansion phase. The high frequency and widespread distribution of haplotypes 2 and 8 (shown in Table 7.2 and illustrated in Figure 7.2), for example, are likely to represent ancestral haplotypes within the southeastern region in which population specific haplotypes have evolved or become subsequently restricted (e.g. haplotypes 1 & 13 on the Manhood Peninsular, haplotypes 4, 5 & 6 in North Kent and haplotype 10 in East Sussex and discussed in more detail below). This geographical association also corresponds with the higher divergence in terms of branch length and nucleotide differences observed with haplotype 3 obtained from the Norfolk (L-NF) population, which is located 150 km from the nearest other sampling location. Two haplotypes (14 and 11, Figure 7.4), that were obtained from the study area, however, showed notable divergence from other regional haplotypes, which cannot be explained by their geographical association or biogeographical history and thus warrant further exploration.

The most notable divergence was observed with haplotype 14, obtained from the River Darent population near Dartford (L-RD), which, after replicated sequencing and alignment, represented a minimum of 30 mutational steps from the ancestral haplotypes present within the southeastern region. The well-

supported assignment of this lineage to the Scottish clade, which consists of populations that are highly divergent from English/Welsh populations, is likely due to separate post-glacial colonisation events (Piertney *et al.,* 2005). The most plausible explanation is that a River Darent bloodline was introduced as part of the restocking or reintroduction programmes that have been widely implemented across the study region. The closest potential source population known to have been augmented with captive bred stock is located approximately three kilometres from the sampling site at Dartford Park, where mitigation works forced the temporary holding of 130 water voles in captivity during 2004 (Gow, 2012b). Whilst it was originally proposed that the progeny of this population would be released back into the site after habitat restoration was complete, the final release did not occur until 2009 and 2010. It is therefore plausible that there was an admixture of this stock with other bloodlines during this time, particularly when the loss of diversity in captive bred environments is expected to threaten population fitness and post-release viability (Tracy *et al.,* 2011). Without access to accompanying studbook records, this scenario can only be hypothesised at this time. However, given that the admixture of Scottish and English lineages risks not only the loss of genetic heritage in the area but also the loss of population fitness resulting from outbreeding depression, the presence of this lineage within lowland England is a concern.

Considerable geographic divergence was also observed for haplotype 11, which was present in the reintroduction site (NL-AW) at Arundel Wildfowl and Wetland Trust in West Sussex. This haplotype, although clustered within the English/Welsh clade, showed a closer affiliation to ancestral lineages derived from the western counties than those observed in the southeastern region. This is particularly evident in the Minimum Spanning Tree where haplotype 11 is clearly separated from the southeastern lineages that are clustered by their genealogical association. This haplotype is likely to represent one of seven bloodlines that had been captive bred and introduced into the site in 2005 and the most divergent of three identified across the seven individuals sampled from the population in this study. Given the high diversity of lineages that were observed in this population, over five years after their original release, it is evident that haplotypes that are typically lost post release due to a high

mortality of introduced individuals (Tracy *et al.,* 2011), have at least been partly retained. This suggests that either the survival of released individuals was high or that the diversity of lineages that were introduced resulted in increased population growth rates. Similar increases in demographic performance have been observed in other mammal populations when they have been augmented with new genetic lineages (Frankham *et al.,* 2011; Fredrickson *et al.,* 2007; Edmands, 2007) and occur when recessive deleterious alleles that result from inbreeding and cause reductions in a population's fitness become masked in heterozygotes when new alleles from new populations are introduced (Heber *et al.,* 2012; Fredrickson *et al.,* 2007). This strategy is thus useful for reintroduction purposes when the number and genetic diversity of founding populations is limited yet the outcome of post release population growth and expansion is required (IUCN/SSC, 2013). Interestingly however, the haplotypes observed at the release site were not found within samples obtained from the two populations (L-HB, L-PB) located >6.5 km within the same river corridor suggesting that migration from the site is limited either by the predator proof fencing surrounding the reintroduction site or by distance. This is surprising given that water voles in the area were considered on the brink of extinction (Booth, 1998) prior to the reintroduction at Arundel, but showed a significant increase in distribution after 2005, which was notable up to 10 km from the release site (Baker *et al.,* 2009). A possible scenario is that migration is male biased and therefore the introduced lineages have provided genetic rescue to extant populations within the river valley but have gone largely undetected given that mitochondrial DNA is maternally inherited. This is further supported by the fact that the only haplotype observed at the other two populations (Haplotype 8) occurs frequently amongst other extant populations across Sussex and Kent (see Table 7.2), suggesting it is an ancestral haplotype in the southeastern region and represents a relic population. These inferences should be considered with caution however, given that only a small number of individuals were sampled from each of these sites. Although the reintroduction of water voles at Arundel was successful, it is a matter of controversy whether the introduction of new lineages that have no geographical association to release sites is a suitable strategy for species conservation given their contribution to the loss of locally derived genetic heritage.

Amongst the other haplotypes identified and affiliated to the southeastern region, a wide variation in haplotype and nucleotide diversity was observed between the study sites reflecting differences in the population age, demography and intra-population connectivity and thus the distribution and conservation of mitochondrial lineages. Whilst this partly reflects variation in sampling intensity between populations, comparatively low variation in haplotype number was observed in the multiple samples obtained from study sites located in the Arun river Valley (discussed above), the Pett Levels (NL-PV), Stodmarsh (NL-SM) and the London Wetland Centre (NL-LW). Only three haplotypes were identified across these populations, two of which (haplotypes 2 and 8) commonly occur at high frequencies suggesting that these are ancestral lineages and thus represent relic populations in the study area. Whilst this corresponds to the historical distribution of most of the populations considered here, it is known that the NL-LW population consisted of individuals that were translocated from a mitigation site on the Thames catchment, suggesting that there was historical migration between Sussex, Kent and London river catchments (discussed in more detail below). The relatively low nucleotide diversity observed within these sites could suggest that a loss of alleles resulting from populations becoming small and more isolated by the contemporary landscape changes, or that historical gene flow between the sites was effective in homogenising any genetic divergences that may have evolved. Given that both Stodmarsh and the Pett Levels are considered as national and regional strongholds for water voles, respectively, where the abundance and connectivity of populations is thought to have been relatively unaffected by factors causing populations declines elsewhere (Strachan *et al.,* 2011), it seems that the homogenising effects of historical gene flow may be more indicative of the pattern in variation observed here.

In contrast, high variation was observed within natural populations located on Elmley (L-EM), the North Kent Marshes (NKM) (L-OM) and the Manhood Peninsular (MHP) (L-CC, L-MM) (Table 7.3). This is likely to be a result of both local and regional processes. Firstly at a local scale, these areas are recognised as either national (Elmley & NKM) or regional (MHP) strongholds for water voles and as such the relatively high diversity in mitochondrial lineages

observed here is likely to be attributable to the presence of relatively large effective population sizes that have been maintained through metapopulation management. Secondly, from a regional perspective, these sites are located within discrete river catchments, which are relatively isolated in relation to the interconnected river network that covers much of Sussex and west Kent. This may have not only facilitated the effective control of mink due to the reduction in defensible waterways in these areas (Zalewski *et al.,* 2009) and thus helped to maintain large and genetically diverse effective populations, but their geographic location would also be expected to invoke higher genetic divergence as a result of decreased rates of immigration, a process that is commonly observed in marginally located sites (Qiang & JinZhong, 2011; Eckert *et al.,* 2008). This is supported by the presence of unique haplotypes (1, 7 & 13 on the MHP and 4, 5 & 6 within Elmley & NKM) and interlineage divergence (haplogroups 4, 5 and 6 shown in Figures 7.3 & 7.4) that has partitioned the mtDNA variation of these populations from the other study sites.

Based on these inferences, the pattern of phylogenetic structure observed amongst water voles in the southeast is consistent with the distribution of interconnected river networks, which suggest that these have acted in facilitating gene flow. Similar patterns have been observed in microsatellite variation amongst water vole populations occupying distinct river catchments in Scotland (Stewart *et al.,* 1999 b) and in Norway (Melis *et al.,* 2013) and are common in other small vertebrate populations affiliated with wetland habitats, where genetic divergence can be exacerbated by topographical relief (Storfer *et al.,* 2010). This may also be the case with water voles given the well-supported inter-lineage divergence of populations from Elmley (L-EM), the NKM (L-OM) and Dartford Marshes (L-DM), which are located north of the Kent Downs. However given that there is a closer affiliation of the other North Downs population, NL-SM, with Sussex haplotypes suggests that river connectivity was a greater influence on gene flow historically.

7.5 Conclusions

This is the first study to use the regional patterns evident in the water vole phylogeny to glean insight into the processes that have shaped the current distribution and diversity of natural and reintroduced populations, for the purpose of directing the conservation management of captive and wild populations both locally and nationally. This study also revealed that there was a high diversity in geographically affiliated mitochondrial lineages conserved within southeastern England at the local scale. This was surprising given that up to 99% of historical populations have at one time been lost from areas within the study site. However, the implementation of national and regional measures to conserve metapopulations, particularly in North Kent and the MHP, have effectively conserved divergent lineages which represent their genetic heritage and biogeographic history. And so it is apparent that, at least at the level of the mitochondrial DNA, there remains a pattern of geographic structure that is consistent with historical isolation, rather than an artefact of recent reintroduction. This raises the issue that geographic variation could still be preserved at the local scale, which may be desirable for maximal conservation as well as mitigating the effect of local adaptation and outbreeding depression. The pattern of phylogenetic structure revealed by the mitochondrial control region diversity suggests that the conservation management of water voles should focus upon discrete river catchments in order to minimize genetic erosion whilst preserving subspecific distinctiveness. This concurs with the study by Piertney *et al.* (2005) in which significant mtDNA variation was observed between regional populations across the UK and suggested that the appropriate scale of management be at the watershed level.

This study has highlighted, however, that the implementation of reintroduction and restocking programmes for conservation and mitigation purposes poses an inherent threat to the genetic heritage and diversity of water vole populations, due to the release of divergent lineages that have no geographical association to receptor sites. Of notable concern is the possible release of Scottish derived lineages within lowland England which, given their glacial separation, could result in reductions to local population fitness due to the potential disruption that

genetic exchange would cause to coadapted gene complexes (Frankham *et al.,* 2011). This undoubtedly warrants further investigation, using additional samples that were obtained from this site to determine the prevalence of this lineage within the local population. However, it has highlighted that there is a fundamental failure in current legislation to license the introduction of captive bred water voles and to ensure appropriate admixing in captivity to secure the viability of the release stock and that of the natural populations in which it may interbreed.

This study has highlighted the usefulness of phylogenetic approaches for understanding past and contemporary processes that have shaped the current distribution and genetic diversity of water vole populations in southeastern England, which is critical for directing future conservation measures that aim to ensure their long-term viability and adaptive potential. It has also identified the genetic signature of reintroductions and as such could provide a useful post release tool for monitoring population expansion and admixture.

Chapter Eight: General discussion, methodological considerations and conservation implications

8.1 Background to study

Human landscape modification is one of the most ubiquitous and serious threats confronting the long-term persistence of species worldwide. As natural habitat continues to be altered in size and shape, species are becoming increasingly confined to small, geometrically altered patches that are embedded within perceptively hostile environments. For effective management that will secure the long-term viability of species, a quantitative understanding of the implications of habitat loss and fragmentation on species viability is required. A considerable body of research has already contributed to understanding how populations and species are perturbed by the loss and fragmentation of natural habitat, revealing that it can provoke changes in biological processes at almost every ecological level from the genesis and behaviour of individuals, to population dynamics, persistence and the ultimate extinction of species (Temple *et al.,* 2006). Perhaps one of the most concerning and reoccurring of themes, however, is that species do not respond to landscape change in an obviously deterministic manner: knowledge of key population parameters appear to be only weakly predictive. This restricts the ability of conservation biologists and wildlife practitioners to identify causal processes so that adequate projections can be made and incorporated into remedial conservation strategies that will secure the long-term viability of species that have become threatened by anthropogenic processes of landscape modification.

The European water vole is a species of high conservation importance in the UK where it has undergone one of the fastest documented declines of any British mammal during the past century (Strachan & Jefferies, 1993; Lawton & Woodroffe, 1991). The causes of decline have primarily been driven by landscape change, which has seen large areas of extensive wetlands being lost through the process of land drainage for agriculture and development (Jefferies *et al.,* 1989). The progressive intensification of land use has also resulted in semi-natural vegetation along river systems becoming increasingly narrow,

patchily distributed and bounded by unfamiliar and hostile land use types (Rushton *et al.,* 2003). As such, many water vole populations are now fragmented and confined to linear remnants of formerly more extensive wetland systems, where they are postulated to be at increased risk of predation by feral American mink that have colonised British river systems over the past 60 years (Baretto *et al.,* 1998b). Consequently, conservation strategies have been implemented that aim to re-establish populations within their historical range through human-mediated enhancements to wetland habitat and by reintroducing populations where natural colonisation is unlikely to occur. However, there is currently limited knowledge on the conservation value of non-linear wetlands for water voles, specifically whether they may provide suitable refugia from mink and what the appropriate criterion is for delineating areas that require separate management for securing genetic diversity and heritage.

