# Know your enemy: A molecular approach to determine how the pygmy shrew (*Sorex minutus*) in Ireland succumbs to the invasion of the greater white-toothed shrew (*Crocidura russula*).

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Rising early each morning
We get out our traps,
And exploring prickly ditches
We set out our traps

The species is decided
On close inspection,
Each bagged and recorded
For later dissection,

We sat down with our bottles
And sharpened little knives,
And recorded every detail
Gaining knowledge of their lives,

This island is heaven
I think it does suit us,
Said *Crocidura russula*
And *Sorex minutus*

Stephen Browett – “Friendly Shrews of Belle Ile” (2017)

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## Candidate Declaration

All the work presented in this thesis is my own. It involved collaborations with multiple colleagues:

Jon Yearsley (JY) helped to calculate the required sample sizes for gaining ethical approvals.

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# Abbreviations

**CR-Ire**: *Crocidura russula* from Ireland

**CR-Bel**: *Crocidura russula* from Belle Île

**RFC**: Random Forest Classifier

**LGM**: Last Glacial Maximum

**MOTU**: Molecular Operational Taxonomic Unit

**POO**: Percentage of Occurrence

**RRA**: Relative Read Abundance

**SFB**: Segmented Filamentous Bacteria

**SM-Ire**: *Sorex minutus* from Ireland

**SM-Bel**: *Sorex minutus* from Belle Île

# Abstract

Ireland's smallest resident mammal, the pygmy shrew (*Sorex minutus*) is rapidly being displaced by the invasive greater white-toothed shrew (*Crocidura russula*). The presence of this invasive shrew was first recorded in 2007 and they have been expanding their range at a rate of ~5km per year. Considering these two species co-inhabit other regions of Europe, this raises the question of why they seemingly cannot coexist in Ireland. This study applies DNA (and rRNA) metabarcoding to shrew gut contents to investigate the roles of resource competition and gut microbial community structure in this species replacement event. This was applied to over 300 shrews of both species sampled across radial transects in Ireland, two seasons, and a natural 'control' site in Belle Île (France) where both species occur together in high abundance. The results show that during the initial stage of colonisation there is little resource competition between the species allowing their ranges to overlap. Over time, interspecific competition increases as the long-term established populations of *C. russula* switch their diet to the core prey of *S. minutus*. This could be a result of *C. russula* exhausting local invertebrate resources, which could be damaging to Ireland's invertebrate community structure. The Belle Île population of *S. minutus* can co-exist with *C. russula* by exploiting locally abundant key taxa, but the Irish *S. minutus* are not adapting their diet in response to the invader. In addition, Irish *S. minutus* have a different microbiome structure with a significantly reduced abundance of microbes associated with host immunity which may have left them susceptible to newly introduced stresses. No novel pathogens were detected in the invasive population of *C. russula*. This diet-microbiome analyses demonstrates that Irish *S. minutus* have not adapted to compete with another shrew species and could be completely replaced by *C. russula* in Ireland over time. This multi-faceted approach on this invasive system has demonstrated that subtle differences between populations of shrews can have significant effects on their ability to co-exist.

# Chapter 1 - Introduction

**Note:** Several sections within this Chapter appear in Browett SS, O'Meara DB, McDevitt AD (2020) Genetic tools in the management of invasive mammals: recent trends and future perspectives. *Mammal Review* 50: 200 – 210. Doi: 10.1111/mam.12189

## 1.1. Species Introductions

For millions of years, flora and fauna have been expanding their ranges, colonising new areas and causing changes in community compositions (Ricciardi, 2007). The more recent 20,000 years have seen colonisations (or re-colonisations) of mammals into the British Isles as the ice sheets receded from the Last Glacial Maximum (LGM) and opened up corridors into new areas (Searle, 2008a; Montgomery *et al*., 2014). The most iconic example of range expansions is probably humans (*Homo sapiens*) leaving Africa and colonising the entire world over a period of only tens of thousands of years (Templeton, 2002). The constant travels, expansion and development of human society has led to us bringing other species with us into new areas, such as Polynesian rats (*Rattus exulans*) into New Zealand around 1280 A.D. (Wilmshurst *et al*., 2008; Hulme, 2009). Although the colonisation of new areas by flora and fauna (including humans) is not a recent phenomenon, it has gained a huge amount of attention by researchers because of the increased rate and dispersal distance at which new introductions are happening due to increased globalisation (Hulme, 2009).

Hulme *et al*. (2008) covers various modes of introduction of species into new areas by humans. These include intentional introductions, such as the harlequin ladybird (*Harmonia axyridis*) which originated in Asia but was introduced to various countries (such as North America) as a form of crop pest control by predating on aphids (Roy *et al*., 2016). The grey squirrel (*Sciurus carolinensis*) was intentionally introduced multiple times into parts of the British Isles from North America as they were considered an ornamental species, but they subsequently expanded to cover the majority of the UK and Ireland (Signorile *et al*., 2016). Many organisms have been accidentally introduced through various means, such as an initial intentional introduction (for captive purposes) and subsequent escape of organisms. This form of accidental introduction happened with many plant species after an increase of cultivating foreign plants for ornamental value in botanical and private gardens (Hulme, 2015). For example, *Lantana camara* was brought into India, Africa and Europe from South America for ornamental purposes

but expanded into wild habitats where it is now abundant (Taylor *et al*., 2012). The North American mink (*Neovison vison*) escaped from multiple European captive fur farms to establish populations that expanded their ranges in multiple countries (Bonesi and Palazon, 2007). Introduced organisms can simultaneously co-introduce novel pathogens into new areas (Hulme, 2014). Rodents such as the black rat (*Rattus rattus*) and Norway rat (*R. norvegicus*) are likely to be responsible for the dissemination of the blood-borne pathogen *Bartonella sp.* to multiple countries (Hayman, McDonald and Kosoy, 2013).

The increasing rate at which species are introduced into new areas outside their native ranges corresponds with developments in global trade and transport (Hulme, 2009; Seebens *et al*., 2017). There was an increase of introductions corresponding to the industrial revolution, with another increase in the last 35 years due to later advancement in transport efficiency and globalisation (Hulme, 2009). Marine organisms can be transported over large distances in ballast water of cargo ships (Seebans, Gastner and Blasius, 2013). Even recent developments of trading items over the internet has seen a spike in transporting flora and fauna to new regions across the globe (Lenda *et al*., 2014). At the current rate of economic and technical development, these introductions show little evidence of slowing down (Seebens *et al*., 2017). The ever-increasing shipping rates around the world have led to predictions of 3 to 20 times higher risk of further species introductions by the year 2050 (Sardain, Sardain and Leung, 2019). In addition to human transport, climate change is also having an influence on the rate that organisms are redistributing themselves to follow their preferred environmental conditions (Pecl *et al*., 2017). We will continue to see large changes in biodiversity and community structures as species introductions continue to happen at an ever-increasing pace.

## 1.2. Introduction Phases

Every species introduced into a new region must over-come various barriers to establish a population in new regions, many of which are unsuccessful (Cassey *et al*., 2005; Lockwood, Cassey and Blackburn, 2009; Zenni and Nuñez, 2013). Based off a previously developed framework (Blackburn *et al*., 2011), Hoffmann and Courchamp (2016) describe the various stages and barriers that a species must go through to successfully establish a population in a new area, regardless if the introduction is

human caused or not (**Figure 1.1**). Newly arrived organisms must be capable of finding the required resources to survive such as food, water and safety from predators. A sufficient number of individuals of an ideal sex ratio must also arrive in a new area to facilitate reproduction (Simberloff, 2009). The more individuals released into an area (including multiple introductions), the higher the chance of becoming an established and self-sustaining population (Lockwood, Cassey and Blackburn, 2009). This relationship between the number of introduced individuals and establishment success is referred to as propagule pressure (Lockwood, Cassey and Blackburn, 2009). Propagule pressure is reliant on various factors, such as genetics. If only a small number of individuals arrive into a new area, they go through a population bottleneck which leads to high levels of inbreeding and reduced genetic variability for adapting to the new environment (Dlugosch and Parker, 2008; Dlugosch *et al*., 2015; Barker *et al*., 2019). Alternatively, introduced individuals may have 'pre-adapted' genotypes suitable for the new ecosystem which increases the likelihood of a successful establishment (Rius and Darling, 2014; Bock *et al*., 2015). Propagule pressure and establishment success can also be dependent on environmentally stochastic events such as the suitability of the area for the species survival (i.e. releasing a non-native fish in the desert will not be successful; Simberloff, 2009). Another factor is the condition of the introduced population influenced by method of transport (Verling *et al*., 2005). A long journey with limited resources will result in weaker individuals with a lower chance of survival in new terrain. It must be noted that propagule pressure alone is not enough to fully predict the outcome of an introduced species (Blackburn *et al*., 2013).

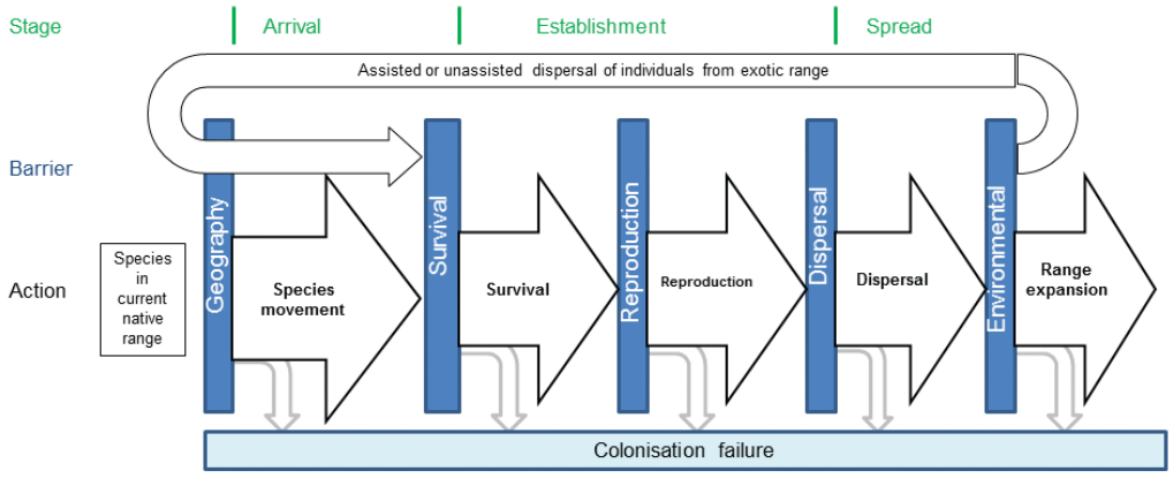



**Figure 1.1.** The stages/phases involved in the introduction, establishment and expansion of alien species in new regions. Figure taken from Hoffman and Courchamp (2016).

If an introduced species is successful in establishing a population that begins to increase in size, they will expand out and 'disperse' into new areas and begin the cycle all over. This framework by Hoffmann and Courchamp (2016) accounts for these populations naturally dispersing to adjacent areas, or subsequent introductions into new regions again. The rapidly expanding population of non-native cane toads (*Bufo marinus*) in Australia is an example (Phillips *et al*., 2006). Over time, introduced species can 'naturalise' and become an integrated part of the ecosystem, such as the pygmy shrew (*Sorex minutus*) (McDevitt *et al*., 2009) and red deer (*Cervus elaphus*) (Carden *et al*., 2012) in Ireland.

## 1.3. Terminology

Studying how organisms enter a new area and what effects they have on the new area is commonly referred to as 'Invasion science' (Richardson and Ricciardi, 2013). Various terms have been used to describe these introduced species (or populations) over the years, such as 'invasive' (Tompkins, White and Boots, 2003), 'alien' (Blackburn *et al*., 2014), 'invasive alien' (Roy *et al*., 2012), 'introduced' (Barrios-Garcia and Ballari, 2012) and more (Valéry *et al*., 2008). Valéry *et al*. (2008) discuss in detail the various criteria that have been used to classify an introduced species as invasive based off various criteria such as the distance travelled, the geographic barriers crossed and the environmental impact of that population. However, the existence of many terms and what they've been used to define can cause confusion within the field of ecology, let alone invasion science (Blackburn *et al*., 2011). For example, the term 'invaded' has been used to describe colonisation of North America by Eurasian mammals millions of years ago (Leigh, Dea and Vermeij, 2014). 'Invasive' was also used to describe movement of marine species from the Red Sea to the Mediterranean via a man-made canal (Azzurro *et al*., 2016), or mammals directly brought into new areas by humans (Doherty *et al*., 2016). In addition, some groups consider referring specifically to the population in question rather than the species, based on the premise that a species could be non-native in one place, but not another (Pereyra, 2016).

Other less accusatory terms become questioned, such as 'non-native', so some studies have asked to define the term used in each article (Richardson *et al*., 2000). Pereyra (2019) define a non-native as a species or population occurring outside of their native range. They do however highlight the difficulty in describing a species that

has crossed a barrier only possible with human intervention as non-native, compared to a species with high dispersal capabilities (such as birds) that do not occupy particular areas within their dispersal range. Should we consider species entering new areas on their own as non-native? This can become complicated further when considering populations that have naturally dispersed due to climate change, which has increased rates of species introductions (Ricciardi, 2007; Pecl *et al*., 2017). These species could be considered to disperse in response to human intervention, classifying them as non-native even though they relocated via their own abilities.

Despite what the 'correct' definition or classification of these introduced species is, it is a fact that some introduced populations can cause a range of significantly negative effects to biodiversity and the economy. Considering the possible negative effects and the increasing rate of introductions, invasion biology is a necessary discipline to understand and mitigate these impacts. The difference between the terminology can be considered an ethical concern (Hoffmann and Courchamp, 2016). Perhaps these non-native species only need to be classified according to the extent of their impact on the local ecosystem (Blackburn *et al*., 2014; Jeschke *et al*., 2014; García-Díaz *et al*., 2020). The International Union for Conservation of Nature (IUCN) classifies an alien population as 'invasive' if they have a harmful impact on the local ecosystem, economy or human health (IUCN, 2000; Pyšek *et al*., 2020). As this work will concentrate on the negative impacts of introduced species, the term 'invasive' will be used hereafter.

## 1.4. Negative Impacts and Management of Invasive Species

Although they can provide economic benefits (Roy *et al*., 2016), the severe negative impacts of invasive species are now considered to be one of the greatest contributors to species extinctions world-wide (Bellard, Cassey and Blackburn, 2016; Catford, Bode and Tilman, 2018). Invasive mammal predators (such as canids and mustelids) contribute significantly to biodiversity loss, as they have been recorded to have negatively impacted on 596 threatened and caused 142 extinct species world-wide (Doherty *et al*., 2015, 2016). Invasive species can threaten native populations through resource competition. For example, after the South American lantana plant (*L. camara*) was introduced to India and South Africa for ornamental purposes during the mid-19th century, it began out-competing local vegetation, including crops and is toxic to many wild animals and livestock (Bhagwat *et al*., 2012). The harlequin ladybird (*H. axyridis*)

is an Asian insect that has been intentionally introduced to multiple European countries as a crop pest control method (Brown *et al.*, 2008), but began to spread to other areas and cause the decline of multiple native ladybird species in Europe through competition and direct predation (Roy *et al*., 2012). Direct predation can have significant impacts on biodiversity, such as feral populations of domestic cats (*Felis silvestris catus*) which have been recorded to predate on over 175 endangered vertebrates across their invasive distribution (Medina *et al*., 2014). The introduction of animals into novel environments comes with the risk of co-introducing pathogens or parasites that local fauna have not yet developed resistance to (Paziewska *et al*., 2011). Mammalian invasions in Europe are likely to have been responsible for the transport of pathogens responsible for zoonotic diseases' such as salmonellosis, toxoplasmosis, and leptospirosis (Hulme, 2014), and for the dissemination of the plague across continents via rodent introductions (Gage and Kosoy, 2005).

The concern of biodiversity change by invasive species is due to the link from alpha and beta diversity to 'ecosystem functions' (the processes involved of passing energy and nutrients through taxa interactions) and 'ecosystem services' (the benefits that ecosystems provide to humanity; Cardinale *et al*., 2012). Although other factors play a role in ecosystem functioning, high biodiversity likely indicates more productive ecosystem functions and services (Cardinale *et al*., 2012; Hooper *et al*., 2012; van der Plas, 2019). Ecosystem functions are complex and not always fully understood, meaning the full extent of any negative effects caused by invasive species may not be appreciated until later on (Pauchard *et al*., 2018). The loss of biodiversity via species extinctions is an irreversible occurrence, meaning that reduced ecosystem services due to extinctions caused by non-native populations can be difficult to restore (Chapin *et al*., 2000; Pyšek *et al*., 2020). In addition, species diversity influences an ecosystems stability and resistance to environmental change, such as climate change (Chapin *et al*., 2000). Shuffling of biodiversity structure and disruption to ecosystem functioning can have knock-on effects on economic efficiency and human health, such as disruption to taxa important for crop pest control (Pecl *et al*., 2017). There are also particular hotspots around the world that have higher alien population richness, mainly islands and coastal regions (Dawson *et al*., 2017).

Islands have been known hotspots for the introduction of invasive species with significant impacts for some time (Simberloff, 1995). An island's vulnerability could be a result of relying on high volumes of imports from foreign lands with an increased risk

of accidental introductions (Dawson *et al*., 2017). Another possibility is a higher rate of successful establishment. Some islands (such as New Zealand) are home to endemic fauna that have never experienced mammal predators, and are therefore naïve to them (e.g. don't practice more evasive behaviour) and provide easy prey resources (Courchamp, Chapuis and Pascal, 2003). The domestic cat (*F. silvestris catus*) has been introduced to the majority of the world's islands, subsequently having negative impacts on local populations (estimated hundreds of species) through direct predation or competition (Medina *et al*., 2011, 2014). The size of islands can also influence the impact. Considering introduced rats, larger islands have a higher chance of containing native predators or competitors that could supress the introduced population while smaller islands would lead to denser populations and exacerbated impacts on the native prey fauna (McCreless *et al*., 2016). Islands are suggested to be high on the priority list for conservation effort against invasive species because of their many endemic species and they house 40% of the worlds threatened species (Tershy *et al*., 2015).

Invasive species can have massive economic costs through agricultural impacts. Introduced European rabbits (*Oryctolagus cuniculus*) in Australia are shown to be responsible for over-grazing of local vegetation, reducing the forage available for domestic livestock (Cooke, 2012). In response to invasive species, plans and policies are put into place to prevent their entry and reduce or eliminate their impact, which comes at a cost. Total costs of economic loss and control efforts for Australia in 2011 – 2012 was estimated at approx. AUS$13.6 billion (Hoffmann and Broadhurst, 2016). Crop and forest production in the US lose an estimated US$40 billion per annum due to invasive insects and pathogens (Pimentel, Zuniga and Morrison, 2005). Countries that are the largest producers of agricultural output are most affected by economic costs from invasive pests and pathogens (Paini *et al*., 2016). The European Union alone spends approximately €12 billion annually on the control and management of invasive species and on mitigating their adverse impacts (Browett, O'Meara and McDevitt, 2020). The most efficient method to reduce impacts from invasive species if to prevent their entry in the first place. This can be done using extremely vigilant checks at borders, which Australia now sets an example (Hoffmann and Broadhurst, 2016).

If invasive species make it across the border into a novel area, the invasive pathway/route must be identified to prevent entry of additional propagules. In the absence of direct evidence indicating the routes of invasion (such as records from

interception at ports), indirect methods such as the analyses of genetic data from invasive populations and putative sources becomes vital (Searle, 2008b; Gargan *et al*., 2016). An invasive species that is beginning to establish a population will typically go through a period of slow population growth (called the 'lag' phase) before a rapid increase in abundance (Mack *et al*., 2000). Control efforts must be implemented during this initial lag phase to have the best chance of successful eradication before it exponentially grows and spreads (Larkin, 2012). This is where early detection methods such as environmental DNA (eDNA) can be useful (Browett, O'Meara and McDevitt, 2020), particularly for invasive mammals as they are difficult to eradicate due to their high reproductive output and ability to rapidly re-disperse (Doherty *et al*., 2015). There is plenty of evidence that control and eradication measures can be successful (Dawson *et al*., 2015), but controlling an invasive population that has made it past the lag phase and rapidly grown in density can be extremely costly. The invasive spiny water flea (*Bythotrephes longimanus*) surpassed the lag phase and established a large population in large North American lakes (such as Lake Mendota, Wisconsin) which lead to severely reduced water clarity. Control efforts was estimated up to US$163 million to return the water clarity to acceptable levels (Walsh, Carpenter and Vander, 2016).

Different invasions require different control strategies that are determined through research and trials. The preferred method of eradicating the plant *L. camara* from its invasive range (e.g. South Africa) is physical removal, which can be costly and time consuming (Bhagwat *et al*., 2012). The cottony cushion scale (*Icerya purchasi*) is an invasive insect in the Galapagos that can damage plant health (including endemic species) in dense populations (Calderón Alvarez *et al*., 2012). After reviewing various control methods, it was decided to use a biological control of introducing the cardinal beetle (*Rhodolia cardinalis*) to supress *I. purchase* through predation and has shown promising results with minimal effects on native wildlife (Hoddle *et al*., 2013). Follow-up impact studies are incredibly important for monitoring the effects of biological control measures and assessing the predictions based off pre-release trials (Calderón Alvarez *et al*., 2012). In areas with multiple functionally equivalent invasive species (such as multiple rat species), removing one of them may result in invasion from another functionally similar non-native species (Russell, Sataruddin and Heard, 2014).