A rapidly emerging field that has wide applications for species conservation is ecological genetics that combines ecological fieldwork with laboratory genetics to quantify the effects that habitat loss and fragmentation has on species persistence. The combination of demographic and genetic studies can reveal important information about population size and demographic stability, social structure and kinship and the rate and scale of dispersal that may otherwise be left unanswered or would be logistically difficult to measure. Furthermore, it allows for important historical and contemporary divisions of genetic varition to be identified so that genetic diversity and heritage can be secured, through management, to enable species to adapt to the selective pressures of contemporary landscapes and a changing climate.

This thesis presents the results of a comprehensive study that has combined genetic and demographic analyses to study water vole populations occupying different wetland habitats in south east England. The study had two primary aims. The first was to investigate patterns in the demography, kin structure and genetic diversity of water vole populations occupying linear and non-linear wetlands to inform on the conservation value of different wetland systems for water voles. The second was to investigate historical and contemporary factors that have influenced the spatial patterns in microsatellite and

mitochondrial variation to inform on suitable units for both human mediated and naturally facilitated recolonisation of the species. To aid in the collection of genetic samples from wild water vole populations, this thesis also presents the results of a pilot study that investigated the effectiveness of non-invasive hair capture tubes for obtaining DNA samples for microsatellite analyses.

8.2 Patterns in demography, kin structure and genetic diversity of populations occupying different wetlands in southeast England

The study populations of water voles showed considerable variation in their demographic patterns which reflect different ecological conditions and responses to environmental perturbations that occurred during the study period. For instance, the relative abundance of water voles at sites NL-AW and L-EM was significantly higher than other study sites which can be accrued to the protection of these populations from mink, whilst low survival rates characterised site NL-SM which concurred with reports of mink within the study area during the duration of study. Furthermore, spring droughts in 2012 and extensive flooding over the winter of 2012/3 resulted in variable population growth rates and significant variations in the age structure, relative abundance of young of the year and both the operational and young of the year sex ratios. The high variation observed amongst the study populations limited the ability to detect significant variations in demographic patterns between linear and non-linear habitats and potential bias would have accrued from those sites where high numbers of captures provided more robust estimates of demographic parameters.

Despite this, by combining the demographic field data with genetic analyses, evidence from this study suggests that habitat linearization does influence patterns of demography, kin structure and genetic variation. This study found that water vole populations occupying linear wetland systems were characterised by a significantly lower proportion of overwintered females in spring which reflected both higher losses of females and increases in the proportion of males between populations over winter. Results from both

demographic and kin structure analyses demonstrated that lower abundances of overwintered females in linear systems resulted in higher natal philopatry in females. This is shown by the significantly higher proportion of sexually mature females that were estimated as close relatives (1st or 2nd order kin) in linear sites in autumn, and by the significantly higher proportion of both breeding and young of the year females that were observed in the autumn cohorts. The accumulation of related females is proposed by Chesser (1991) to be a prerequisite for kin selection and Lambin & Krebs (1991) argue that the social organisation across *microtus* species is based on female biased kin clusters which are most prevalent at the end of the breeding season. The patterns of water vole populations in linear systems are partly in concordance with this, whereby related females accumulate during the breeding season as a result of female natal philopatry. However, this did not consistently result in the formation of female kin clusters, as between 20% and 40% of neighbouring breeding females were estimated to be closely related. This may partly be explained by the increased competition for resources, as the proportion of neighbours estimated to be closely related was negatively influenced by the relative abundance of females in both habitat types. Increases in relative abundance of overwintered females also negatively correlated with the relative abundance of young of the year that were observed in the linear populations in autumn. Competition for space and female territoriality are characteristics of the social organisation of water voles at the beginning of the breeding season (Strachan *et al.,* 2011; Lambin & Krebs, 1991) and the results of demographic patterns and kin structure in linear populations are in support of this.

In non-linear wetlands, the overwintered cohort consisted of a significantly higher proportion of breeding females which positively influenced the relative abundance of young of the year that were observed in populations in autumn. This concurs with the expectation in this study, that competition for resources would be less pronounced in non-linear systems, resulting in lower dispersal rates. However, the proportion of closely related breeding females in non-linear wetlands was significantly lower than in linear sites, which suggests that decreased dispersal did not result in kin clusters amongst breeding females. One factor that may contribute to this pattern is that sexual maturation of young

within the year of birth was significantly reduced when breeding female abundance increased in non-linear wetlands. Thus, kin clusters may have been more prevalent between breeding females and their non-breeding relatives. Similar patterns have been observed in meadow vole *(Microtus pennsylvanicus)*, whereby the average maturation rate of young in the year of birth was negatively related to density of females (Boonstra *et al.,* 1989). Postponing maturation may reduce the risk of competition, the risk of inbreeding, or both (Lambin, 1994). In this study, the significantly higher proportion of males that were observed in the autumn breeding and young of the year cohorts in populations occupying non-linear wetlands suggests that sexual suppression of female young of the year could be a strategy that avoids female kin mating with their fathers or opposite sex sibs. Inbreeding avoidance is considered a strong mechanism that leads to sex biased dispersal and is commonly undertaken by males near attainment of sexual maturity (Lambin, 1994). The results of this study concur with this, as female water voles had a 4.3 times higher probability of being recruited into the breeding cohort and relatedness amongst breeding males was generally lower than between breeding females in autumn. However, opposite sex breeding pairs estimated to be close relatives were observed in populations occupying both linear and non-linear sites, which reflects that natal dispersal by males is not always the rule in water voles or that daughters have sexually matured whilst their father was still alive. Potential father daughter mating does occur as a result of female philopatry in meadow voles (Boonstra, 1989), white footed mice, *Peromyscus leucopus* (Wolff *et al.,* 1988) and Townsend's vole, *Microtus townsendii* (Lambin & Krebs, 1991 b) and results of this study suggest this may also be the case for water voles, particularly in populations occupying linear wetland systems where significant inbreeding coefficients were observed. Incidences of inbreeding are likely to have accrued in linear populations where a significantly higher proportion of breeding adults were estimated to be close relatives and where between 50% and 75% of breeding females were closely related to one or more breeding male within the same populations. As there was no evidence of sexual suppression in female water voles from linear sites, this is likely to be evidence of father daughter matings. Inbreeding is expected to be elevated in fragmented habitat where restricted dispersal and small populations result in

elevated genetic similarity among potential mates and where the shape of the patch can reduce spatial avoidance and thus the ability to select for less-related mates (Banks *et al.,* 2007). This may be the case for linear systems where there was no evidence to suggest that closely related opposite sex adult water voles spatially avoided each other and where movement is restricted along the bank edge, which is not the case in non-linear habitat types.

Despite the increased risks of inbreeding in linear habitat types, water vole populations occupying both linear and non-linear wetlands maintained comparable levels of genetic diversity and both allelic richness and heterozygosity did not differ significantly by habitat type, nor was it influenced by the perimeter area ratio of the patch. This indicates that the survival, or migration, of voles is sufficient to prevent a significant loss in genetic diversity in the study populations. There was also tentative evidence from this study to suggest that the mechanisms in which genetic diversity is maintained may vary between habitat types. Populations in linear habitat types were characterised by significant temporal changes in genetic composition, which are characteristic in populations where the turnover of individuals is high. Thus, the loss of genetic diversity that would be expected to be lost through drift, from populations comprised of few breeding females in spring, is counterbalanced by the immigration of new individuals, which results in significant temporal variation in genetic structure. Conversely in non-linear wetlands, three of the four populations showed temporal stability in genetic structure, suggesting the increased survival of females that has been proposed here, may contribute to the continuation of genetic structure amongst annual groups.

This study proposed that both mortality and dispersal, which are two important mechanisms that shape the demographics and dynamics of water voles, were likely to vary between populations occupying linear and non-linear wetlands. There was evidence to suggest that overwinter survival of females may be higher in non-linear habitat types resulting in temporal stability in genetic structure. Dispersal was found to be greater in linear habitat types, however, contrary to expectations, higher levels of female philopatry were also observed, suggesting that related females aggregate rather than risk dispersal into an

unfamiliar matrix. Whilst female philopatry was also observed in non-linear wetlands, the sexual suppression of young would have resulted in fewer breeding female kin clusters and a lower risk of inbreeding when compared with linear systems.

8.3 Historical and contemporary patterns in genetic variation amongst water vole populations

Despite the widespread decline of water voles across the study region, this study revealed fine-scale phylogenetic and microsatellite structure that reflect the historical heritage of water voles in the southeast of England and how these have been influenced by contemporary processes. Phylogenetic analyses revealed that water vole populations in southeast England form part of a genetically divergent clade within the English water vole phylogeny that suggests a historical population expansion that diverged following the retreat of the Pleistocene ice sheet. Of the 14 haplotypes identified, 12 reflected a close genealogical association to each other and their geographic origin to the southeast which is particularly encouraging given the contemporary pressures of landscape change and colonisation across the region by mink. The phylogeographic patterns across the study sites were consistent with river topology, with divergent haplogroups being differentiated by disconnected river catchments and potentially the topographical relief of the North and South Downs. This pattern is consistent with the microsatellite variability that was significantly differentiated by river catchment and thus provides evidence that is consistent with Piertney *et al.* (2005) that management units for water voles should be at a watershed level. This study has also shown that contemporary conservation management of stronghold populations has been effective at conserving genetic heritage and diversity of mitochondrial lineages in the North Kent Marshes, Dartford Marshes and the Manhood Peninsular in Sussex.

8.4 The influence of reintroductions on patterns of genetic diversity *within* and *between* populations

Reintroductions within the study area made an unexpected and considerable contribution to the observed patterns in genetic variation. Two of the study

sites, NL-AW and NL-LW, had approximately 200 water voles introduced in 2005 and in 2001 respectively and exhibited significantly higher heterozysity and allelic richness in microsatellite loci relative to the natural popualtions. Furthermore, high haplotype diversity was also observed at one of the two sites. This is of notable interest as reintroduced populations can often exhibit a loss of genetic diversity due to the reduced survival and reproductive capabilities of founders (Kekkenon & Brommer, 2015). This is the first study to assess the genetic diversity of reintroduced populations and provides evidence that human-mediated measures to repopulate water voles are a viable option and when doing so, approximately 200 individuals released into high quality habitat is likely to be sufficient for securing genetic diversity in the longer term.

However, results from the spatial patterns in microsatellite variability indicate that reintroductions and translocations of water voles foregoing mitigation works may have contributed to the genetic homogenisation of geographically disparate populations. Furthermore, captive breeding between divergent lineages that have no geographical affiliation to water voles in the study area was evident from the phylogenetic analyses. This not only risks the loss of genetic heritage to south eastern populations which may be important to their long-term adaptability, but could potentially result in outbreeding depression, particularly amongst the natural population on the River Darent (L-RD) where a Scottish bloodline was observed. The source and potential spatial extent of this anomaly, however, warrants further investigation and thus the insinuation that this has resulted from captive breeding, can only be inferred in the context of this thesis.

8.5 Methodological advancements and limitations

Study designs are often a trade-off between effort and cost, and the ability to obtain sufficient samples to generate unbiased, precise estimates of parameters of interest. Thus an important part of any study is identifying suitable field and analytical methods and determining the sample size required to accurately detect significant patterns to provide an accurate approximation of the truth.

One of the first aims of this study was to investigate the effectiveness of non-invasive hair capture tubes for obtaining DNA from water voles and is presented in Chapter Three. This was the first study to apply this method to study water voles and thus the main focus was to refine the technique at each step, from the tube design, the field method protocol and the extraction of DNA and genotyping proficiency. The tube design and field collection of hair proved successful and efficient, obtaining 91 samples of hair with shafts from 15 different locations and taking just four man hours for servicing 25 tubes per site. The effort in terms of trap nights for obtaining water vole hair using the tubes was comparable to live capture techniques, however, when considering the costs, license requirements and man hours spent live trapping, the non-invasive tubes proved to be a more efficient and cost effective method that is widely accessible to amateur researchers and volunteers. A major limitation to this method, however, is that the quantity and quality of DNA can become compromised, reducing sampling efficiency and the ability to obtain accurate genotypes from individuals. Thus, the application of real time quantitative PCR in this study was a relatively novel, yet essential, stage in identifying DNA degradation and quantity. This not only economized laboratory time and expenditure but also allowed for samples that may have led to genotyping errors to be eliminated from the dataset. Nearly half of the hair samples obtained from the hair-capture tubes were unusable due to sample degradation and of those with intact fragments of target DNA, 47% yielded low quantities and were thus at risk genotyping errors. Despite these limitations, this technique was used to successfully obtain mtDNA from water voles in Norfolk, North Kent and Sussex allowing for the phylogeographic patterns of water voles to be determined across a large geographical scale (presented in Chapter Seven). There is therefore, a realistic application of these non-invasive hair capture tubes for genetic studies on water voles and by optimising field protocols, DNA extraction techniques and by isolating shorter polymorphic fragments of DNA, both the accuracy and efficiency of this technique can be improved.