The choice of control method may also have to depend on the preference of different societal groups. For example, horses *(Equus caballus*) were introduced to the

Australian Alps in the 1890's and damaged aquatic and wet land habitats through trampling and grazing (Robertson *et al*., 2019). Although there were scientifically sourced recommendations towards culling these animals for the conservation of native flora and fauna, pressure from the general public prevented the cull from taking place, despite the damage they clearly cause (Höbart, Schindler and Essl, 2020). Education can play a key role in invasive species control as groups may be more willing to pay towards mitigation and management measures if they are fully aware of the negative impacts of the invasive species in question (Junge *et al*., 2019).

While the majority of studies highlight the negative impacts of invasive species, it must be stressed that not all alien populations have significantly negative impacts on the recipient ecosystem and its biodiversity (Moles *et al*., 2012; Jeschke *et al*., 2014). Impacts can be beneficial or harmful to the local ecosystem (Davis *et al*., 2011; Heimpel and Cock, 2018), each with varying degrees of severity (Blackburn *et al*., 2014). For example, after introducing the wild boar (*Sus scrofa*) to Argentina from Europe for hunting purposes, both positive and negative effects have been reported (Caruso *et al*., 2018). While their rooting behaviour alter soil horizon structures, nutrient retention and plant community structures, the animals provide a hunting alternative for native wildlife and prey for native predators (Barrios-Garcia and Ballari, 2012; Caruso *et al*., 2018). Because of the variety of different impacts caused by introduced species, a definition of 'impact' is difficult, so it is recommended that when talking about impacts, they are clearly described (Jeschke *et al*., 2014).

## 1.5. Invasive Species in Ireland

### 1.5.1. Ireland

Many aspects of invasions are difficult to predict, such as how likely they will establish a viable population and spread, or what impacts they will have on the novel ecosystem (Griffen *et al*., 2020). Some studies have utilised experimental set ups to try and predict invasive species impacts on full scale ecosystems (Lohrer and Whitlatch, 2002). These kinds of experimental predictions can be difficult to fully interpret, meaning there is a call out for extensive studies on real world invasive systems. Ireland is an island that has seen multiple species introductions with various impacts on its local ecosystems (Searle, 2008a; Montgomery *et al*., 2014). The combination of some of invasive species in Ireland show compounding effects on local fauna (termed 'invasional meltdown'; Simberloff and Holle, 1999; Montgomery, Lundy and Reid, 2012). Some of

these invaders are still expanding their range on Ireland (White *et al*., 2013; McDevitt *et al*., 2014) and is therefore an ideal study site for studying the ecology of ongoing invasions.

Ireland was likely the first island of size to become isolated from the rest of mainland Europe following the LGM (19 – 23,000 years ago), which resulted in a reduced representation of fauna compared to Britain and the rest of mainland Europe (Searle, 2008a). Many animal species may simply not have had time to reach Ireland before it was isolated by the Irish Sea. It has been suggested that a reduced level of biodiversity provides more 'unoccupied niches' and may influence an ecosystem's vulnerability to invasion by exotic species (Chapin *et al*., 2000), which may be influential in Ireland. The increased level of international transport and trade in the last few decades have also seen an increase of species introductions (Caffrey *et al*., 2008, 2011; Greene, McElarney and Taylor, 2015). For example, construction of the Shannon Hydroelectric Scheme in the 1920s required the import of materials from Germany to Ireland, which likely provided the introduction route for the bank vole (*Myodes glareolus*) (Stuart *et al*., 2007). Invasive species have resulted in negative impacts on Ireland's ecosystem and even begin replacing local fauna (Gurnell *et al*., 2004). One such example is the introduction of the greater white-toothed shrew (*Crocidura russula*) into Ireland a number of years prior to its discovery in Ireland in 2007 (Tosh *et al*., 2008). Its first discovery in Ireland was in raptor pellets during a survey. Their invasion is recent, so more work is needed to fully understand their ecology in Ireland and their impact on the local fauna (McDevitt *et al*., 2014; Montgomery, Montgomery and Reid, 2015). The range expansion of *C. russula* in Ireland and their subsequent impacts on the local fauna is the focal point of this work.

### 1.5.2. *Crocidura russula* and *Sorex minutus*

*Crocidura russula* is an average sized shrew (mean 11g in weight and 115.8mm in total length) native to Northern Africa but crossed the Gibraltan strait to mainland Europe before the LGM (Brändli *et al*., 2005; Cosson *et al*., 2005). Since then, the species has expanded its range across mainland Europe and several Mediterranean islands (Lo Brutto, Arculeo and Sarà, 2004; Brändli *et al*., 2005; Cosson *et al*., 2005). Despite going through population bottlenecks and high levels of inbreeding, *C. russula* can retain normal fecundity or reproductive output (Duarte *et al*., 2003) and has

repeatedly colonised new areas from only a small number of individuals (low propagule pressure) (Vogel, 1999; Gargan *et al*., 2016). *Crocidura russula* have a broad insectivorous diet (partly facilitated by their relatively high bite force; Cornette *et al*., 2015), predating on various groups of invertebrates of different sizes such as shelled Gastropoda, Isopoda, Araneae, Acari and occasionally consuming small vertebrates (Brahmi *et al*., 2012; Biedma *et al*., 2019). This consumption of vertebrates is mainly seen in their home range in Algeria, claimed to be most likely opportunistic behaviour or scavenging (Brahmi *et al*., 2012). During its Europe-wide expansion *C. russula* has shown highly competitive capabilities by displacing other species of shrews (Cornette *et al*., 2015). Examples include the island of Sein (France), where *C. russula* has completely replaced the lesser white-toothed shrew (*C. suaveolens*) (Pascal, Lorvelec and Vigne, 2006). Additionally, over the last 20 years, the species has been implicated in the replacement of the bicoloured white-toothed shrew (*C. leucodon*) in Switzerland (Vogel *et al*., 2002).

The invasive population of *C. russula* in Ireland is likely a result of a single accidental introduction as a stowaway with horticultural imports from mainland Europe (McDevitt *et al*., 2014; Gargan *et al*., 2016). Some of these imported goods come in the form of root balls that could contain enough water and prey to sustain a small number of shrews during the journey. To sustain unfavourable conditions such as scarce resources, *C. russula* can enter a state of torpor, drop body temperature and will huddle together with others to reduce energy use (Genoud and Hausser, 1979; Genoud, 1985). The invasive population established itself inland in the South of Ireland and began a rapid radial expansion, estimated at approx. 5.5 Km per year, likely facilitated by the homogenous landscape and connecting hedgerows acting as corridors for expansion (McDevitt *et al*., 2014). This species has been recorded producing up to four litters from March to September with litter sizes ranging from 2 to 11 offspring (Bouteiller and Perrin, 2000; S Churchfield, 2008) (**Table 1.1**). The range of *C. russula* in Ireland comprises of a large core population with some outlier populations (see **Figure 1.3**; sites O1 – O3). Given the timing of discovering these populations and restricted ranges, McDevitt *et al*. (2014) considered these originating from the core population, through human-assisted dispersal. This was supported further by genetic analysis using cytochrome *b* (Gargan *et al*., 2016). In 2013, *C. russula* occupied a large area (~7600km$^2$) of southern Ireland (McDevitt *et al*., 2014) (**Figure 1.3**).


**Figure 1.2** Comparison of the two species of shrew currently in Ireland. The pygmy shrew (*S. minutus*) and the greater white-toothed shrew (*C. russula*). Bottom image is of cadavers next to a ruler set in millimetres to show size difference. Weights can be found in **Table 1.1**.

The introduction of *C. russula* was originally proposed to benefit wildlife in Ireland by providing locally threatened predators, such as barn owls (*Tyto alba*), with an additional prey source (Tosh *et al*., 2008). Over time it became clear that the expanding range of *C. russula* in Ireland was associated with the local disappearance of the pygmy shrew (*S. minutus*) (Montgomery, Lundy and Reid, 2012; McDevitt *et al*., 2014; Montgomery, Montgomery and Reid, 2015). At the edge of the invasive range, *S. minutus* is still present and appears to co-exist with *C. russula* (see light grey area in **Figure 1.3B**). After the *C. russula* population has become well established over time, the *S. minutus* population is no longer found (see dark grey area in **Figure 1.3B**). The negative

association with *C. russula* and *S. minutus* is stronger than any habitat effects measured (McDevitt *et al*., 2014). It has been suggested that the combined presence of *C. russula* and *M. glareolus* (also invasive) is associated with the disappearance of *S. minutus* (Montgomery, Lundy and Reid, 2012), but McDevitt *et al*. (2014) show a strong negative association between the abundance of *S. minutus* in response to the presence of *C. russula* alone. The sudden disappearance of *S. minutus* after *C. russula* have a well-established population suggests that the impact is rapid.

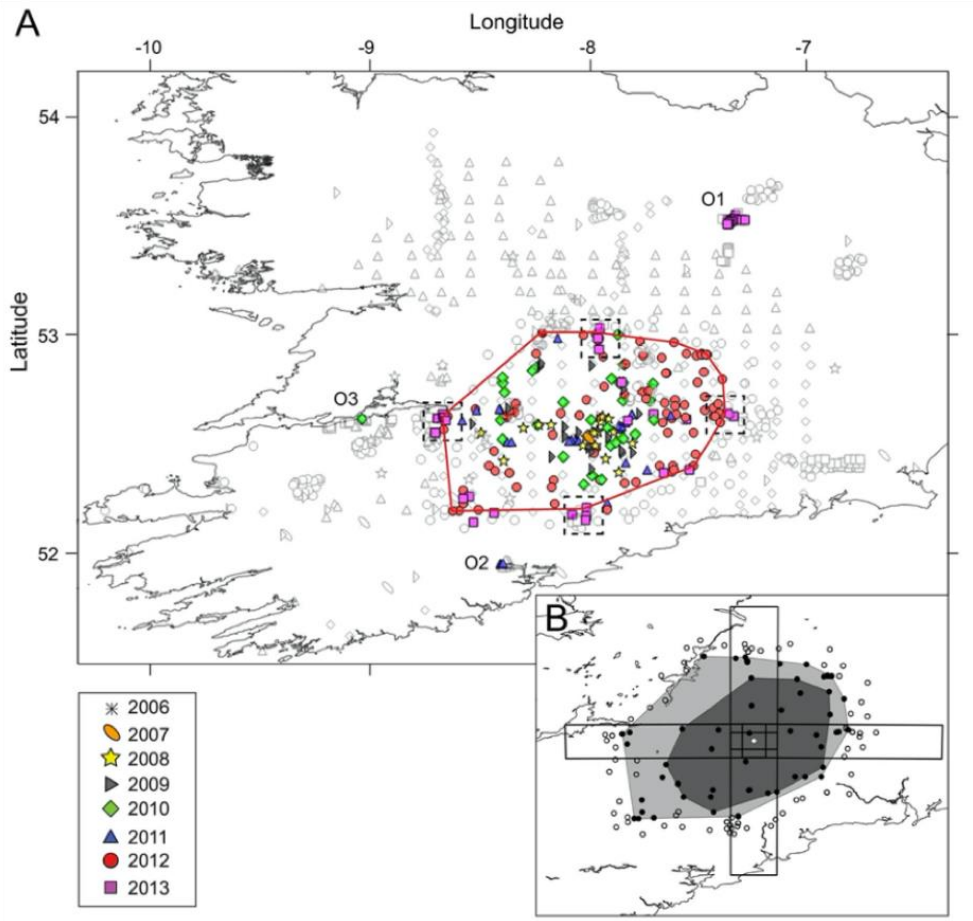



**Figure 1.3.** Range of *C. russula* in Ireland according to the last official survey by McDevitt *et al*. (2014). A) shows the trapping sites that observed *C. russula* coloured according to trapping year. Filled shapes represent sites with positive *C. russula* sightings (via live-trapping) while empty shapes represent negative sightings. Sites O1 – O3 represent the outlier populations (see main text for details). B) This is the core range of *C. russula* where *S. minutus* is also found. The light grey area contains sites where *S. minutus* is still present. The dark grey area is where *S. minutus* has disappeared. Figure taken from McDevitt *et al*. (2014).

The distribution of *S. minutus* ranges from the British Isles and into mainland Europe as far west as central Siberia (McDevitt *et al*., 2010, 2011) and co-exists with up to four other species of shrews. In Europe for example, they have been found alongside *S.*

*araneus, S. coronatus, S. caecutiens, Neomys fodiens, N. anamalus, C. leuodon* and *C. russula* (Churchfield and Sheftel, 1994). Although *S. minutus* co-exists with various other species, they represent only between 2.9% - 4% of shrews trapped compared to other sympatric species in France (Yalden, Morris and Harper, 1973; Butet, Paillat and Delettre, 2006). On the other hand, *C. russula* seems to be the most abundant species in multi-species communities according to number of individuals trapped, making up to 70% of trapped shrews in mainland France (Yalden, Morris and Harper, 1973). These multi-species communities of shrews in mainland France show that *S. minutus* and *C. russula* can be sympatric in more diverse/complex communities, but there is an island off the Western coast of France called Belle Île that *S. minutus* and *C. russula* appear to be the only two shrews currently co-existing in relatively high abundance alongside the bank vole (*M. glareolus*) and wood mouse (*Apodemus sylvaticus*) (McDevitt *et al*., 2014). Belle Île is the largest of Brittany's islands at approx. 84 $km^2$ with no large mammals other than livestock and a similar hedgerow system to Ireland. While both Belle Île and Ireland boast similar hedgerow systems and small mammal communities, the notable difference is the co-inhabitancy of *C. russula* and *S. minutus* (with *S. minutus* being more abundant than in mainland European populations).

The pygmy shrew's colonisation history of Ireland has been under debate for years (Mascheretti *et al*., 2003; McDevitt *et al*., 2009, 2011; Montgomery *et al*., 2014). Although, mitochondrial DNA, Y chromosomal and microsatellite data was used to show that *S. minutus* was most likely introduced from Britain to Ireland by humans during the Holocene (McDevitt *et al*., 2009, 2011). Although they were introduced by humans, they are the only species of shrew that is considered 'native' to Ireland and has been the only species of shrew present on the island since the LGM prior to the arrival of *C. russula*. They are a protected species in Ireland under the Wildlife (Amendment) Act, 2000. Following the invasion of *C. russula*, the direct contributing factors allowing them to simultaneously colonise Ireland and completely replace *S. minutus* have not been fully explored (Montgomery, Lundy and Reid, 2012; McDevitt *et al*., 2014). If this rate of expansion and replacement continues, *S. minutus* may disappear from mainland Ireland by 2050 and is therefore of immediate conservation concern (McDevitt *et al*., 2014).

Each invasion should be assessed to determine management priorities and methods (Boltovskoy, Sylvester and Paolucci, 2018). Attempts to eradicate invasive shrews from various Mauritius islands have been successful on smaller islands (~2 ha), but

unsuccessful in larger islands (~25 ha) (Seymour *et al*., 2005). Eradication of *C. russula* at this stage of the invasion is likely not feasible (McDevitt, 2019). There are multiple islands off the coast of Ireland that *S. minutus* still inhabits (Churchfield, 2008). Due to the large size of these islands (>87 ha), eradication of invasive species would also likely be unsuccessful. Therefore, preventative measures should be put into place to make sure *C. russula* does not reach these islands.

**Table 1.1** Comparison of physical and ecological attributes of *S. minutus* and *C. russula*. Sources: 1 – (Current study); 2 – (Churchfield, 2008); 3 – (Grainger and Fairley, 1978); 4 – (Ellenbroak, 1980); 5 – (Yalden, 1981); 6 – (Cantoni and Vogel, 1989); 7 – (Bouteiller and Perrin, 2000); 8 – (Cosson *et al*., 2005).

| | *Sorex minutus* | *Crocidura russula* | Source |
|---|---|---|---|
| **Ave. Weight** | 3.34g | 11g | 1 |
| **Ave. Length** | 91.4mm | 115.8mm | 1 |
| **Diet** | Invertebrates | Invertebrates. May take small vertebrates | 2,3 |
| **Territorial** | Yes | Only during breeding season | 4,5,6 |
| **Social** | No | Yes | 4,6 |
| **Breeding Season** | March - October | March - September | 7,2,3 |
| **Litter Size** | 2-7 | 2 - 11 | 2,3 |
| **Distribution** | Western Europe, Northern Europe, Scandinavia | Northern Africa, Southern Europe, Western Europe, Mediterranean Islands | 5,8 |

## 1.6. Potential Drivers of Shrew Species Replacement in Ireland

### 1.6.1. Resource Competition

Resource competition is a possible driver for the invasion-extinction event between *C. russula* and *S. minutus* in Ireland (McDevitt *et al*., 2014). Niche separation facilitates the sympatric existence of *S. minutus* with larger species of shrews in mainland Europe (Churchfield and Rychlik, 2006), although *S. minutus* represents between 2.9% - 4% of total shrews in some areas (Yalden, Morris and Harper, 1973; Butet, Paillat and Delettre, 2006). By being the only shrew species to successfully colonise Ireland thousands of years ago (McDevitt *et al*., 2009, 2011), *S. minutus* has likely experienced

a competitive release opportunity (reduced resource competition) and has changed physiologically and behaviourally over time. The shape of their skulls and mandibles have become distinct from mainland populations over time, possibly in response to resource use (Vega *et al*., 2016). Controlled experiments have shown *S. minutus* to increase predation on larger invertebrates in the absence of larger competitors such as the common shrew (*S. araneus*; Dickman, 1988). The 'island rule' states that insular populations of small mammals show a trend for increasing in size compared to their mainland counterparts (Lomolino *et al*., 2013). After their arrival to Ireland, *S. minutus* may have increased in size due to the island rule and become dependent on larger prey species in the absence of larger competitors. Considering *C. russula* also goes for larger prey taxa and both species are surface foragers (Ellenbroak, 1980; Brahmi *et al*., 2012), they may have a high niche overlap in Ireland. The considerable bite force and broad diet of *C. russula* gives them a competitive advantage that has already out-competed and displaced the smaller lesser white-toothed shrew (*C. suaveolus*) across Europe (Cornette *et al*., 2015; Biedma *et al*., 2018). The rapid arrival and dispersal of a superior competitor may leave *S. minutus* unable to alter their behaviour and diet in response to successfully co-exist in Ireland within such as short period of time.

Mammals are notoriously elusive, making their diet difficult to document through direct observations (particularly small species), so that morphological diagnostics of prey remains from stomach contents and faeces are a popular method (Brzeziński *et al*., 2018). This methodology produces biased results due to variable degradation rates between species and body parts (i.e. soft body parts degrade faster than hard body parts), and residual body fragments that are found are difficult to identify to species level (Deagle, Kirkwood and Jarman, 2009). Stable isotope analysis shows promise, but has difficulties identifying individual prey species when isotopic signatures naturally vary between geographic locations (Chibowski *et al*., 2019).

Genetic tools require DNA to be extracted from faeces or gut contents using appropriate extraction kits capable of removing inhibitors associated with the digestive tract. Species-specific primers and PCR are straight-forward and cost-effective methods to measure predation rates of a single species of interest (Waraniak, Blumstein and Scribner, 2018). However, invasive mammals can have a variable diet between native and introduced ranges (Ballari and Barrios-García, 2014), making it difficult to predict what they will consume in their introduced range. This would be particularly difficult for shrews that have a broad range diet of invertebrates. DNA

metabarcoding is a promising method that can identify multiple dietary components of hundreds of individuals and increases prey detection from 2% using morphological diagnostics to 70% using metabarcoding (Pompanon *et al*., 2012; Egeter, Bishop and Robertson, 2015).

Not only can DNA metabarcoding accurately document an animal's impact on local resources, but it can also reduce ambiguity. Previous assessments of the impact of invasive rats (*R. rattus*) on endemic amphibians in New Zealand relied on abundance estimates of native frog species in comparison with arrival patterns of the invasive rat (Egeter, Robertson and Bishop, 2015). Inconsistencies between observers caused doubt, but DNA metabarcoding clarified the rat's consumption of New Zealand's native frog species and its contribution to the population declines (Egeter *et al*., 2019). The sensitivity achieved from next-generation sequencing methods allows multiple prey items to be identified to the species level and generates a comprehensive account of multiple animals' resource use and overlap. Telfair's skink (*Leiolopisma telfairii*) was introduced to Ile aux Aigrettes, Mauritius, Indian Ocean, for conservation purposes, but unexpectedly met potential threats from the invasive Asian musk shrew (*Suncus murinus*). Species-specific primers showed the two species did not predate on one another (once adulthood was attained), but DNA metabarcoding identified significant prey overlap and resulted in the suggestion that controlling shrew populations would benefit the skink population (Brown *et al*., 2014).