There is a wide application of genetic tools for studying ecological processes in wild populations and an important consideration is obtaining samples that will

be representative of the total population being studied. In genetic studies, it is particularly important that the sampling design will obtain DNA from enough individuals so that all informative alleles at frequencies that are representative of the study population can be identified. In Chapter Three, a power analysis using 50 genotyped water voles at eight microsatellite loci, identified that a sample size of 35 would be required to detect all alleles at a real frequency of $\geq$0.05, and that 25 individuals would maximise the probability of identifying significant spatial and temporal genetic differences. Sample sizes of this magnitude were rarely obtained from the study populations using live capture or non-invasive techniques. In some instances, particularly in linear wetlands where sampling along the bank allows for a complete identification and intensive sampling of colonies, the number of individual genotypes obtained, was thus likely to be representative of the genetic composition of the populations. However, in non-linear habitat patches, sampling was largely hindered by access constraints, which prohibited setting traps in a grid configuration to sample from individuals whose movement was not constrained along the bank edge. For three of the four non-linear study sites, the water vole samples obtained were unlikely to be representative of the population and reduced the power to detect patterns in both genetic composition (Chapter Six) and genetic relatedness (Chapter Five). Combining both non-invasive tubes and live capture may serve to increase sampling efficiency in non-linear wetlands and reduce the level of error associated with small sample sizes.

8.6 Key Findings

The main findings of this study were as follows:

8.6.1 Chapter Three

- Non-invasive hair capture tubes are an effective method of obtaining hair samples from wild populations of water voles for DNA extraction.
- Sampling efficiency is optimised during September; however, this may reduce effective sampling of dispersed or dispersing young of the year due to the size of the tube which would thus limit the capabilities of this method to investigate patterns in local gene flow.
- There was no correlation found between the time samples were left in the field and the genotyping success rate.
- Quantitative real time PCR provides accurate determination of DNA yield and quality that economizes laboratory time and expenditure and thus is a recommended tool for studies that employ non-invasive genetic tools.
- With improvements to the study design and DNA extraction techniques, remote hair capture tubes offer a more cost effective, resource reduced and logistically feasible alternative to live trapping for collecting genetic information from water voles.

8.6.2 Chapter Four

- Considerable variation in the population dynamics was observed across the eight study populations reflecting differences in the local ecological conditions.
- Across all populations, the probability of recruitment into the breeding cohort was 4.3 times higher for females than for males and was positively correlated with weight.
    - There was evidence that increases in the relative abundance of breeding females in non-linear wetlands reduced the probability and weight at which YOTY females became sexually mature.
- Survival estimates were highly variable between populations but concur with those reported in previous studies on water voles.

- Apparent survival estimates were significantly higher in linear wetlands than non-linear wetlands but did not account for variation between sexes and age cohorts.
- Non-linear populations had a significantly higher proportion of breeding females in the overwintered population in spring compared with linear sites, which were generally male biased.
    - Higher overwinter survival estimates and proportion of recaptures of previously marked individuals, relative to summer in non-linear wetlands, suggests more females survive overwinter than during summer.
    - Male biased spring populations occupying linear habitat types resulted from both immigration of males and higher losses of females from the autumn cohorts.
- Increases in the relative abundance of overwintered females significantly reduced the relative abundance of YOTY remaining in linear wetlands by autumn, suggesting density dependent regulation. This pattern was not observed in non-linear wetlands where a positive relationship between spring female abundance and autumn young of the year abundance was observed.
- Linear wetland populations had a significantly higher proportion of females in the autumn operational and YOTY cohorts, suggesting that females may be more philopatric in linear wetlands. This concurs with the accumulation of related females presented in Chapter Five.
- Considerable variation in weather conditions during the study period contributed to the patterns of demography observed.
    - Spring droughts in 2012 resulted in a significantly lower relative abundance of YOTY, compared with 2013, and lower adult abundances resulted in significantly more YOTY per overwintered adult being observed in 2012.

- Flooding overwinter of 2012/13 resulted in a significantly lower proportion of females being observed in the spring cohorts in 2013 compared with 2012, which increased the proportion of female YOTY that were observed in the 2013 autumn population.
- Summer population growth estimates were generally higher in 2013 compared with 2012, suggesting that spring droughts may have a larger influence on demographic performance of water voles than flooding.

8.6.3 Chapter Five

- The use of pedigree data to differentiate between commonly used relatedness estimators are an essential step in determining the most reliable estimates to be used for analysing sociality in wild populations.
- Spatial patterns in genetic relatedness revealed that sociality is present in water vole populations occupying both linear and non-linear wetlands.
    - Between 48% and 87% of adult water voles were estimated to have one or more sexually mature, close relatives within the same population.
    - Significantly lower overall relatedness than would be expected from a population comprised of unrelated individuals was observed, suggesting that populations were established from largely unrelated individuals in spring.
- Female water voles are more philopatric than males, as shown by the higher relatedness estimates between females, but natal dispersal by male water voles is not always the rule.
- Spatial affiliation between sexually mature female kin was observed across all populations where between 10% and 50% of neighbours were estimated to be 1^{st} or 2^{nd} order kin.
    - Variation in the extent of spatial affiliation amongst female water voles suggests that female philopatry does not consistently result in the formation of kin groups.

- Tolerance between sexually receptive female relatives was reduced at higher abundance, suggesting that territorial females will tolerate mature kin when resources are not saturated, but will be forced to disperse or be reproductively suppressed when competition for resources is increases.
- A significantly higher proportion of breeding adults and breeding female water voles were estimated to be close relatives in linear wetland systems, suggesting female philopatry is higher in linear compared with non-linear wetland populations.
- Habitat confinement of edge effects in linear wetlands are suggested to contribute to the higher relatedness amongst females and between opposite sex pairs when compared with non-linear sites.
- Between 20% and 75% of breeding females were related to one or more sexually mature males in five of the six study sites.
    - There was no evidence to suggest that closely related opposite sex pairs spatially avoid each other.
    - Significant inbreeding coefficients at two of the three linear sites where 50% and 75% of females had a sexually mature close relative within the populations, implies that breeding between close relatives is not avoided.
    - It may be more beneficial to remain within natal populations and risk breeding with relatives than risk dispersal into a hostile surrounding matrix.

8.6.4 Chapter Six

- Genetic diversity was comparable between water vole populations occupying linear and non-linear wetlands, suggesting that either migration and/or survival of water voles is sufficient at the study sites for maintaining genetic diversity.
- Populations in linear habitats exhibited temporal instability in genetic composition suggesting a high turnover of individuals resulting from seasonal demographic instability and migration.

- Significant deviations from Hardy Weinberg Equilibrium were observed in two of the three populations occupying linear sites.
    - The positive inbreeding coefficients concurred with annual populations that had been affected by overwinter flooding and thus the accumulation of related individuals following low spring densities caused inbreeding and/or a temporal 'Walhund Effect'.
- Temporal stability in three of the four non-linear populations suggests that higher survivorship may result in annual groups with a higher proportion of shared genes, which thus contributes to the maintenance of genetic diversity.
- Reintroduced populations at Arundel (NL-AW) and London Wetland Centres (NL-LW) showed significantly higher levels of heterozygosity and allelic richness with extant populations, suggesting both schemes were successful.
    - Although a similar number of founders were released into a comparable amount of suitable habitat, NL-AW showed higher genetic diversity than NL-LW and concurs with the phylogenetic diversity presented in Chapter Seven.
    - Differences in allelic diversity and heterozygosity between the reintroduced populations may reflect unknown differences in microsatellite variation within the founding population or be as a result of higher reproductive success at Arundel (NL-AW), which can aid in maintaining genetic diversity.
    - Releases of approximately 200 water voles into high quality habitat are suggested to be sufficient in maintaining high genetic diversity.
- Although the scale of management required to conserve genetic integrity is beyond the scope of this study, the results revealed that genetic variation is partitioned amongst river catchments and potentially further differentiated by more cryptic boundaries such as land use types and/or the degree of river catchment connectivity.

    - Unique alleles identified potentially relic populations that have been maintained by conservation measures on the Manhood Peninsular in Sussex and in populations from North Kent, which concurs with mitochondrial patterns described in Chapter Seven.
    - Significant genetic differentiations between two populations (L-HB and NL-AW) that are located within the same river catchment suggest gene flow is not effective at a scale of 6.5 km.
- Reintroductions and translocations foregoing mitigation works have contributed to geographically disparate population becoming genetically homogenised and resulted in populations not conforming to an 'isolation by distance' pattern of genetic variation.
- Genetic diversity of water voles in southeast England is lower than populations in Northern Scotland which suggests there may be different patterns in reproductive success or that reduced effective population sizes have resulted from habitat loss and predation of populations by mink.

8.6.5 Chapter Seven

- High diversity in geographically affiliated mitochondrial lineages was found to have been conserved within southeastern England at a local scale.
    - Patterns of phylogeographic structure reflect a process of historical patterns of gene flow, which are associated with both river topology and elevation and represent the genetic heritage and biogeographic history of populations.
- The phylogenetic structure suggests that, as shown in Chapter Six and in concordance with previous research by Piertney *et al. (*2005), the conservation management of water voles should be focussed on discrete river catchments in order to minimize genetic erosion, maintain genetic heritage and secure the adaptive flexibility of the species in the longer term.

- Contemporary conservation measures of stronghold sites have assisted in the conservation of genetically divergent populations in North Kent (NKM) and the Manhood Peninsular (MHP) in Sussex. This divergence is in concordance with the presence of unique alleles within these populations, as shown in Chapter Six.
- The genetic signature of reintroduction and restocking programmes has contributed to the diversity and divergence which has been observed amongst populations.
    - Nevertheless, the introduction of divergent lineages, through human mediated programmes, could pose an inherent threat to the genetic heritage and diversity of populations if there is no previous geographical association with the receptor site and leads to genetic homogenisation as shown in Chapter Six.
- The presence of a haplotype from a water vole captured in Dartford (L-RD) that is affiliated to the Scottish water vole phylogenetic clade is of particular concern and may be attributed to captive breeding between populations from two evolutionary significant units.
- The usefulness of phylogenetic studies as an approach for understanding historic and contemporary processes has been highlighted.
    - This has a critical role to play in future conservation measures aimed at ensuring the long-term viability and adaptive potential of populations.
    - Future phylogenetic studies can be used to identify genetic structure of reintroductions and provide a useful post release tool that could be combined with non-invasive hair capture tube methods, described in Chapter Three, to monitor population expansion and admixture.

8.7 Conservation implications

The results of this study have provided a wide range of information on the population processes, which promote, or may negate, the viability of water vole populations both locally and, potentially, nationally. This information should be used to inform future conservation management strategies that aim to re-establish populations within their historical range through human mediated enhancements to wetland habitat and by reintroducing populations where natural colonisation is unlikely to occur.

This study has revealed that genetic variation is partitioned amongst river catchments and may be further differentiated by more cryptic boundaries such as land use type and the degree of river catchment connectivity.

- It is recommended that, in concordance with previous suggestions by Piertney *et al.* (2005), conservation management of water voles should be at a watershed level.
- It is further recommended that genetic tools should be utilised in establishing localised restrictions to gene flow and differentiation between source and receptor populations when translocations of water voles are undertaken.

There was considerable variation in climatic conditions throughout the study period, with a drought in the spring of 2012 and overwinter flooding between 2012 and 2013. Whilst not conclusive, there were observed differences in demographic parameters between 2012 and 2013, which may be associated with these climatic conditions. As such, ongoing management objectives should consider climate change based resilience for water voles. The south and south east of England are projected to experience an increase in the frequency of droughts and periods of water stress associated with climate change, which the rest of the UK is expected to be relatively unaffected by (IPCC, 2013; Met Office, 2011).

- As such, the importance in the preservation and creation of habitat based around permanent water features within the south east is a major conservation priority to mitigate against the pressures of climate change.

- Additionally, whilst there remains uncertainty in regards to summer flood events, it is predicted that winter flooding will increase across the UK (IPCC, 2013; Met Office, 2011). It is therefore recommended that additional satellite habitat is maintained or created at floodplain boundaries in order to provide suitable short to medium term habitat refugia for water voles.

Careful consideration should be given to the process of reintroducing water voles into the landscape as part of mitigation works or as part of wider programmes of re-establishment. Both captive breeding and translocation of water voles requires more stringent guidelines that help to preserve the genetic heritage, diversity and adaptive flexibility that are important to the long-term persistence and conservation of biodiversity.

The connectivity between linear wetlands should be maintained to ensure that migration remains effective to prevent the loss of genetic diversity and risk of inbreeding that characterises water vole populations in linear watershed systems. Maintaining wide riparian margins and altering vegetation cuts so that a gradual transition from riparian vegetation into the adjoining habitat types may aid in reducing edge effects and increasing migration between colonies occupying linear systems. The creation of non-linear wetlands would provide a valuable addition to many lowland areas and will help to provide refugia for water voles from over winter environmental perturbations and from predation. Furthermore, this study concurs with research by Carter and Bright (2003) that suggests that non-linear habitats are more likely to promote the establishment of stronghold populations of water voles that will provide a source for recolonisation.