Metabarcoding projects for dietary studies require some important considerations before they are started. The first is targeting the appropriate genetic region for the target taxa in the diet, such as vertebrates, invertebrates or plants (Kress *et al*., 2015). To know the full diet of an omnivorous invader (e.g. wild boar), multiple regions are required for the full taxonomic range within their diet (De Barba *et al*., 2014). Alternatively, highly degenerative (non-specific) primers can be used to capture a wider range of prey taxa, but this can result in over-representation of higher-quality host DNA (Zeale *et al*., 2011). The broader the primers' taxonomic range, the more likely the chance of amplifying non-target taxa and reducing the amount of information on a species' diet. Blocking primers can mitigate host DNA amplification, but require more time to design and test, as they may also block the amplification of some target prey taxa (Su *et al*., 2018). The high sensitivity of PCR and high-throughput sequencing can also result in the detection of taxa through secondary predation (i.e. detecting the food of the food; Sheppard *et al*., 2005). Another difficulty is the inference of biomass or the

number of prey individuals from molecular diet analysis (Deagle *et al*., 2019). Estimates of prey proportion can be biased towards certain taxa due to differential degradation rates of tissue types (Thomas *et al.*, 2014). There are multiple ways to determine the importance of certain taxa within a predator's diet, such as frequency of occurrence or relative abundance (reviewed by Deagle *et al*., 2019). Due to these considerations, preliminary tests should be performed using some form of pilot study before a full-scale analysis.

### 1.6.2. Pathogens and Microbial Communities

Invasive species can bring with them novel pathogens which can have impacts on local biodiversity (McGeoch *et al*., 2010). These introduced pathogens can 'spill over' and infect native populations (Telfer and Bown, 2012). Exposure of local fauna to novel strains of pathogens can be hazardous as they have not yet had a chance to develop some form of resistance (Inoue *et al*., 2009; Berglund *et al*., 2010; Paziewska *et al*., 2011). For example, the invasive grey squirrel (*S. carolinensis*) brought the adenovirus into the British Isles, which causes high mortality rates of native red squirrel (*S. vulgaris*) populations (Everest *et al*., 2014). The invasive raccoon (*Procyon lotor*) experienced several introductions into Europe from North America and has increased the geographic range of the raccoon roundworm (*Baylisascaris procyonis*), a gastrointestinal parasite (Osten-Sacken *et al*., 2018). This roundworm has been recorded to infect ~130 vertebrate species, showing the increased risk of invasive raccoons to local wildlife (Page, 2013). Multiple European populations of the white-clawed crayfish (*Austropotamobius pallipes*) have been infected with the pathogen *Aphanomyces astaci* (the crayfish plague) which was brought in with invasive populations of multiple American crayfish species (Collas *et al*., 2016). The invasive *C. russula* has the potential to co-introduce novel pathogens that could then infect *S. minutus* and increase their mortality rate. *Crocidura russula* has already been shown to host a novel strain of *Leptospira spp.* that could impact native small mammal populations, such as *S. minutus* (Nally *et al*., 2016).

Some invasive species arrive into a new region without a natural enemy in the form of a pathogen, which gives them an advantage over their native range where the pathogen may reduce their health (known as the 'enemy release hypothesis'; Strauss, White and Boots, 2012). The invasive population of *C. russula* may have lost

pathogens because only a small subset of individuals without the infection were introduced (Torchin *et al*., 2003). Various species of invasive invertebrates have been found to host a lower prevalence of pathogenic microbes in their invasive range compared to their native range (Roy *et al*., 2011).

Genetic tools are becoming more highly regarded for disease management in wildlife (DeCandia, Dobson and vonHoldt, 2018), currently used to verify morphological identification of pathogens/parasites (Bagrade *et al*., 2016) and accommodate for difficulties in recreating optimal cell growing conditions for morphological identification (Nally *et al*., 2016; Guglielmini *et al*., 2019). PCR techniques can be used as a detection tool to quantify prevalence/infection levels in mammalian hosts (Mayer-Scholl *et al*., 2014; Heuser *et al*., 2017). PCR detection has been a reliable tool in showing the role of various invasive small mammals in Puerto Rico hosting zoonotic *Leptospira spp.* and the imposed risk to humans and wildlife alike (Benavidez *et al*., 2019). However, these genetic methods only target specific bacterial taxa. High-throughput sequencing has paved the way rRNA gene metabarcoding techniques that can identify whole microbial community compositions of hundreds of samples (Kozich et al., 2013). Metabarcoding can thus give the advantage of early detection of various pathogens potentially present in the invasive *C. russula* population that may be of concern to local wildlife. Bacterial pathogens have not been explored much in shrews using 16s metabarcoding techniques, but this technique has identified pathogenic genera such as *Salmonella* and *Yersinia* in the gastrointestinal system of other insectivores (Sun *et al.*, 2020). By targeting the 16S region, there are less complications in terms of co-amplifying host DNA that is associated with DNA metabarcoding in dietary studies of mammals (see previous section).

By using 16S rRNA metabarcoding to scan for pathogens in the gastrointestinal system of *C. russula* and *S. minutus*, the shrews' bacterial microbiomes can also be characterised. The diversity of host-microbiomes can regulate disease susceptibility, primarily through competitive exclusion and the production of antibiotics that target invasive pathogens (Daskin and Alford, 2012; Bahrndorff *et al*., 2016; Rebollar *et al*., 2016). Various groups of bacteria are also associated with resistance to specific pathogens (e.g. Jani and Briggs, 2014). Additionally, the gut microbiota has been recorded to have direct interactions with host immune system, providing signals for immune responses (Chow *et al*., 2010). Although extensively researched in invasive and native plant hosts (Kowalski *et al*., 2015), there remain many unknowns for the

potential of invasive animals disrupting the host-microbiome of native animals. Resource competition, or potential introduction of novel pathogens (Nally *et al*., 2016), may have a downstream effect on the *S. minutus* microbiome composition, thus affecting their fitness (Alberdi *et al*., 2016). Indeed, high microbiome diversity has shown to aid mitigation of disease infections through processes such as competitive exclusion and antibody production (Bahrndorff *et al.*, 2016; Antwis and Harrison, 2017), so we can identify the likely susceptibility of *S. minutus* to any emerging disease.

As host-associated microbiomes can act as host phenotypes that evolve quickly in response to the environment, Alberdi *et al*., (2016) argue that the microbiota may play an important role in adaptation. This invasion scenario gives us multiple opportunities to expand our knowledge of adapting microbiomes of successful invaders. Different traits can benefit individuals from different environments (Ley *et al*., 2008a), therefore understanding the diversity and composition of microbial taxa hosted by *C. russula* at different stages of the invasion may provide insight into their adaptive potential (Fietz *et al*., 2018). Invading a new area can be strenuous as the animals may have to adapt when coming from an environmentally different origin. A diverse and structurally stable microbiome may be one of the *C. russula* attributes to their high dispersal abilities.

## 1.7. Overarching Aims of the Thesis

Many studies on resource competition to date between native and invasives rely on laboratory settings (Richter-Boix *et al*., 2013) and although extensively researched in invasive and native plant hosts (Kowalski *et al*., 2015), there remain many unknowns for the potential of invasive animals disrupting the host-microbiome of native animals. The *C. russula* invasion in Ireland is still ongoing. By following a similar strategy as McDevitt *et al*. (2014), sampling shrews from different invasion zones (see **Figure 1.3B**) can allow us to identify the prey and microbial composition of *C. russula* and *S. minutus* at different stages of the invasion. Sampling outside the *C. russula* range will represent ‘before’ the invasion while sampling at the edge of the range will represent ‘during’ the invasion and sampling further inside the invasive range will represent ‘after’ the invasion. Shrews have also been sampled from the island Belle Île, France. Belle Île is ecologically similar to Ireland, with an identical small mammal community and both *C. russula* and *S. minutus* co-exist in a high abundance with no other shrew species (McDevitt *et al*., 2014). Belle Île can thus act as a ‘natural control’ site to

compare samples from there and Ireland. Shrews of both species were sampled from Belle Île just as in Ireland, but at a smaller scale. To account for differing population densities (due to breeding) and food availability, previous studies suggested that dietary/niche overlap studies in shrews should cover Summer and Winter cohorts (Churchfield and Rychlik, 2006). Sampling will therefore occur over two seasons.

To accurately identify what prey and microbes are present in the shrew's guts at the time of trapping, DNA (and rRNA) metabarcoding was applied to the gut contents of samples. Due to the complications associated with DNA metabarcoding for dietary studies (see **Section 1.6.1**), a protocol was first trialled and optimised on a subset of samples before applying the technique to all shrew samples.

## 1.8. Chapters and Research Questions

### Chapter 2

The choice of primers and protocol used in DNA metabarcoding for dietary analyses can affect ecological conclusions of studies (Alberdi *et al.*, 2018). DNA metabarcoding was performed on a subset of shrew samples using multiple primers (that target either invertebrates specifically or both invertebrates and vertebrates), PCR conditions and bioinformatic parameters to determine the optimal protocol for characterising the diet of *C. russula* and *S. minutus*. We hypothesised that there would be an optimal primer for detecting a wide range of taxa consumed by both species of shrews.

### Chapter 3

The DNA metabarcoding protocol optimised in Chapter 2 was applied to a full-scale study of >300 samples to determine what *C. russula* and *S. minutus* are eating in Ireland and Belle Île. The dietary information gained from the DNA metabarcoding was used to determine if there is a significantly higher level of resource overlap between *C. russula* and *S. minutus* in Ireland compared to Belle Île. A higher level of resource overlap in Ireland would suggest that resource competition is a contributing factor to the disappearance of Ireland's mainland population of *S. minutus* in response to the presence of *C. russula*.

## Chapter 4

Metabarcoding of the 16S region was used to characterise the gut microbial community of the same shrew samples as Chapter 3 to address multiple hypotheses. The invasive population of *C. russula* may have introduced novel gastrointestinal pathogens to Ireland and the native fauna. Novel pathogens may negatively affect the health of *S. minutus* and contribute to their decline in numbers in Ireland. This data can also determine if there are structural differences in the microbial community of *S. minutus* in Ireland compared to the Belle Île population. The microbiome composition of Irish *S. minutus* could potentially leave them more susceptible to novel stresses (such as novel pathogen introduction or new competition) in Ireland compared to Belle Île.

During the range expansion of *C. russula* in Ireland, the microbiome structure of both species may be altering. The *C. russula* microbiome is possibly changing during the range expansion through dispersal and population sub sampling effects. In addition, the *S. minutus* microbiome could be changing in response to the *C. russula* invasion as a result of stress or direct interspecific interactions. The microbiomes of invasive mammals have not been rigorously examined in the literature yet, therefore this provides important insight into the microbiome of small mammals during invasions. The microbiome can be affected by the individual's diet, therefore this chapter was closely linked to Chapter 3 to infer if the diet is affecting microbiome results that could be interpreted as a result of the invasion itself.

# Chapter 2 – Evaluation of different primer sets to optimally characterise the diet of two species of shrew

**Note:** This chapter is part of the larger study Browett SS, Curran TG, O'Meara DB, Harrington AP, Sales NG, Antwis RE, O'Neill D, McDevitt AD (2021) Primer biases in the molecular assessment of diet in multiple insectivorous mammals. *BioRxiv,* Doi: 10.1101/2021.01.18.426998

## 2.1. Introduction

In a constantly changing environment, knowledge of complex food webs is vital for our understanding of ecosystem functioning and biodiversity conservation. The advent of Next-Generation Sequencing (NGS) technology has revolutionized the analyses of trophic interactions (Deagle *et al*., 2019; Browett, O'Meara and McDevitt, 2020). DNA metabarcoding uses NGS technology for the simultaneous identification of entire species communities using a standardised region of DNA, and has become a widely adopted method for investigating animal diets from faecal samples or gut contents (Pompanon *et al*., 2012; Nielsen *et al*., 2018). Small mammals such as shrews are too elusive to directly observe them hunt, which makes DNA metabarcoding an ideal method to characterise their diet. Despite the significant developments and improvements afforded by DNA metabarcoding over the last decade, the technique still has certain limitations for documenting an animal's diet. These include problems in describing diverse diets (e.g. omnivorous species); assigning sequences to appropriate taxonomic levels with incomplete or poor reference databases; false negatives/positives for species detections and host co-amplification (Piñol *et al*., 2015; Alberdi *et al*., 2018; Deagle *et al*., 2019).

Several of these limitations are particularly evident when studying the diets of mammalian insectivores in terrestrial environments. Invertebrates are a massively diverse and widely distributed group (Stork, 2018) which means designing primers to describe broad insectivore diets via DNA metabarcoding can be challenging. Primers can be designed to detect specific groups of invertebrates (Saitoh *et al*., 2016) while others are capable of detecting a wide range of taxa (Corse *et al*., 2019). Designing new primers is not always required because over the last decade, a range of primers capable of simultaneously detecting hundreds of invertebrates have already been designed and tested (see Elbrecht, Leese and Nichols, 2017; Piñol, Senar and

Symondson, 2019 for list of popular choices). However, designing and evaluating the efficiency of these highly degenerative (i.e. non-specific) primers has largely been restricted to analyses performed *in silico* (Piñol, Senar and Symondson, 2019) or on malaise traps and mock communities (Braukmann *et al*., 2019; Elbrecht *et al*., 2019). While these have been essential steps in primer design and have led to the ability to detect a wide range of invertebrate species, they may not account for some of the potential biases within a dietary context such as over-representation of higher-quality host DNA (Zeale *et al*., 2011). The broader the range of taxa that can be detected by a primer, the more likely the chance of co-amplifying host DNA, resulting in reduced details on the animal's diet. This will make documenting the diet of shrews challenging as they predate on a broad range of invertebrates (Churchfield and Rychlik, 2006; Churchfield, 2008a, 2008b). Host co-amplification has been deemed beneficial to some dietary studies by simultaneously detecting a wide range of prey taxa and confirming the predator species from faecal samples (Galan *et al*., 2018; Tournayre *et al*., 2020). If host amplification is not desirable, blocking primers can be designed to preferentially bind to the host DNA but with modifications (such as a C3 spacer on the 3' end) that inhibit elongation and amplification of host DNA during PCR (Liu *et al.*, 2019). Blocking primers can therefore mitigate against host DNA amplification but require more resources to design and ensure they don't also block amplification of some target prey taxa (Su *et al*., 2018).

In terms of insectivorous mammalian predators, bats have been the dominant subject of primer testing and comparisons, likely given attention due to their ecological importance and their significant role in the suppression of pests that are implicated in the spread of disease and negatively impact agriculture (Bohmann *et al*., 2018; Galan *et al*., 2018; Baroja *et al*., 2019). They have not only served as a key study group for primer comparisons, but also for methodological approaches such as sampling design, Molecular Operational Taxonomic Unit (MOTU) clustering thresholds and mitigating contamination/errors (Alberdi *et al*., 2018, 2019). Although investigations into the diets of ground-dwelling and semi-aquatic mammalian insectivores using DNA metabarcoding are less frequent, several recent studies have been undertaken to compare primer combinations and host/diet detection (Brown *et al*., 2014; Esnaola *et al*., 2018) or focus on resource overlap between various insectivores (Brown *et al*., 2014; Biffi *et al*., 2017a). It has been acknowledged that the best primer combination for detecting invertebrate prey in one system may not be the best for another (Tournayre *et al*., 2020).

The recent introduction of the greater white-toothed shrew (*C. russula*) into Ireland and their negative impact on the local population of pygmy shrews (*S. minutus*) has gained recent attention (McDevitt *et al*., 2014). *Sorex minutus* is present across Europe, but they have been the only species of shrew in Ireland for over 5000 years (McDevitt *et al*., 2011). *Crocidura russula* is a rapidly spreading species that, since crossing the Gibraltan strait from northern Africa before the Last Glacial Maximum (LGM), has been expanding their range across Europe and has colonised several Mediterranean islands (Cosson *et al*., 2005). A small number of *C. russula* that likely originated from France established a population within Ireland around the year 2000 (Tosh *et al*., 2008; Gargan *et al*., 2016). The expansion of *C. russula* across southern Ireland has now led to the displacement of the local populations of *S. minutus* (McDevitt *et al*., 2014). Both species of shrews are insectivores and successfully co-inhabit other regions such as the island Belle Île, France. Considering that Ireland and Belle Île are ecologically similar islands, with similar small mammal communities, it has been puzzling why both species can co-inhabit Belle Île, but not Ireland. One possibility is resource competition in Ireland (McDevitt *et al*., 2014). To determine if *C. russula* is out-competing *S. minutus* for resources in Ireland, DNA metabarcoding can be used to determine what they are eating.

DNA metabarcoding has not yet been applied to characterise the diet of *S. minutus* and *C. russula*, therefore it is uncertain of which would be the optimal primers to use. *Sorex minutus* have a diet consisting of 12 identified Orders from multiple hard-part dietary analyses, with Araneae, Coleoptera and Opiliones highly represented across different parts of the species range (Meharg, Montgomery and Dunwoody, 1990; Churchfield and Rychlik, 2006). A recent metagenomics study (not to be confused with the metabarcoding approach used here) on five individuals identified the importance of Lepidoptera and Acari (Ware *et al*., 2020). Detailed studies of the *C. russula* diet are scarcer, but Lepidoptera larvae, Araneae and Isopoda are important components of their diet (Churchfield, 2008b). In addition to invertebrates, lizards/geckos have been recovered from *C. russula* stomach samples in its African range, but it is unclear if this is due to predation or scavenging (Brahmi *et al*., 2012). This finding suggests that there is the possibility that the invasive population of *C. russula* may predate on local vertebrate fauna in Ireland. The use of host blocking primers will likely block out other vertebrate taxa (Su *et al*., 2018) and remove any chance to determine if *C. russula* are predating on native vertebrate fauna in Ireland. As this is essential information on the *C. russula* invasion in Ireland, blocking primers will not be used here. To over come

any host amplification, sequencing depth can be increased as a cost-effective alternative to using blocking primers (Piñol *et al*., 2014).

Highly degenerative primers are needed to detect the expected wide range of a shrew's diet (Churchfield and Rychlik, 2006; Churchfield, 2008a, 2008b). This chapter directly compared two widely used primer pairs (Zeale *et al*., 2011; Gillet *et al*., 2015) targeting the mitochondrial COI region (chosen due to its high taxonomic coverage, resolution and well-defined reference database; Clarke *et al*., 2017; Elbrecht and Leese, 2017). These primer pairs differ in terms of prey identified (dietary constituents) and predator (host) amplification (Esnaola *et al*., 2018; Aldasoro *et al*., 2019). The objective was to apply a DNA metabarcoding pipeline using these two primers on a small number of *S. minutus* and *C. russula* samples to determine which primer set will return the most information about their diet, or if both primers should be used in combination. The pipeline was tested on DNA that had been extracted from the gut contents of dissected shrew specimens, meaning species identity is known for each sample and host-amplification is not desirable. The optimal primers amplify a wide range of invertebrate taxa, with minimal co-amplification of host DNA. The protocol established here is applied to a full-scale study to find out if there is a significant level of prey resource competition between *S. minutus* and *C. russula* in Ireland.

## 2.2. Methods

### 2.2.1. Lab Methods

#### DNA Extraction

*Crocidura russula* and *S. minutus* samples were trapped in Ireland and Belle Île and killed by cervical dislocation following guidelines set out by Sikes (2016). Appropriate licenses and permits had been issued (see **Section 6.4**). Entire gut tracts were removed and frozen in absolute ethanol at -20$^{o}$C until extraction. Gut tracts were defrosted on ice, removed from ethanol and air dried. Dissection was performed on disposable bench covers and tools were cleaned and flamed in between each sample to avoid cross-contamination. DNA was extracted from the gut contents using the Qiagen Power Soil Kit, with the protocol altered according to Alberdi *et al*. (2018). All DNA extractions were subsequently diluted in molecular grade water down to 10 – 15 ng/µl. Full details on sample collection, dissection and DNA extraction are provided in Chapter 3, **Section 3.2.1**.

#### PCR

For this primer trial, 12 *C. russula* and 15 *S. minutus* samples were chosen from the full dataset of ~300 shrews sampled for **Chapter 3** (see **Table S2.2** for details of chosen samples). These samples are intentionally distributed across sample sites in Ireland and Belle Île, and across seasons. Each sample was used with two sets of primers to compare their performance. The first primer pair (hereafter referred to as the 'Zeale' primers) amplifies a 157 bp section of the 'Folmer region' of COI (Zeale *et al*., 2011) and have been successfully used to capture arthropod communities from the faeces of various bat species (Clare, Symondson and Fenton, 2014), Pyrenean desmans (*Galemys pyrenaicus*) (Esnaola *et al*., 2018) and simulated datasets (Piñol, Senar and Symondson, 2019). Zeale primers are well suited to certain groups of Arthropoda but are predominantly used on bats faeces. Here we determined their viability in determining the diet of terrestrial insectivores.