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Spatial Data Sources

Hampshire and Isle of Wight Wildlife Trust (2014), Water vole reintroduction site locality, spatially referenced list. Created using ArcGIS Version 10.1, Redland, CA: Environmental Systems Research Institute, Inc, 1992-1998

Office of National Statistics Boundary Datasets (2015), NISRA: Website www.nisra.gov.uk.

Ordnance Survey MasterMap Topography Layer. Updated 17th December 2011, Ordnance Survey (GB), Using: EDINA Digimap Ordnance Survey Service > http://digimap.edina.ac.uk>, Downloaded 24 October, 2012

Strategi [SHAPE: geospatial data], scale 1:250000, Tiles: GB Updated: 29th November 2013, Ordnance Survey (GB), Using: EDINA Digimap Ordnance Survey Service > http://digimap.edina.ac.uk>, Downloaded 21 October, 2014.

Strategi [SHAPE: geospatial data], scale 1:50000, Tiles SU, TQ, TR, Updated: 17July 2014, Ordnance Survey (GB), Using: EDINA Digimap Ordnance Survey Service > http://digimap.edina.ac.uk>, Downloaded October 21, 2014.

Appendix One: Copies of Natural England water vole licenses issued to the author for this study.

LICENCE

Miss Rowenna Baker

Wildlife Licensing Unit
Natural England
First Floor
Temple Quay House
2 The Square
Bristol, BS1 6EB

T 0845 601 4523
F 0845 601 3438

www.naturalengland.org.uk

Licence number: **20120780**

WILDLIFE & COUNTRYSIDE ACT 1981 (as amended).

This licence is granted under the following legislation - Section 16 (3) (c) of the Wildlife & Countryside Act 1981(as amended). Natural England hereby authorises **Rowenna Baker** hereinafter referred to as "the licensee", and the following accredited agent(s), to carry out the activities detailed on the attached annex:

Persons appointed by the Licensee.

This Licence is valid from **08 March 2012** to **31 October 2012** inclusive, and is granted under the following conditions:

[1] While engaged in work permitted by this licence the licensee must have access to a copy of this licence and produce it to any police officer or any Natural England officer on demand.

[3] No agent of the licensee is permitted to carry out work under this licence unless he or she is in possession of a letter signed by the licensee appointing him or her by name as the duly accredited agent of the licensee for the purpose of this licence, and shall carry with him or her the said letter and a copy of this licence and shall produce them to any police officer on demand. The licensee is responsible for all activities carried out under this licence including those undertaken by any accredited agent.

[4] The licensee or his/her agent may employ assistants provided they work under the personal supervision of the licensee or agent.

[5] No work shall be carried out under this licence on a National Nature Reserve or Marine Nature Reserve except with the prior written permission of Natural England's appropriate area team.

[8] A report of the work carried out under this licence must be sent to Natural England at the above address within two weeks of the expiry date. To renew your licence from the date it expires a request must be sent to Natural England at least three weeks prior to expiry with a report form completed to that date.

[10] This licence is granted subject to compliance with the conditions specified. Anything done otherwise than in accordance with the terms of this licence may constitute an offence. Before exercising this licence the licensee should sign and date it in the space provided.

[11] This licence may be modified or revoked at any time by Natural England.

[14] Any wild animal taken under this licence shall be liberated as detailed in the annex of this licence.

Notes:

[202] Nothing in this licence confers any right of entry onto land or property.

[217] All equipment used for the purposes of this licence shall be so constructed and maintained as to avoid cruelty and distress to the species named on this licence.

[231] The licensee may photograph the animals as an incidental part of licensed work provided that no additional disturbance is caused thereby.

[249] All animals retained in captivity should be kept in accordance with the provisions of the Animal Welfare Act 2006.

[257] Good practice, as detailed in the Water Vole Conservation Handbook (2nd Edition), shall be followed.

[270] The scientific name for the Water Vole was recently changed. In England the new scientific name is Arvicola amphibius. However the law applies to the name given in the legislation (in this case the Wildlife & Countryside Act 1981 (as amended)) therefore for the purpose of this licence the Water Vole will be referred to as Arvicola terrestris.

Matt Shore

Date: **08 March 2012**

Signed:
FOR AND ON BEHALF OF NATURAL ENGLAND

PLEASE SIGN TO STATE THAT YOU HAVE READ AND UNDER[illegible] LICENCE.

Date: _____ - _____ - 2012 Signed RJBaker _____

Annex to Licence Number 20120780 (Details)

Species: Water Vole (Arvicola terrestris)
Item:
Number:
Activities: • Disturb and take
Methods: • Hand & appropriate trap
Purposes: • Conservation
County:
- East Sussex
  - Pannel Nature Reserve Reedbed Site & Rye and Pett Levels
- Greater London
  - WWT London Wetland Centre , Queen Elizabeth's Walk, Barnes, London, SW13 9WT
- Kent
  - Elmley Marshes RSPB Nature Reserve, Settingbourne, ME12 3RW & Thames and Medway Canal, Gravesend & Stodmarsh NNR, Grove Ferry, CT3 4BP & Rye and Pett Levels
- West Sussex
  - WWT Arundel Wetland Centre BN18 9PB & Houghton Bridge, Houghton, Amberley & Chichester Canal, Chichester & Arun Valley Catchment & Chichester Coastal Plain

Notes: Trapping will continue until no further water voles have been trapped and no new field signs found for three full days. Any lactating females which are trapped will be released at the site of capyure and trapping suspended in the immediate vicinity until it is estimated that the young have become independent.

LICENCE

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Licence number: **20123101**
Supersedes licence number: 20120780

WILDLIFE & COUNTRYSIDE ACT 1981 (as amended).

This licence is granted under the following legislation - Section 16 (3) (a) & (b) of the Wildlife & Countryside Act 1981(as amended). Natural England hereby authorises **Rowenna Baker** hereinafter referred to as "the licensee", and the following accredited agent(s), to carry out the activities detailed on the attached annex:

Persons appointed by the Licensee,

This Licence is valid from **22 September 2012** to **31 October 2013** inclusive, and is granted under the following conditions:

[1] While engaged in work permitted by this licence the licensee must have access to a copy of this licence and produce it to any police officer or any Natural England officer on demand.

[3] No agent of the licensee is permitted to carry out work under this licence unless he or she is in possession of a letter signed by the licensee appointing him or her by name as the duly accredited agent of the licensee for the purpose of this licence, and shall carry with him or her the said letter and a copy of this licence and shall produce them to any police officer on demand. The licensee is responsible for all activities carried out under this licence including those undertaken by any accredited agent.

[4] The licensee or his/her agent may employ assistants provided they work under the personal supervision of the licensee or agent.

[5] No work shall be carried out under this licence on a National Nature Reserve or Marine Nature Reserve except with the prior written permission of Natural England's appropriate area team.

[8] A report of the work carried out under this licence must be sent to Natural England at the above address within two weeks of the expiry date. To renew your licence from the date it expires a request must be sent to Natural England at least three weeks prior to expiry with a report form completed to that date.

[10] This licence is granted subject to compliance with the conditions specified. Anything done otherwise than in accordance with the terms of this licence may constitute an offence. Before exercising this licence the licensee should sign and date it in the space provided.

[11] This licence may be modified or revoked at any time by Natural England.

[14] Any wild animal taken under this licence shall be liberated as detailed in the annex of this licence.

[184] Any trap used under the authority of this licence must be of a size and design suitable for the humane live capture of the target species and when set to capture must be inspected at least twice per day.

Notes:

[202] Nothing in this licence confers any right of entry onto land or property.

[217] All equipment used for the purposes of this licence shall be so constructed and maintained as to avoid cruelty and distress to the species named on this licence.

[231] The licensee may photograph the animals as an incidental part of licensed work provided that no additional disturbance is caused thereby.

[257] Good practice, as detailed in the Water Vole Conservation Handbook (3rd Edition), shall be followed.

[270] The scientific name for the Water Vole was recently changed. In England the new scientific name is Arvicola amphibius. However the law applies to the name given in the legislation (in this case the Wildlife & Countryside Act 1981 (as amended)) therefore for the purpose of this licence the Water Vole will be referred to as Arvicola terrestris.

Matt Shore

Date: **24 September 2012**

Signed:
FOR AND ON BEHALF OF NATURAL ENGLAND

PLEASE SIGN TO STATE THAT YOU HAVE READ AND UNDERSTO ENCE.

Date: _____ - _____ - 2012 Signed: ___ RJBaker _____

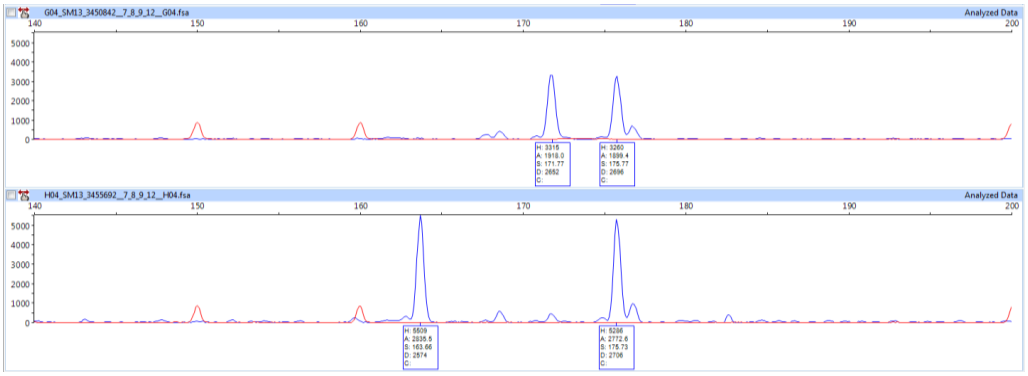
Annex to Licence Number 20123101 (Details)

Species: Water Vole (Arvicola terrestris)
Item:
Number:
Activities: • Disturb and take
Methods: • Hand & appropriate trap
Purposes: • Science and Education
• Ringing or marking or examining any ring or mark
County: • East Sussex
  • Pannel Nature Reserve Reedbed Site & Rye and Pett Levels
• Greater London
  • WWT London Wetland Centre, Queen Elizabeth's Walk, Barnes, SW13 9WT. River Darent, Dartford. Stretch of river between Acacia Island approx TQ545736 to Hawley Meadow TQ554172
• Kent
  • Elmley Marshes RSPB Nature Reserve, Settingbourne, ME12 3RW & Stodmarsh NNR, Grove Ferry, CT3 4BP & Rye and Pett Levels
• West Sussex
  • WWT Arundel Wetland Centre BN18 9PB & Houghton Bridge, Houghton, Amberley & Chichester Canal, Chichester & Arun Valley Catchment & Chichester Coastal Plain

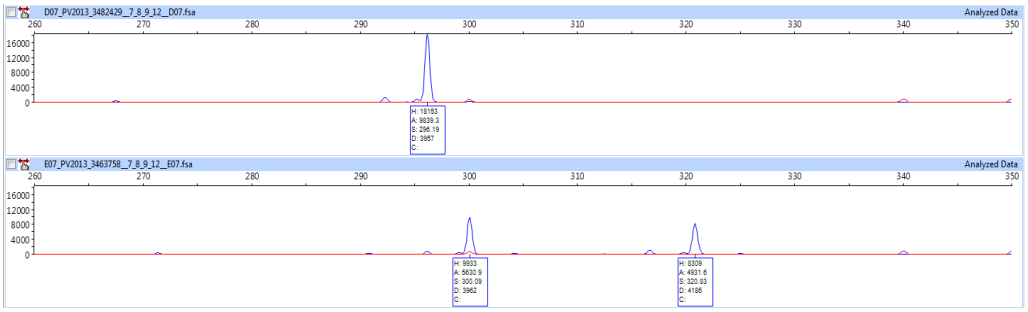
Notes: Prior to release at the site of capture, water voles may be examined and weighed, marked using PIT tags and a small hair pluck may be taken from them for future DNA analysis.