The second primer pair (hereafter referred to as the 'Gillet' primers) amplify a 133 bp fragment of the 'Folmer region' of COI (Gillet *et al*., 2015). They are modified versions of the forward LepF1 (Hebert *et al*., 2003) and EPT-long-univR (Hajibabaei *et al*., 2011). This primer set has been successfully used in studies examining insectivore diets such as bats (Galan *et al*., 2018), Eurasian water shrew (*Neomys fodiens*) and

*G. pyrenaicus* (Biffi *et al*., 2017a, 2017b; Esnaola *et al*., 2018). They are a more degenerative primer pair than Zeale, shown to amplify up to 30 invertebrate orders (Biffi *et al*., 2017a). A wide taxonomic range will be required as shrews are shown to have a wide range diet of invertebrates (Pernetta, 1976).

The Zeale and Gillet primers were synthesized with a set of 24 unique 8 base pair multiplex identifier (MID) tags on the 5' ends of both the forward and reverse primers (**Table 2.1**). A different set of 24 unique barcodes were applied to each primer pair (i.e. Gillet primers used a different unique set of 24 MID tags than the Zeale primer pair). In addition, a varying number of N's (A, T, C or G) were added to the 5' end of the primers (**Table 2.1**). These N's help 'stagger' the fragments during sequencing to aid in creating variability and improve sequence quality. The number of N's varied between 2 and 4, with a total of 6 N's collectively between the forward and reverse primers. Using these forward and reverse primers/MID tags in different combinations will allow the multiplexing of hundreds of samples.

Baseline PCR conditions were taken from Esnaola *et al*., (2018) who tested 4 primer sets on the diet of the *G. pyrenaicus*, including the Gillet primer set and the Zeale primer set. The volumes of reagents, primers and DNA were altered in various combinations from their protocol, as well as the cycling conditions (see **Table S2.1** for details on tested conditions). Optimal reaction volumes and cycling conditions were chosen based on the strongest bands at the target amplicon size on a 1.2% agarose gel, with the weakest signal from non-target bands (such as primer dimer).

The final PCR reaction mix for both Gillet and Zeale primer sets contained 12.5 µl Qiagen Multiplex PCR Mastermix, 1 µl of each primer (5 µm), 7.5 µl of molecular grade water and 3 µl of DNA template (at 5 – 10 ng/µl). PCR conditions for the Zeale primers included an initial denaturation at 95˚C for 15 minutes, followed by 40 cycles of 95˚C for 20 seconds, 55˚C for 30 seconds and 72˚C for one minute followed by a final extension at 72˚C for seven minutes. PCR for Gillet primers involved an initial denaturation at 95˚C for 15 minutes followed by 10 cycles of 94˚C for 30 seconds, 49˚C for 45 seconds and 72˚C for 30 seconds, followed by 30 cycles of 95˚C for 30 seconds, 47˚C for 45 seconds, 72˚C for 30 seconds followed by a final extension of 72˚C for 10 minutes (**Table 2.2**). Two PCR blanks were included as negative controls and the success of reactions were determined by electrophoresis on a 1.2% agarose gel.

**Table 2.1.** Primer sequences. The primers include a variable number of N's at the 5` end to aid in variability for sequencing quality. The X's highlighted in red represent the 8bp MID tags for multiplexing samples.

| Alias | Primer Name | Full Sequence (5` - 3`) | Length (bp) | Source |
|---|---|---|---|---|
| Zeale | **F:** ZBJ-ArtF1c | NN(N)(N)XXXXXXXXAGATATTGGAACWTTATATTTTATTTTTGG | 157 | Zeale et al 2011 |
| | **R:** ZBJ-ArtR2c | NN(N)(N)XXXXXXXXWACTAATCAATTWCCAAATCCTCC | | |
| Gillet | **F:** LepF1 (modified) | NN(N)(N)XXXXXXXXATTCHACDAAYCAYAARGAYATYGG | 133 | Gillet et al 2015 |
| | **R:** EPT-long-univR (modified) | NN(N)(N)XXXXXXXXACTATAAAARAAAATYTDAYAAADGCRTG | | |

**Table 2.2.** Optimal PCR cycling conditions determined for these samples.

| Zeale Primers | | | Gillet Primers | | |
|---|---|---|---|---|---|
| **Cycles** | **Temp (ºC)** | **Time** | **Cycles** | **Temp (ºC)** | **Time** |
| | 95 | 15 mins | | 95 | 15 mins |
| 40 X | 95 | 20 secs | 10 X | 94 | 30 secs |
| | 55 | 30 secs | | 49 | 45 secs |
| | 72 | 60 secs | | 72 | 30 secs |
| | 72 | 7 mins | 30 X | 94 | 30 secs |
| | | | | 47 | 45 secs |
| | | | | 72 | 30 secs |
| | | | | 72 | 10 mins |

### Normalising and Cleaning PCR products

For the primer trials, PCR products were normalised by using band strength in the agarose gel as a proxy for concentration. 10µl for bright bands, 12.5µl for medium bands and 15µl for faint bands. Before library preparation (i.e. the ligation of sequencing adapters onto PCR products), a bead clean was performed to purify the PCR products. To remove unwanted fragments smaller than 100bp, a left-side bead clean was performed using MAGBio HighPrep™ PCR Clean-up System beads at a 1.1x ratio. To remove fragments larger than 300bp, a right-side bead clean was performed using a 0.8x ratio of beads to DNA template.

### Adapter Ligation and Sequencing

Sequencing adapters (Illumina TruSeq dual-index adapters) were ligated onto amplicons using the NextFlex PCR-free DNA-seq kit (for Illumina Platforms) following the manufacturers protocol. The final library for the primer trial was quantified by qPCR using the NEBio library quant kit for Illumina. The library was run on a single MiSeq run using a V2 300 cycle kit, loaded at 9pM with a 1% PhiX spike-in.

A separate library from a project characterising the diet of 24 lesser horseshoe bats (*Rhinolophus hipposideros*) was also sequenced on this run. The MiSeq could produce enough reads for both projects, therefore both libraries were included in a single run to save costs for both projects. The bat project amplified invertebrate DNA from faecal samples using Gillet and Zeale primers under the same conditions as the shrew samples described here. Post PCR cleaning and library preparation was performed using the same protocol but using an adapter with a different unique index for downstream demultiplexing.

### 2.2.2. Bioinformatics

#### Sequence Processing

Sequence processing and taxonomic assignment was performed on the shrew and bat samples together. Sequence quality was examined using FastQC (Andrews, 2010). No trimming of sequences was required. Processing of raw sequence reads was performed using Obitools metabarcoding software (Boyer *et al*., 2016). Paired-end sequences were aligned using Obitools *illuminapairedend*, discarding any reads with a phred quality score below 40. Sequences were demultiplexed using Obitools *ngsfilter* according to the unique dual MID tag combination for each sample. Sequences between 128bp and 138bp in length were retained for the Gillet primer datasets. Sequences between 152bp and 162bp were retained for the Zeale primer set. Unique sequences were combined using Obitools *obiuniq*. Chimeras were detected using the *uchime denovo* method (Edgar *et al*., 2011) in Vsearch (Rognes *et al*., 2016) and subsequently removed.

Sequences were clustered into Molecular Operational Taxonomic Units (MOTUs) using Obitools *sumaclust*. Empirically testing different similarity values for clustering sequences is highly recommended (Alberdi *et al*., 2018). To empirically test the number of MOTUs identified and taxonomically assigned at different similarity thresholds, the dataset was explored using clustering thresholds between 95% and 98%. Singletons are MOTUs represented by a total of one sequence read in the entire dataset. These were removed prior to taxonomic assignment to aid computation time.

#### Taxonomic assignment

Sequence MOTUs were taxonomically assigned using *blastn* against the ncbi genbank database. Sequences required at least 80% identity and 90% alignment for a match. The top 25 matches were returned, and the most common taxid (taxonomy identifier) was assigned to that MOTU. MOTUs required at least 98% identity for species level assignment (Clare, Symondson and Fenton, 2014; Arrizabalaga-Escudero *et al*., 2018). While some studies include species assignment at identity levels below 98% (Shutt *et al*., 2020), here taxonomic assignment of MOTUs are restricted to different levels depending on the percentage identity to the reference database sequence. MOTUs between 95% and 98% were restricted to genus level assignment. MOTUs between 93% and 95% were restricted to family level assignment. MOTUs between 90% and 93% were restricted to order level assignment.

MOTU sequences were blasted against the GenBank database because of its larger depository. In addition, a recent study shows that for insect taxa, GenBank performs on par for identification to Barcode of Life Database (BOLD; Meiklejohn, Damaso and Robertson, 2019). However, MOTUs with low taxonomic resolution were manually blasted against the BOLD database to increase resolution of dataset.

Before fully discussing the Gillet and Zeale primers, it should be noted that the highly degenerative Leray-XT primers (Wangensteen *et al*., 2018) were also tested on 10 shrew samples. Apart from PCR conditions, the methodology was identical to that described here. The Leray-XT primers produced >99% reads belonging to the host with the remaining reads primarily belonging to other non-prey such as bacteria. The Leray-XT primers are useful for bulk samples, but lack practicality when used in the presence of high-quality host DNA (Kemp *et al*., 2019). The Leray-XT dataset was therefore discontinued and is not presented here.

**MOTU and Sample Filtering**

MOTUs of which more than 0.1% of the total reads were found in the PCR blanks were removed. At this point, the bat samples from the separate project were removed from further analysis. All MOTUs belonging to non-prey taxa (such as vertebrates and parasites) were removed. Samples with fewer than 1000 reads were removed. To avoid the inclusion of false positive taxa, MOTUs were removed from each sample if they were represented by less than 0.01% of the total reads of that individual sample (Alberdi *et al*., 2018). Using a percentage threshold will account for variable

sequencing depth of samples, rather than an absolute threshold such as 5 reads (Kemp *et al*., 2019) or 20 reads (Shutt *et al*., 2020).

To determine the coverage of samples, rarefaction curves were generated using the R package vegan (Oksanen *et al*., 2019). In addition, the *depth_cov()* function in the *hilldiv* R package (Alberdi and Gilbert, 2019) was used to clarify if sufficient read depth was obtained for each sample, using the qvalue = 1 (equivalent to Shannon diversity measure).

**Combining Primers**

Because Zeale and Gillet primers amplify slightly different regions of COI, sequences were clustered into MOTUs separately. It is therefore possible that the Zeale and Gillet primers may be able to detect two separate MOTUs of unknown species to the same genus level. For example, primer A detects MOTU-A without the reference sequence to assign to species, but it can be assigned to the genus level. Primer B detects MOTU-B without the reference sequence to assign to species, but it can be assigned to the same genus as MOTU-A. There is no way to definitively determine if both these MOTUs are two different species, or the same species split into two MOTUs. The latter case can artificially inflate diversity measures. To compare diversity measures between primers, the more conservative method is applied to agglomerate MOTUs to the highest taxonomic resolution available. Each sample will be represented by Gillet, Zeale and both primers combined (referred to hereafter as ‘Both’).