Appendix Two: Examples of water vole DNA profiles, by microsatellite loci, obtained from capillary electrophoresis and displayed using Peak Scanner™ software

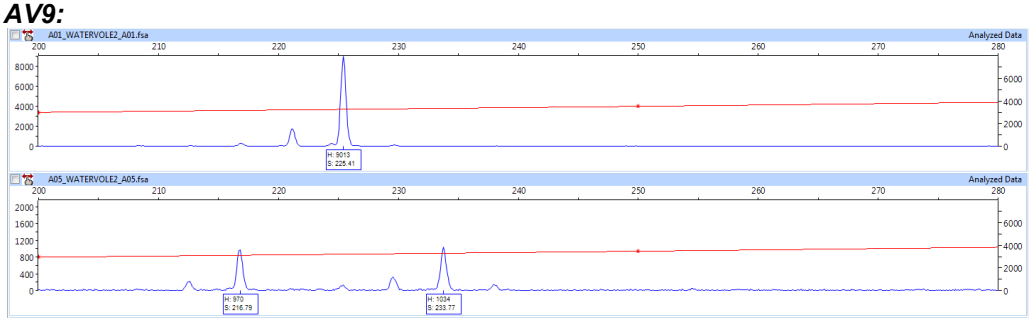
AV7:



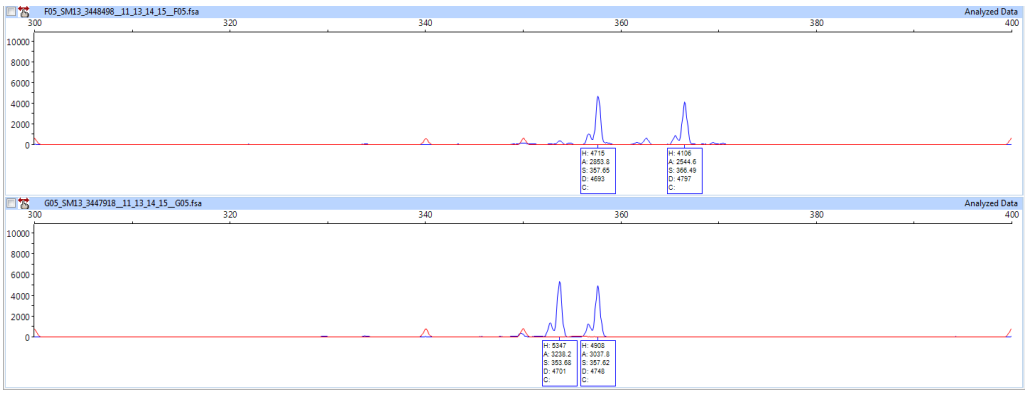
AV8:



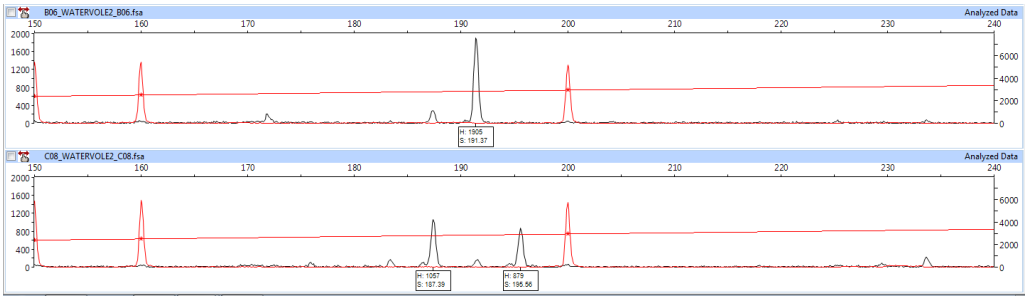
AV9:



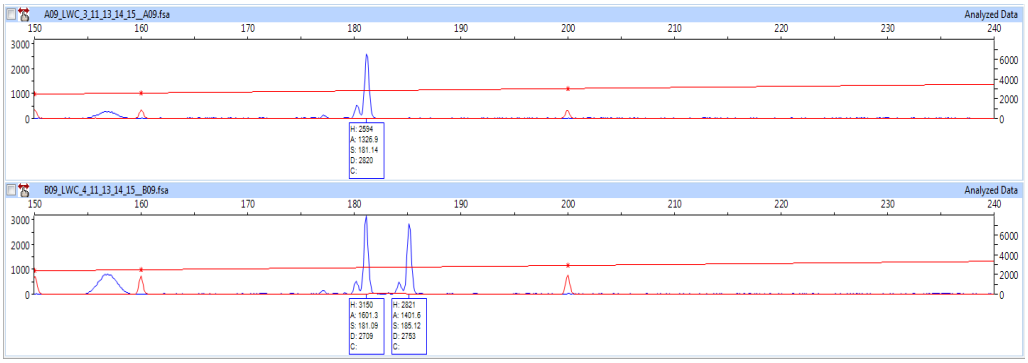
AV11:



AV12:

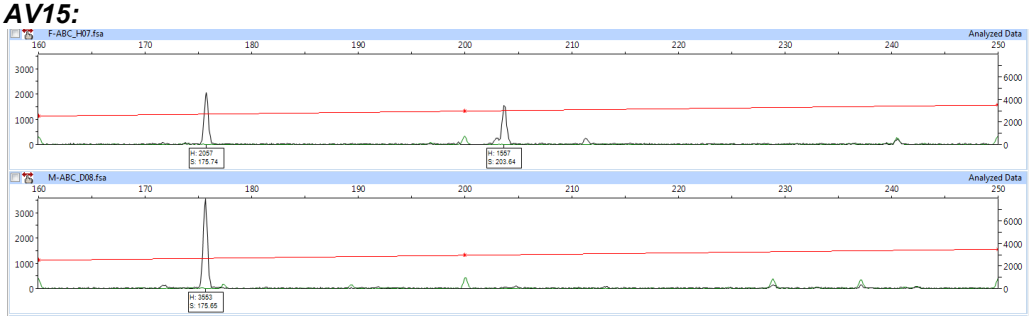


AV13:

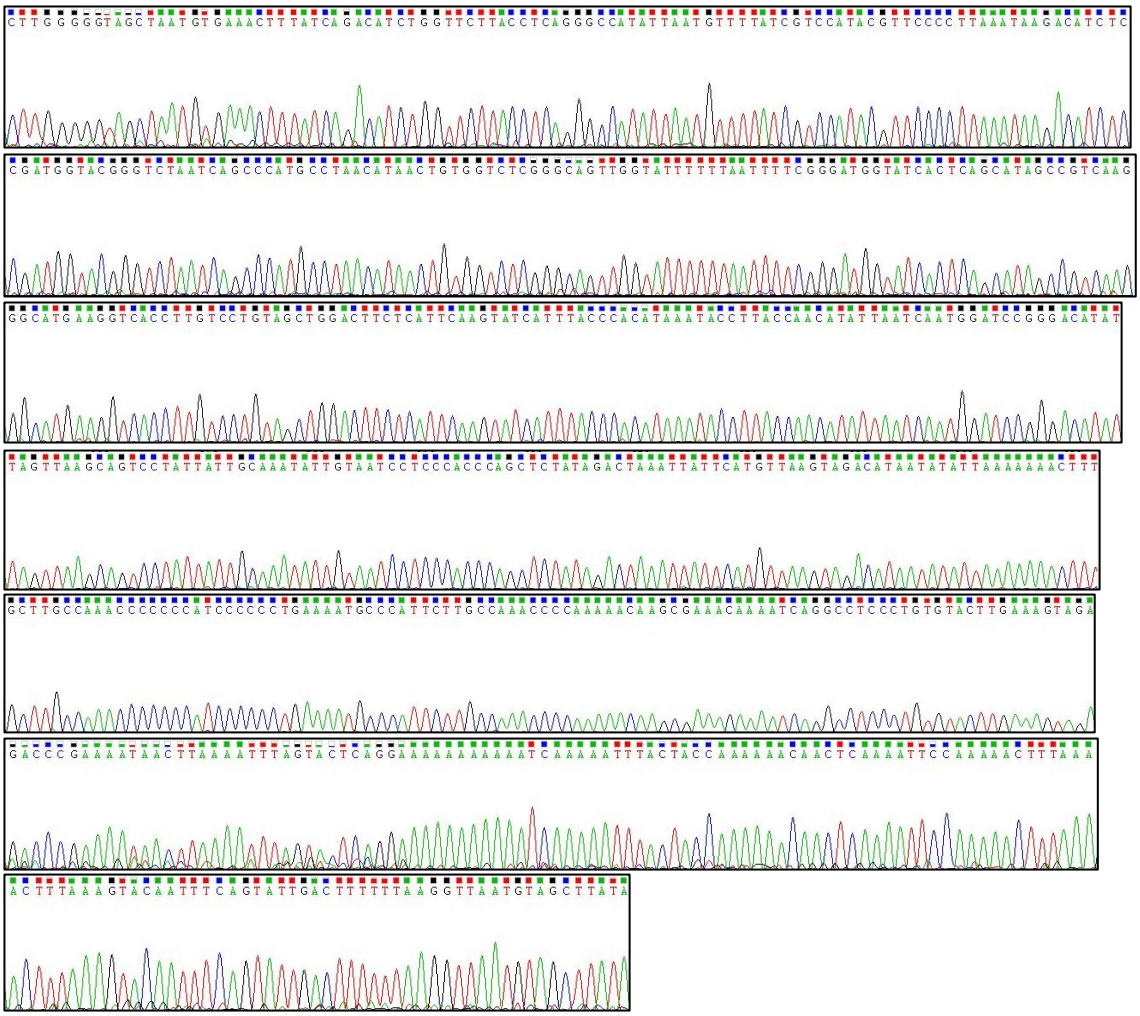


AV14:

AV15:



Appendix Three: Example of an electropherogram of water vole mtDNA sequence



N.B. First letter of each row is a replicate of the last letter of the preceding row.

Appendix Four: Summary table of water vole breeding weights (g) by sex (M = male, F = female), study site and trapping session. Missed capture sessions are denoted by a dash (-).

		NL-AW		NL-SM		NL-PV		NL-LW		L-HB		L-EM		L-CC		L-RD	
	Weight (g)	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
Spring 2012	Mean	244.3	258.9	196.3	220.0	185	215.0	-	-	244.2	255.2	-	-	211.5	254.4	-	-
	Standard Deviation	32.4	10.4	16.6	18.4	0	0.0	-	-	25.3	19.3	-	-	37.2	36.8	-	-
	Minimum	198	242	182	207	185	215.0	-	-	206	240	-	-	180	207	-	-
	Maximum	307	279	220	235	185	215.0	-	-	292	291	-	-	255	295	-	-
	N	18	9	4	2	1	1	-	-	9	6	-	-	4	6	-	-
Autumn 2012	Mean	198.2	212.8	217	237	213.0	278.0	126.3	206.7	241.0	234.3	176.6	226.5	188.2	269.3	181	198
	Standard Deviation	27.4	19.6	0	0	26.7	45.3	53.4	38.0	32.6	28.0	46.3	30.8	23.7	17.8	0	0
	Minimum	168	191	217	237	180	246	95	179	210	202	101	186	150	250	181	198
	Maximum	254	233	217	237	245	310	188	250	275	251	247	260	216	290	181	198
	N	11	5	1	1	4	2	3	3	3	4	12	6	6	4	1	1
Spring 2013	Mean	223.6	249.2	216	210.9	-	-	-	-	196.0	246.1	200.0	253.4	-	-	-	-
	Standard Deviation	38.6	28.6	0	19.2	-	-	-	-	45.3	26.2	0	18.2	-	-	-	-
	Minimum	130	185	216	187	-	-	-	-	133	202	200	233	-	-	-	-
	Maximum	270	300	216	245	-	-	-	-	250	295	200	279	-	-	-	-
	N	13	14	1	7	-	-	-	-	6	14	1	5	-	-	-	-
Autumn 2013	Mean	199.9	211.2	-	190	241.0	240.8	184.7	220.3	207.3	249.8	185.8	222.1	204.0	224.0	-	-
	Standard Deviation	36.5	31.8	-	0	21.8	44.6	69.1	30.3	53.1	35.6	38.3	24.1	62.2	29.7	-	-
	Minimum	137	182	-	190	226	183	105	188	135	196	115	187	160	203	-	-
	Maximum	274	305	-	190	266	292	229	248	340	302	234	258	248	245	-	-
	N	23	18	0	1	3	6	3	3	13	8	22	8	3	2	-	-

Appendix Five: Allele frequencies of water vole populations to accompany Chapter Five

Locus 1: AV7											
155	159	163	167	171	175	179	183	187	191		
0.0135	0.0946	0.0649	0.1432	0.3324	0.2243	0.0757	0.0351	0.0081	0.0081		
Locus 2: AV8											
280	284	288	292	296	300	304	308	312	316	320	
0.0357	0.0192	0.0852	0.0604	0.0852	0.1841	0.1181	0.3077	0.0385	0.0247	0.0412	
Locus 3: AV9											
221	225	229	233	237	241	245	249	253	257	261	265
0.0359	0.1547	0.0249	0.1215	0.1022	0.2928	0.1547	0.0359	0.0193	0.0166	0.0331	0.0083
Locus 4: AV11											
312	326	330	334	338	342	346	350	354	358	362	366
0.0056	0.0056	0.0141	0.0141	0.0847	0.0424	0.0028	0.0424	0.3757	0.1215	0.0678	0.0339
370	374	378	382	386	390						
0.1158	0.0169	0.0226	0.0198	0.0028	0.0113						
Locus 5: AV12											
174	178	182	186	190	194	198	202	206	210	226	234
0.1230	0.0628	0.0464	0.1148	0.0902	0.2240	0.1038	0.1639	0.0383	0.0273	0.0027	0.0027
Locus 6: AV13											
174	178	182	186	190	194	198					
0.2539	0.1425	0.2461	0.0959	0.1684	0.0492	0.0440					
Locus 7: AV14											
220	224	228	232	236	240	244	248	256			
0.0130	0.0777	0.2383	0.1684	0.1684	0.2902	0.0311	0.0026	0.0104			
Locus 8: AV15											
176	180	184	188	192	196	200	204	212	216	220	224
0.4707	0.0293	0.0372	0.0399	0.1968	0.0213	0.0239	.0771	0.0745	0.0106	0.0080	0.0027
228											
0.0080											

Table A5.1 *showing alleles (top rows) and their frequencies (bottom rows) of eight microsatellite markers (Locus: #) from a global population of water voles consisting of 203 individuals and including six individuals of known pedigree.*

Locus 1: AV7

	155	**159**	**163**	**167**	**171**	**175**	**179**	**183**	**187**	**191**
NL-AW	0.1563		0.0469		0.1875	0.1719	0.1719			
NL-SM			0.375		0.1667	0.4583				
NL-PV			0.125		0.4688			0.4063		
L-HB		0.0400		0.0600	0.5000	0.3200	0.0400	0.0200		
L-EM		0.4545	0.0152	0.1667	0.1818	0.1364	0.0303		0.0152	
L-CC				0.2000	0.5750	0.025		0.1250		0.075

Locus 2: AV8

	280	**284**	**288**	**292**	**296**	**300**	**304**	**308**	**312**	**316**	**320**
NL-AW	0.0313	0.1875	0.0313	0.3906	0.0625	0.0313	0.0156	0.125			0.313
NL-SM					0.3636	0.0455	0.2727	0.1818	0.1364		
NL-PV	0.0455	0.0455		0.1364	0.2727	0.3182	0.0455				0.1364
L-HB				0.0357	0.0179	0.6250	0.0893	0.1071			0.1250
L-EM				0.0345	0.0690	0.1207	0.2931	0.2241	0.0864	0.0690	0.1034
L-CC			0.7750	0.1750			0.0500				

Locus 3: AV9

	221	**225**	**229**	**233**	**237**	**241**	**245**	**249**	**253**	**257**	**261**	**265**
NL-AW	0.0469	0.1094		0.2344	0.0156	0.2188	0.2969			0.0625		0.0156
NL-SM		0.0385		0.2692		0.5385	0.1538					
NL-PV					0.3667	0.1000	0.4333	0.0667	0.0333			
L-HB		0.4107	0.0714	0.0357		0.4464	0.0179	0.0179				
L-EM		0.0313	0.0156	0.2188	0.2969	0.2344	0.1406	0.0469	0.0156			
L-CC		0.0250			0.0500	0.3500	0.0750	0.1000	0.0250		0.3000	0.0750

Locus 4: AV11

	330	**334**	**338**	**342**	**346**	**350**	**354**	**358**	**362**	**366**	**370**	**374**
NL-AW		0.0469	0.2344	0.2031			0.2031	0.0469	0.1563	0.0156	0.0781	
NL-SM	0.0357	0.0357	0.0357	0.0714			0.1429	0.2143	0.0357	0.1071		0.1786
NL-PV			0.0357			0.3929	0.5000					
L-HB	0.0250	0.1750			0.0750	0.0250	0.0500	0.0500		0.2250	0.3500	
L-EM		0.0345				0.0345	0.6034		0.0345		0.2241	
L-CC				0.1563		0.1250	0.0938	0.3750	0.1563			

	378	**382**	**386**	**390**
NL-AW				0.0156
NL-SM	0.0714	0.0714		
NL-PV			0.0714	
L-HB				0.0250
L-EM	0.0345	0.0345		
L-CC		0.0938		

Table A5.2 *Alleles identified across eight microsatellite loci and frequency of occurrence within each study population analysed in Chapter Five.*

Locus 5: AV12

	174	178	182	186	190	194	198	202	206	210	226
NL-AW	0.2667			0.0667	0.0167	0.2	0.1	0.2667	0.05	0.0333	
NL-SM			0.1071		0.3214	0.0357	0.1429	0.1429	0.1786	0.0714	
NL-PV				0.0667	0.1333			0.8000			
L-HB	0.0682	0.0682		0.2500	0.0455	0.1364			0.0682	0.3409	
L-EM	0.1818		0.1061	0.2273	0.1515	0.1818		0.0606	0.0909		
L-CC			0.0263	0.1316	0.0526	0.3947	0.1579	0.2105			0.0263

Locus 6: AV13

	170	174	178	182	186	190	194	198
NL-AW		0.1724	0.3276	0.1379	0.069	0.2069	0.0862	
NL-SM			0.0714	0.2857	0.3929	0.0714	0.1429	0.0357
NL-PV		0.2333	0.3667	0.1667	0.1667	0.0667		
L-HB	0.0357	0.1607	0.3214	0.4643		0.0179		
L-EM		0.0882	0.0441	0.4412	0.0294	0.3971		
L-CC			0.0588	0.6765	0.2059	0.0588		

Locus 7: AV14

	220	224	228	232	236	240	244	248	256
NL-AW		0.0167	0.25	0.0167	0.1	0.5333	0.0833		
NL-SM			0.0357	0.7143	0.1786	0.0714			
NL-PV		0.0333	0.2667	0.1667	0.1333	0.4000			
L-HB		0.1552	0.500	0.0172		0.2586	0.0172		
L-EM	0.0606	0.3333	0.0303	0.0909	0.3182	0.1667		0.0263	0.1053
L-CC			0.5789	0.0263	0.0263	0.0526	0.1842		

Locus 8: AV15

	176	180	184	188	192	196	200	204	212	216	220	228
NL-AW	0.4839		0.1129		0.0645			0.1129	0.2258			
NL-SM	0.8077	0.0385		0.1538								
NL-PV	0.1071			0.1071	0.2500		0.2857		0.2500			
L-HB	0.5862				0.3448	0.0345		0.0345				
L-EM	0.4853	0.0147	0.1324	0.1176	0.2500							
L-CC	0.2105	0.0789				0.0526	0.0263	0.2895	0.0789	0.1053	0.0789	0.0789

Table A5.2 Continued

Appendix Six: Graphical representation of correlations between genetic relatedness and geographic distance to accompany Chapter Five.

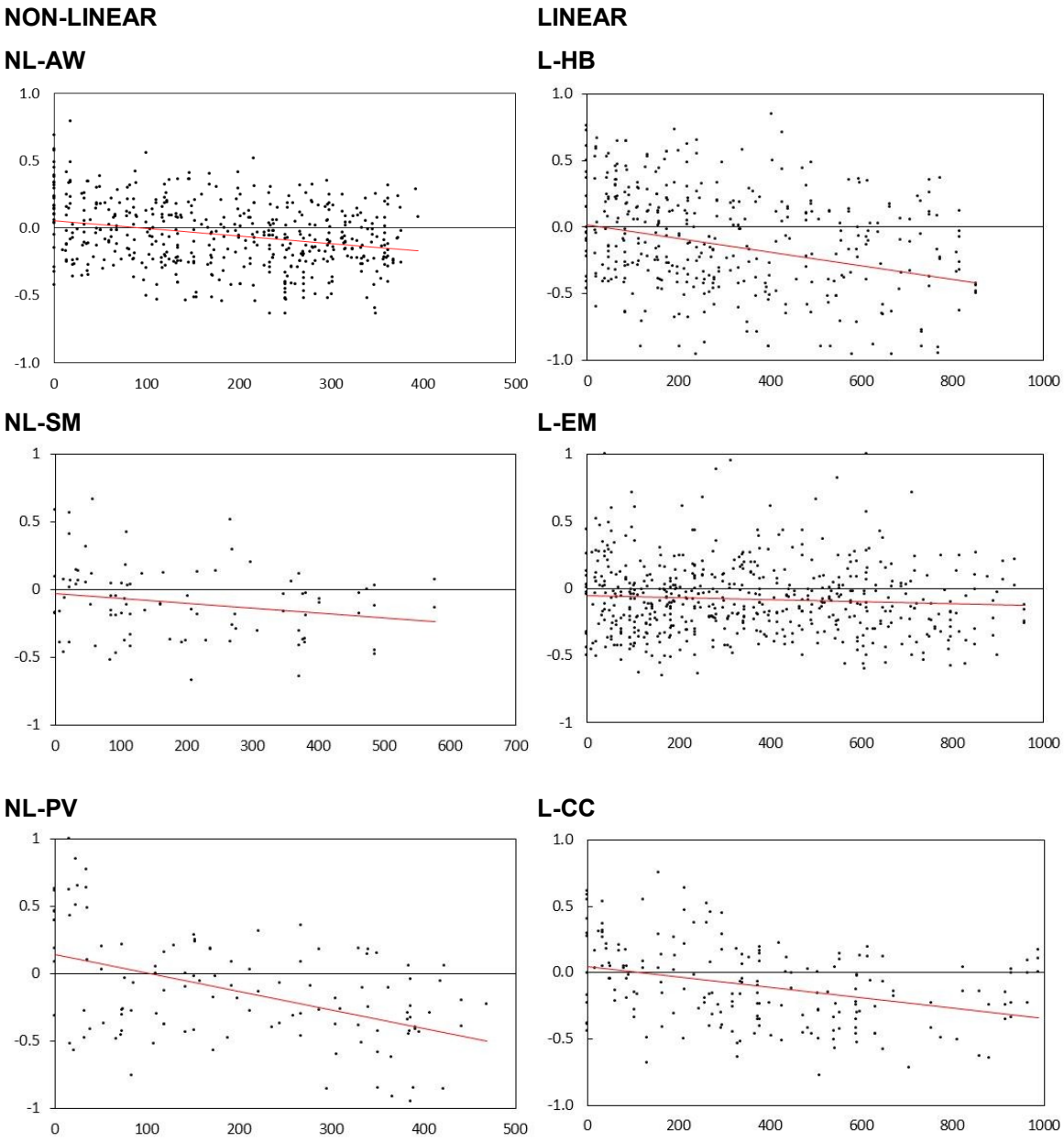


Figure A6.1 *Scatterplots of relatedness coefficients (y-axis) and distance in metres (x-axis) for all pairs of individual water voles by population. Linear trend line is shown in red.*

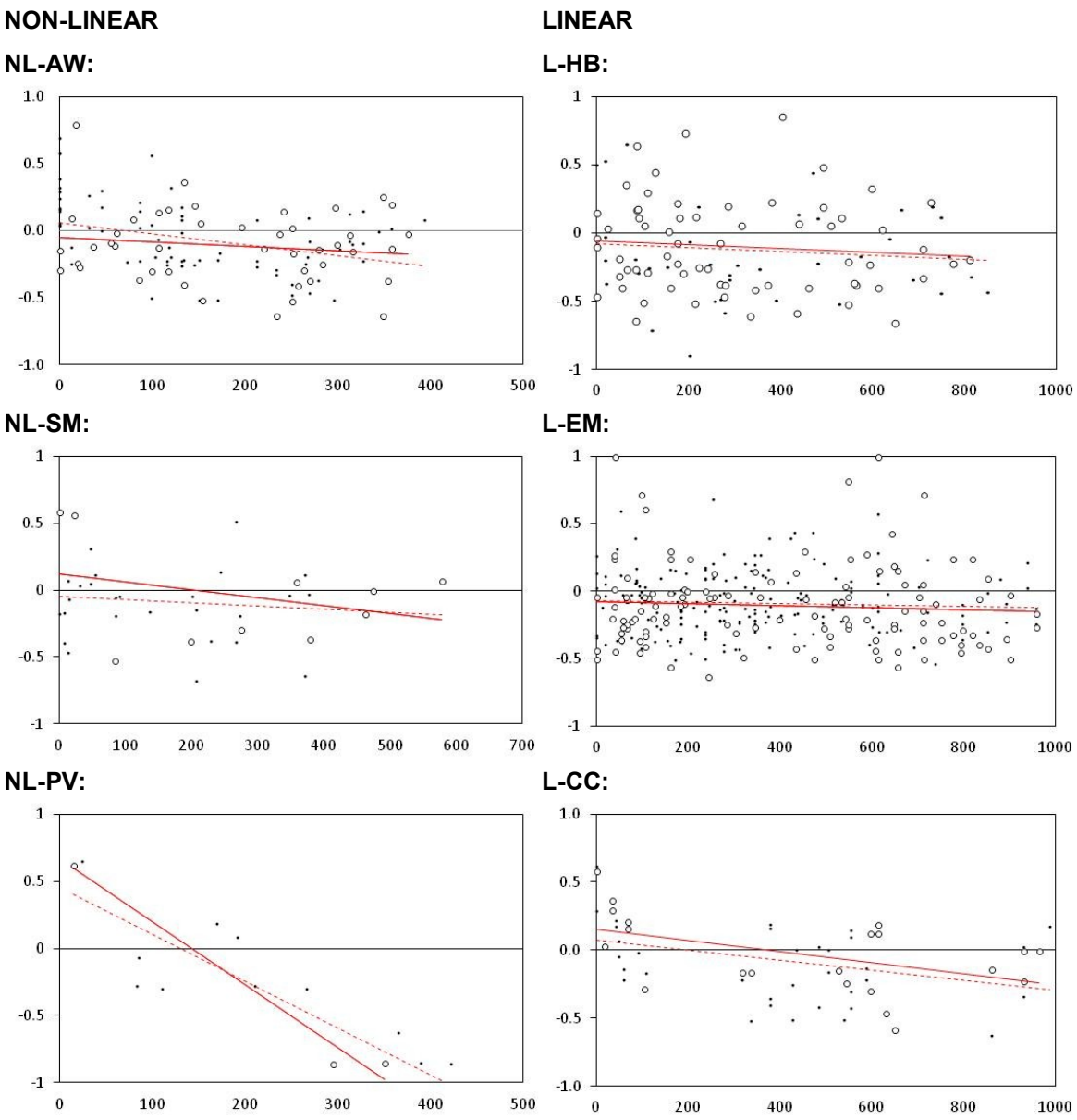


Figure A6.2 *Scatterplots of relatedness coefficients (y-axis) and distance in metres (x-axis) for all pairs of female (solid circles) and breeding female (outlined circles) water voles by population. Linear trend line is shown in solid red for breeding females and dotted red for all females. .*

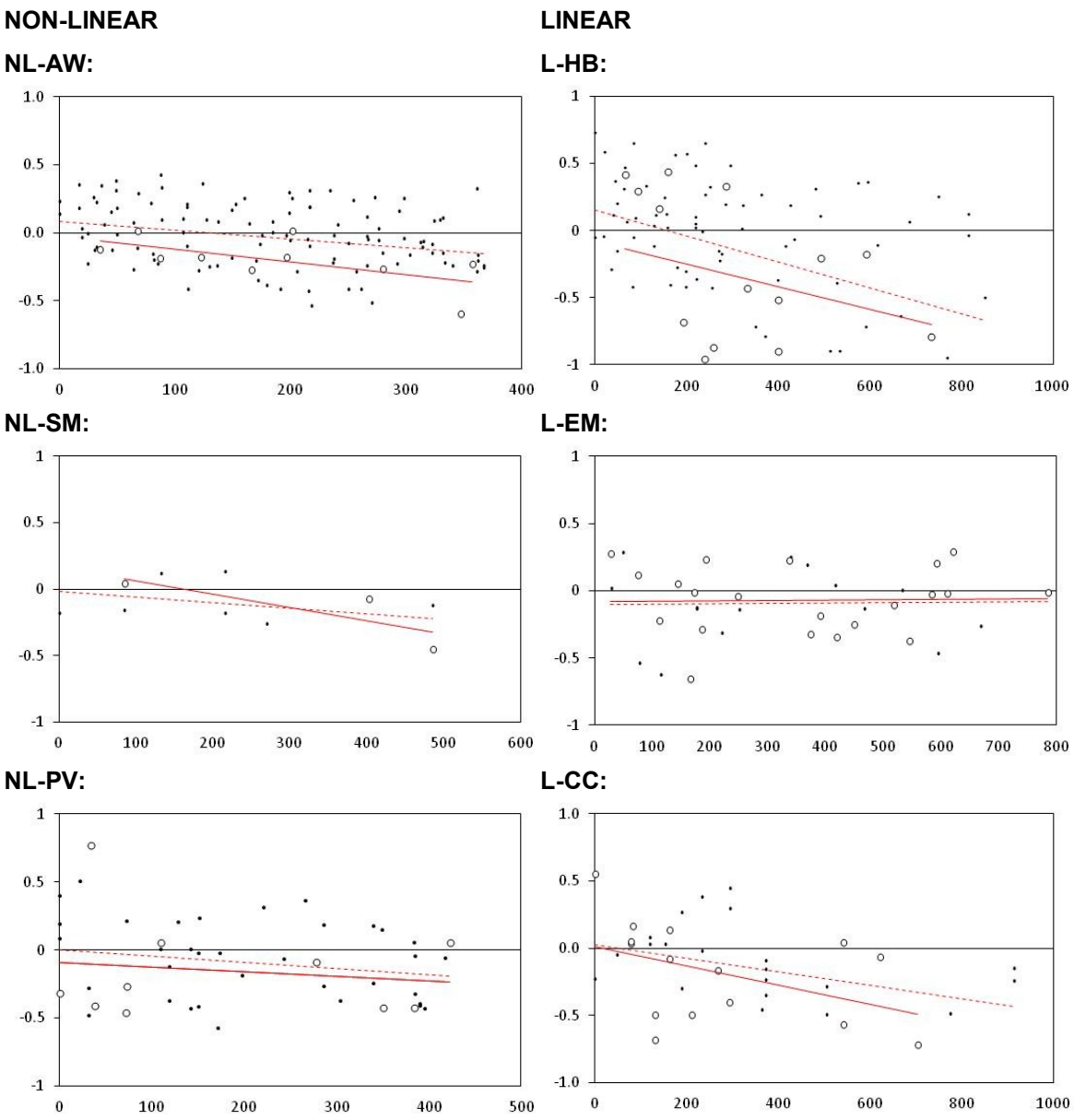


Figure A6.3 *Scatterplots of relatedness coefficients (y-axis) and distance in metres (x-axis) for all pairs of male (solid circles) and breeding male (outlined circles) water voles by population. Linear trend line is shown in solid red for breeding males and dotted red for all males.*

Appendix Seven: Allele frequencies of water vole populations to accompany Chapter Six.

Locus	Allele/n	NL-AW 1	NL-AW 2	NLAW 3	L-CC 3	L-CC 4	L-EM 4	L-HB 1	L-HB 2	L-HB 3	L-HB 4	NL-LW 3	NL-LW 4	NL-PV 3	NL-PV 4	NL-SM 3	NL-SM 4	L-RD 3
AV7	155	0.038	0.000	0.156	0.000	0.000	0.000	0.016	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	159	0.000	0.000	0.000	0.000	0.000	0.455	0.048	0.109	0.091	0.040	0.111	0.000	0.000	0.000	0.000	0.000	0.000
	163	0.029	0.032	0.047	0.000	0.000	0.015	0.000	0.000	0.045	0.000	0.167	0.273	0.031	0.125	0.375	0.182	0.000
	167	0.163	0.210	0.188	0.310	0.357	0.167	0.210	0.152	0.136	0.060	0.389	0.136	0.000	0.000	0.000	0.000	1.000
	171	0.308	0.210	0.266	0.483	0.357	0.182	0.387	0.261	0.455	0.500	0.000	0.000	0.719	0.469	0.167	0.227	0.000
	175	0.337	0.355	0.172	0.017	0.000	0.136	0.306	0.478	0.273	0.320	0.000	0.000	0.000	0.000	0.458	0.500	0.000
	179	0.125	0.177	0.172	0.000	0.143	0.030	0.000	0.000	0.000	0.040	0.333	0.591	0.000	0.000	0.000	0.045	0.000
	183	0.000	0.000	0.000	0.086	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.250	0.406	0.000	0.000	0.000
	187	0.000	0.000	0.000	0.017	0.071	0.015	0.032	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045	0.000
	191	0.000	0.016	0.000	0.086	0.071	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
AV8	276	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250
	280	0.000	0.016	0.031	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.406	0.045	0.000	0.318	0.000
	284	0.000	0.145	0.188	0.000	0.000	0.000	0.000	0.167	0.545	0.000	0.000	0.000	0.219	0.045	0.000	0.045	0.625
	288	0.000	0.113	0.031	0.630	0.000	0.000	0.000	0.000	0.000	0.000	0.222	0.273	0.000	0.000	0.000	0.500	0.125
	292	0.048	0.323	0.391	0.241	0.000	0.034	0.000	0.233	0.273	0.036	0.389	0.273	0.219	0.136	0.000	0.045	0.000
	296	0.144	0.032	0.063	0.037	0.000	0.069	0.000	0.000	0.000	0.018	0.222	0.045	0.000	0.273	0.364	0.045	0.000
	300	0.163	0.065	0.125	0.000	0.000	0.121	0.561	0.400	0.000	0.625	0.167	0.182	0.000	0.318	0.045	0.000	0.000
	304	0.058	0.048	0.016	0.093	0.643	0.293	0.015	0.000	0.091	0.089	0.000	0.000	0.156	0.045	0.273	0.045	0.000
	308	0.500	0.258	0.125	0.000	0.357	0.224	0.409	0.167	0.045	0.107	0.000	0.091	0.000	0.000	0.182	0.000	0.000
	312	0.029	0.000	0.000	0.000	0.000	0.086	0.000	0.000	0.000	0.000	0.000	0.091	0.000	0.000	0.136	0.000	0.000
	316	0.000	0.000	0.000	0.000	0.000	0.069	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.000	0.000
	320	0.058	0.000	0.031	0.000	0.000	0.103	0.015	0.033	0.000	0.125	0.000	0.000	0.000	0.136	0.000	0.000	0.000
AV9	221	0.122	0.043	0.047	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	225	0.122	0.152	0.109	0.017	0.143	0.031	0.516	0.500	0.364	0.411	0.000	0.000	0.000	0.000	0.038	0.000	0.000
	229	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.071	0.214	0.364	0.000	0.000	0.000	0.000	0.167
	233	0.122	0.109	0.234	0.000	0.000	0.219	0.081	0.045	0.136	0.036	0.143	0.000	0.100	0.000	0.269	0.318	0.000
	237	0.000	0.000	0.016	0.034	0.000	0.297	0.000	0.045	0.000	0.000	0.000	0.000	0.467	0.367	0.000	0.000	0.000
	241	0.276	0.304	0.219	0.379	0.643	0.234	0.403	0.409	0.409	0.446	0.214	0.227	0.000	0.100	0.538	0.409	0.167
	245	0.255	0.196	0.297	0.121	0.071	0.141	0.000	0.000	0.000	0.018	0.071	0.227	0.267	0.433	0.154	0.136	0.000
	249	0.051	0.022	0.000	0.086	0.143	0.047	0.000	0.000	0.000	0.018	0.000	0.045	0.000	0.067	0.000	0.136	0.167
	253	0.010	0.000	0.000	0.103	0.000	0.016	0.000	0.000	0.000	0.000	0.071	0.045	0.100	0.033	0.000	0.000	0.000
	257	0.041	0.109	0.063	0.000	0.000	0.000	0.000	0.000	0.091	0.000	0.000	0.000	0.067	0.000	0.000	0.000	0.500
	261	0.000	0.000	0.000	0.207	0.000	0.000	0.000	0.000	0.000	0.000	0.143	0.045	0.000	0.000	0.000	0.000	0.000
	265	0.000	0.065	0.016	0.052	0.000	0.000	0.000	0.000	0.000	0.000	0.143	0.045	0.000	0.000	0.000	0.000	0.000
AV11	326	0.000	0.000	0.000	0.000	0.000	0.000	0.040	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	330	0.008	0.019	0.000	0.000	0.000	0.000	0.060	0.000	0.136	0.025	0.000	0.000	0.000	0.000	0.036	0.050	0.000
	334	0.017	0.019	0.047	0.000	0.000	0.034	0.000	0.022	0.091	0.175	0.000	0.000	0.000	0.000	0.036	0.050	0.250
	338	0.117	0.077	0.234	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.375	0.364	0.154	0.036	0.036	0.100	0.000
	342	0.025	0.038	0.203	0.174	0.071	0.000	0.000	0.000	0.000	0.000	0.063	0.227	0.000	0.000	0.071	0.000	0.375
	346	0.000	0.038	0.000	0.000	0.000	0.000	0.020	0.065	0.000	0.075	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	350	0.008	0.077	0.000	0.174	0.000	0.034	0.000	0.043	0.045	0.025	0.000	0.091	0.231	0.393	0.000	0.000	0.250
	354	0.542	0.288	0.203	0.065	0.286	0.603	0.060	0.261	0.045	0.050	0.563	0.318	0.462	0.500	0.143	0.100	0.000
	358	0.083	0.077	0.047	0.261	0.000	0.000	0.240	0.043	0.182	0.050	0.000	0.000	0.000	0.000	0.214	0.400	0.000
	362	0.100	0.019	0.156	0.196	0.357	0.034	0.000	0.087	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000
	366	0.000	0.115	0.016	0.000	0.000	0.000	0.180	0.370	0.182	0.225	0.000	0.000	0.000	0.000	0.107	0.150	0.125
	370	0.042	0.115	0.078	0.000	0.000	0.224	0.400	0.087	0.273	0.350	0.000	0.000	0.077	0.000	0.000	0.050	0.000
	374	0.008	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.179	0.050	0.000
	378	0.025	0.019	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000
	382	0.000	0.077	0.000	0.130	0.286	0.034	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.050	0.000
	386	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.071	0.000	0.000	0.000
	390	0.025	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.038	0.000	0.000	0.000	0.000
AV12	174	0.255	0.310	0.267	0.000	0.000	0.182	0.000	0.000	0.000	0.068	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	178	0.020	0.000	0.000	0.000	0.000	0.000	0.233	0.042	0.000	0.068	0.278	0.227	0.000	0.000	0.000	0.000	0.000
	182	0.010	0.034	0.000	0.036	0.143	0.106	0.000	0.000	0.000	0.023	0.167	0.045	0.033	0.000	0.107	0.136	0.000
	186	0.098	0.069	0.067	0.107	0.143	0.227	0.167	0.208	0.050	0.250	0.111	0.091	0.000	0.067	0.000	0.000	0.875
	190	0.010	0.017	0.017	0.054	0.000	0.152	0.167	0.021	0.000	0.045	0.000	0.000	0.033	0.133	0.321	0.136	0.000
	194	0.137	0.190	0.200	0.375	0.357	0.182	0.267	0.354	0.400	0.136	0.444	0.636	0.133	0.000	0.036	0.000	0.125
	198	0.265	0.241	0.100	0.196	0.286	0.000	0.000	0.000	0.150	0.000	0.000	0.000	0.000	0.000	0.143	0.136	0.000
	202	0.186	0.069	0.267	0.214	0.071	0.061	0.000	0.000	0.000	0.000	0.000	0.000	0.800	0.800	0.143	0.318	0.000
	206	0.010	0.017	0.050	0.000	0.000	0.091	0.033	0.104	0.050	0.068	0.000	0.000	0.000	0.000	0.179	0.182	0.000
	210	0.010	0.052	0.033	0.000	0.000	0.000	0.117	0.271	0.350	0.341	0.000	0.000	0.000	0.000	0.071	0.091	0.000
	226	0.000	0.000	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	234	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
AV13	170	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	174	0.241	0.113	0.172	0.000	0.000	0.088	0.621	0.083	0.091	0.161	0.389	0.273	0.250	0.233	0.000	0.000	0.375
	178	0.216	0.419	0.328	0.038	0.071	0.044	0.030	0.313	0.455	0.321	0.111	0.273	0.313	0.367	0.071	0.150	0.000
	182	0.138	0.210	0.138	0.712	0.714	0.441	0.152	0.333	0.318	0.464	0.278	0.227	0.094	0.167	0.286	0.200	0.000
	186	0.017	0.032	0.069	0.212	0.071	0.029	0.000	0.000	0.000	0.000	0.222	0.227	0.219	0.167	0.393	0.250	0.000
	190	0.224	0.226	0.207	0.038	0.143	0.397	0.015	0.208	0.136	0.018	0.000	0.000	0.125	0.067	0.071	0.100	0.625
	194	0.026	0.000	0.086	0.000	0.000	0.000	0.182	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.143	0.100	0.000
	198	0.138	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.200	0.000
AV14	220	0.008	0.000	0.000	0.000	0.000	0.061	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	224	0.017	0.097	0.017	0.000	0.000	0.333	0.000	0.000	0.000	0.155	0.000	0.000	0.071	0.033	0.000	0.000	0.000
	228	0.325	0.403	0.250	0.571	0.429	0.030	0.141	0.304	0.100	0.500	0.357	0.409	0.286	0.267	0.036	0.050	0.000
	232	0.183	0.081	0.017	0.018	0.000	0.091	0.031	0.022	0.400	0.017	0.286	0.273	0.179	0.167	0.714	0.650	0.750
	236	0.133	0.065	0.100	0.018	0.071	0.318	0.156	0.196	0.500	0.052	0.214	0.227	0.000	0.133	0.179	0.250	0.250
	240	0.292	0.290	0.533	0.036	0.000	0.167	0.672	0.478	0.000	0.259	0.143	0.091	0.464	0.400	0.071	0.050	0.000
	244	0.042	0.065	0.083	0.161	0.214	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	248	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	256	0.000	0.000	0.000	0.071	0.143	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	264	0.000	0.000	0.000	0.054	0.143	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
AV15	176	0.509	0.500	0.484	0.161	0.071	0.485	0.484	0.375	0.636	0.586	0.250	0.455	0.375	0.107	0.769	0.700	0.875
	180	0.000	0.000	0.000	0.107	0.071	0.015	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.050	0.000
	184	0.055	0.145	0.113	0.000	0.000	0.132	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	188	0.000	0.000	0.000	0.000	0.000	0.118	0.000	0.000	0.000	0.000	0.125	0.045	0.063	0.107	0.192	0.250	0.000
	192	0.173	0.065	0.065	0.000	0.000	0.250	0.375	0.625	0.318	0.345	0.000	0.000	0.281	0.250	0.000	0.000	0.000
	196	0.027	0.016	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.094	0.000	0.000	0.000	0.000
	200	0.000	0.032	0.000	0.018	0.071	0.000	0.000	0.000	0.000	0.000	0.125	0.136	0.156	0.286	0.000	0.000	0.000
	204	0.100	0.129	0.113	0.286	0.143	0.000	0.109	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	208	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125
	212	0.136	0.113	0.226	0.071	0.071	0.000	0.031	0.000	0.045	0.000	0.375	0.318	0.031	0.250	0.000	0.000	0.000
	216	0.000	0.000	0.000	0.143	0.286	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	220	0.000	0.000	0.000	0.089	0.071	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	224	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.045	0.000	0.000	0.000	0.000	0.000
	228	0.000	0.000	0.000	0.089	0.214	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Appendix Eight: Whole sequence alignment of 14 unique haplotypes identified from water vole mtDNA from hair samples obtained from east and south east England.

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                    10        20        30        40        50        60        70        80
                    |---------|---------|---------|---------|---------|---------|---------|
Haplotype: 1,       CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 2,       CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 3,       CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 4,       CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 5,       CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 6,       CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 7,       CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 8,       CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 9,       CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 10,      CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 11,      CGGGCCCATTTTAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 12,      CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 13,      CGGGCCCATTTAAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT
Haplotype: 14,      CGGGCCCATTCTAACTTGGGGGTAGCTAATGTGAAACTTTATCAGACATCTGGTTCTTACCTCAGGGCCATATTAATGTT

                    90        100       110       120       130       140       150       160
                    |---------|---------|---------|---------|---------|---------|---------|
Haplotype: 1,       TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 2,       TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 3,       TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 4,       TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 5,       TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 6,       TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 7,       TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 8,       TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 9,       TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 10,      TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 11,      TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 12,      TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 13,      TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC
Haplotype: 14,      TTATCGTCCATACGTTCCCCTTAAATAAGACATCTCGATGGTACGGGTCTAATCAGCCCATGCCTAACATAACTGTGGTC

                    170       180       190       200       210       220       230       240
                    |---------|---------|---------|---------|---------|---------|---------|
Haplotype: 1,       TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 2,       TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 3,       TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 4,       TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 5,       TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 6,       TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 7,       TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 8,       TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 9,       TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 10,      TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 11,      TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 12,      TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 13,      TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT
Haplotype: 14,      TCGGGCAGTTGGTATTTTTTAATTTTCGGGATGGTATCACTCAGCATAGCCGTCAAGGCATGAAGGTCACCTTGTCCTGT

                    250       260       270       280       290       300       310       320
                    |---------|---------|---------|---------|---------|---------|---------|
Haplotype: 1,       AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 2,       AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 3,       AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 4,       AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 5,       AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 6,       AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 7,       AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 8,       AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 9,       AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 10,      AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 11,      AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 12,      AGCTGGACTTCTCATTCAAGTATCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 13,      AGCTGGACTTCTCATTCAAGTGTCATTTACCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAG
Haplotype: 14,      AGCTGGACTTCTCATTCAAGTATCATTTATCCACATAAATACCTTACCAACATATTAATCAATGGATCCGGGACATATAA
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                330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Haplotype: 1,   TTAAGCAGTTCTATTATTGCAAATACTGTAATCCTCCCACCCAGCTCTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 2,   TTAAGCAGTCCTATTATTGCAAATACTGTAATCCTCCCACCCAGCTCTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 3,   TTAAGCAGTCCTATTATTGCAATTACTGTAATCCTCCCACCCAGCTCTATAGACTAAATTATTCATGTTAAATGGACATA
Haplotype: 4,   TTAAGCAGTCCTATTATTGCAAATATTGTAATCCTCCCACCCAGCTCTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 5,   TTAAGCAGTCCTATTATTGCAAATACTGTAATCCTCCCACCCAGCTTTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 6,   TTAAGCAGTCCTATTATTGCAAATACTGTAATCCTCCCACCCAGCTTTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 7,   TTAAGCAGTCCTATTATTGCAAATACTGTAATCCTCCCACCCAGCTCTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 8,   TTAAGCAGTCCTATTATTGCAAATACTGTAATCCTCCCACCCAGCTCTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 9,   TTAAGCAGTCCTATTATTGCAAATATTGTAATCCTCCCACCCAGCTCTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 10,  TTAAGCAGTCCTATTATTGCAAATACTGTAATCCTCCCACCCAGCTCTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 11,  TTAAGCAGTCCTATTATTGCAAATACTGTAATCCTCCCACCCAGCTCTGTAGACTAAATTATTCATGTTAAATGGACATA
Haplotype: 12,  TTAAGCAGTCCTATTATTGCAAATACTGTAATCCTCCCACTCAGCTCTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 13,  TTAAGCAGTCCTATTATTGCAAATACTGTAATCCTCCCACCCAGCTCTATAGACTAAATTATTCATGTTAAGTAGACATA
Haplotype: 14,  TTAAGCAGTTCTATCATTGCAAATACTGTAATCCTCCCACCCAGCCCTGTAGGCTAAATTCTTCATGTTAGATAGACATA

                410       420       430       440       450       460       470       480
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Haplotype: 1,   ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCC-ATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 2,   ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCC-ATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 3,   ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCCCATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 4,   ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCC-ATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 5,   ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCCCATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 6,   ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCC-ATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 7,   ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCC-ATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 8,   ATATATTAAAAAA-CTTTGCTTGCCAAACCCCCCC-ATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 9,   ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCCCATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 10,  ATATATTAAAAAA-CTTTGCTTGCCAAACCCCCCC-ATCCCCCCCTGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 11,  ATATATTAAAAAA-CTTTGCTTGCCAAACCCCCCCCATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 12,  ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCC-ATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 13,  ATATATTAAAAAAACTTTGCTTGCCAAACCCCCCCCATCCCCC-TGAAAATGCCCATTCTTGCCAAACCCCAAAAACAA
Haplotype: 14,  ATATATCAAAAAA-CTTTGCTTACCAAACCCCCCC-ATCCCCC-TGAAAGTGCCCACTCTTGCCAAACCCCAAAAACAA

                490       500       510       520       530       540       550       560
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Haplotype: 1,   GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 2,   GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 3,   GCGGAACAAAATCAGGCCTCCCCGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTTAGGAAAAAAAAA
Haplotype: 4,   GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 5,   GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 6,   GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 7,   GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 8,   GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 9,   GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 10,  GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 11,  GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAACAGCTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 12,  GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 13,  GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGTAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA
Haplotype: 14,  GCGAAACAAAATCAGGCCTCCCTGTGTACTTGAAAGCAGACCCGAAAATAACTTAAAATTTAGTACTCAGGAAAAAAAAA

                570       580       590       600       610       620       630       640
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Haplotype: 1,   AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTTTAAG
Haplotype: 2,   AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTT-AAG
Haplotype: 3,   AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTT-AAG
Haplotype: 4,   AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTT-AAG
Haplotype: 5,   AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTTTAAG
Haplotype: 6,   AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTTTAAG
Haplotype: 7,   AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTT-AAG
Haplotype: 8,   AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTT-AAG
Haplotype: 9,   AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTT-AAG
Haplotype: 10,  AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTT-AAG
Haplotype: 11,  AATCAAAAATTTACTACCAAAAAACAACTCAAAAATTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTT--AAG
Haplotype: 12,  AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTT-AAG
Haplotype: 13,  AATCAAAAATTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTTTAAAGTACAATTTCAGTATTGACTTTTTTTAAG
Haplotype: 14,  AATCAAAAACTTACTACCAAAAAACAACTCAAAA-TTCCAAAAACTCTAAAGTACAATTTCAGTATTGACTTTTT--AAG
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                    650       660       670       680       690       700       710       720
                    ----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
Haplotype: 1,   GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 2,   GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 3,   GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 4,   GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 5,   GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 6,   GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 7,   GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 8,   GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 9,   GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 10,  GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 11,  GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 12,  GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 13,  GTTAATGTAGCTTATATTAAAGCAAAGCACTGAAAATGCTTAGATGGATGCTATCATCCCATAAACACAAAGGTTTGGTC
Haplotype: 14,  GTTAATGTAGCTTATATAAAAGCAAAGCACTGAAAATGCTTAGATGGATGTTATCATCCCATAAACACAAAGGTTTGGTC

                    730
                    ----|----|-
Haplotype: 1,   CTGGCCTTATA
Haplotype: 2,   CTGGCCTTATA
Haplotype: 3,   CTGGCCTTATA
Haplotype: 4,   CTGGCCTTATA
Haplotype: 5,   CTGGCCTTATA
Haplotype: 6,   CTGGCCTTATA
Haplotype: 7,   CTGGCCTTATA
Haplotype: 8,   CTGGCCTTATA
Haplotype: 9,   CTGGCCTTATA
Haplotype: 10,  CTGGCCTTATA
Haplotype: 11,  CTGGCCTTATA
Haplotype: 12,  CTGGCCTTATA
Haplotype: 13,  CTGGCCTTATA
Haplotype: 14,  CTGGCCTTATA
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