**Alpha Diversity**

The observed species richness and Shannon diversity index were calculated for each sample using the *estimate_richness()* function in the *microbiome* package (Lahti and Sudarshan, 2017). To account for any uneven sequencing depth, each sample was rarefied to the lowest sample read depth. To account for any stochastic results from rarefying samples, each sample was rarefied and the diversity measurement was taken 100 times. The mean diversity was taken for each sample across the 100 measurements. Rarefying will identify the diversity identified from the same number of reads. Significant differences between the diversity of samples amplified with different primers was assessed in R using the non-parametric Kruskal-Wallis test and Dunn’s post hoc test using the Benjamini-Hochberg method to correct for multiple testing (Lavrinienko *et al.*, 2018).

## Beta Diversity

Data was normalised by transforming sequence counts into relative read abundances per sample. The compositional variance within each of these tested groups was measured using the *betadisper()* function in the vegan package. This calculates the multivariate distances of samples to the group centroid. A permutation test for homogeneity of multivariate dispersions was then performed on the multivariate distances to see if there was a similar level of variance (average distance of samples to the group centroid) between each group. This measure of variance is often referred to as the homogeneity of dispersion within groups. A distance matrix was created for the dataset using the Bray-Curtis dissimilarity method. To determine the compositional difference in diet identified between species of shrews and primers used, PERMANOVA's were performed using the *adonis()* function in the vegan package in R. The PERMANOVA examines whether the centroid of one group's distances significantly differs from the centroid of other groups and has been used in previous studies assessing beta diversity measures (Knowles *et al.*, 2019). For each PERMANOVA, MOTUs were also agglomerated to genus, family and order levels.

To visualise differences (or similarities) between species of shrews and the primers used, an NMDS plot was generated using the Bray-Curtis dissimilarity method allowing 5 dimensions (k = 5) to reduce stress on the plot. For these, one outlier sample was removed (W13 – *S. minutus* amplified with Zeale)

## Random Forest Classifier

While different primers will amplify different taxonomic groups, it is desirable to determine which of the tested primers will amplify a greater range of taxa important to characterising that predator species. The random forest classification (RFC) is a supervised learning method that classifies samples (such as prey composition) to their source, estimates the level of importance of each prey item to that classification and determines the accuracy of that classification (Breiman, 2001). Here, RFC models were run to determine which primer amplifies taxa that are most appropriate for classifying samples to predator species, and then again to determine the accuracy of classifying samples to the correct primer used.

RFCs were performed on samples using the *randomForest* R package (Liaw and Wiener, 2002) using 10,000 trees. The out-of-bag (OOB) error was used to measure

the accuracy of classification of samples to their correct group. The most important prey taxa contributing to classification of samples were established using the ‘Mean Decrease Mini’ values.

## 2.3. Results

### Sequencing

The MiSeq produced 18,527,116 sequence reads. The shrew library had a total of 9,170,922 sequences (49.5% of total sequences), and the bat library included in this run had a total of 8,967,124 sequences (48.4% of total sequences). For all shrew samples, a total of 2,805,179 and 4,328,058 high-quality reads were generated for the Zeale and Gillet datasets, respectively. After filtering for read length and chimera removal there were 2,773,715 and 4,241,877 reads retained for the Zeale and Gillet dataset respectively.

### Clustering Threshold Trials

With increasing the clustering threshold for MOTUs, there was a greater number of taxa identified, particularly at the genus and species level (**Figure 2.1A**). Increasing the clustering threshold also showed a decrease in the proportion of MOTUs restricted to higher taxonomic levels such as family and order, while keeping a high proportion identified to genus and species (**Figure 2.1B**). Based off these results and previous metabarcoding studies targeting the COI region (Alberdi *et al*., 2018), the clustering threshold was set to 98% for further analyses.

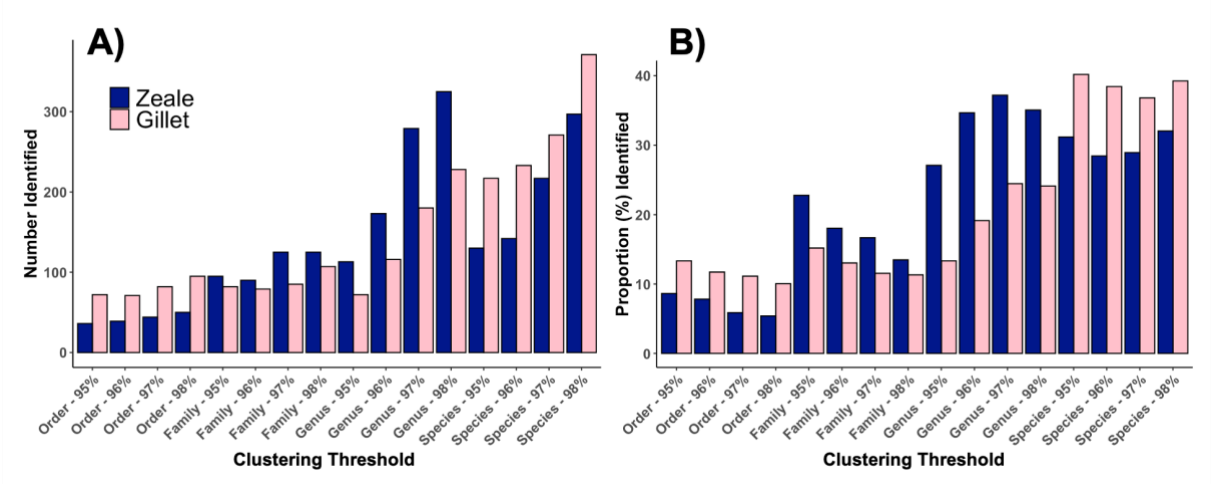



**Figure 2.1.** Comparison of taxa identified with differently clustering thresholds. This was performed with bat samples included. A) The number of MOTUs assigned to order, family, genus and species. B) The proportion of MOTU's identified to different taxonomic levels.

**MOTU and Sample Filtering**

The dataset utilising the sequence clustering threshold at 98% similarity yielded 6,538 and 2,371 non-singleton MOTUs for the Gillet and Zeale dataset, respectively. The Zeale primers returned 180 reads from 2 MOTUs in the Tipulidae family in the negative controls. In the Zeale primer set, there was host amplification in two *C. russula* samples and one *S. minutus* sample (accounting for up to 21 reads).

The Gillet dataset was more susceptible to contamination in the negative controls, containing a total of 8,143 reads for 51 MOTUs. The top 3 contaminant MOTUs belonged to humans (57 reads), bats (5,103 reads) and shrews (2,984 reads, primarily from *Crocidura*). The bat and shrew associated reads in the negative controls accounted for only 0.1% and 0.5% of total reads, respectively. This shows that there is a low rate of cross-contamination between separate libraries sequenced on the same MiSeq run (i.e. there was a low proportion of bat sequences detected in the shrew library), likely due to strong host amplification and/or tag switching using Gillet primers. All other MOTUs in the negative controls for Gillet contained fewer than 6 reads. This level of cross-contamination is comparable to previous studies using these primers (Galan *et al*., 2018). Vertebrate amplification in the Gillet primer set was large, accounting for between ~89% and ~99% of reads in *C. russula* and between 0.82% and ~96% of reads in *S. minutus* (**Figure 2.2**).

The filtered Zeale dataset contained 183 MOTUs across 4 *C. russula* and 11 *S. minutus* with an average read depth of 182,832 per individual. The Gillet dataset contained 371 MOTUs across 7 *C. russula* and 15 *S. minutus* with an average read depth of 64,034 reads per individual. Rarefaction curves indicate that most prey species are detected within each individual between 1,000 and 5,000 reads (**Figure 2.4A**; inset). The *depth_cov()* indicated that the sequencing depth was sufficient for each sample to capture at least 98% of the diversity (q value = 1) of each individual sample.

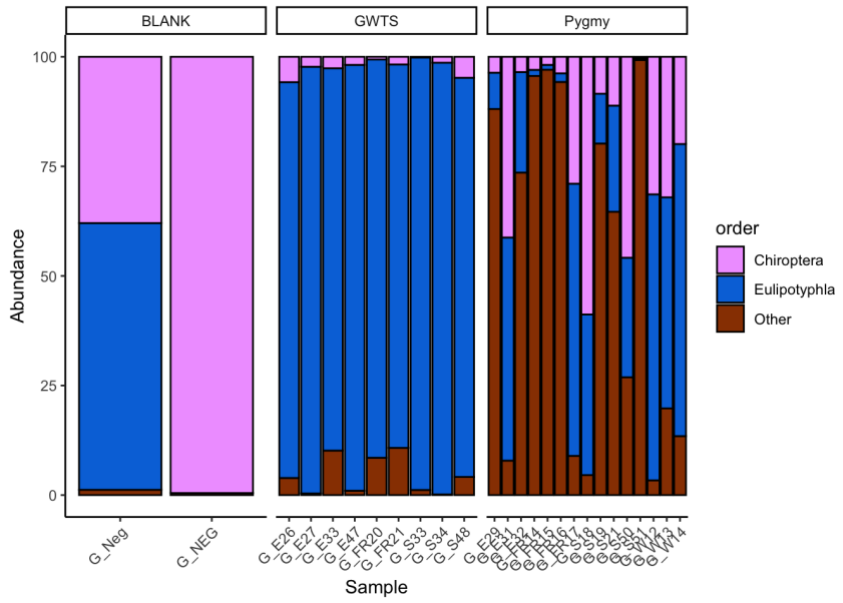



**Figure2.2.** Proportion of host amplification when using Gillet primers. Chiroptera (bat) DNA originates from a separate project on the same MiSeq run. 'Other' represents potential prey reads.

### Taxonomic Range and Composition

The Gillet primers produced more MOTUs (371) than the Zeale primers (183). The majority of MOTUs detected by Zeale belonged to Diptera, Lepidoptera and Coleoptera (**Figure 2.3** and **Figure 2.4A**). The Gillet primers identified MOTUs from a wider range of orders (Gillet 26; Zeale 15), families (Gillet 84; Zeale 51), genera (Gillet 126; Zeale 84) and species (Gillet 123; Zeale 52) (see **Figure 2.4**).

Although there was some overlap in identified taxa, there is a substantially higher number of taxa identified by Gillet than Zeale at all taxonomic levels (**Figure 2.4B**). There are 23 species, 48 genera, 18 families and 3 orders uniquely identified by Zeale. Only the orders Sarcoptiformes, Neuroptera and Blattodea were detected with Zeale primers, but not Gillet. These three orders only take up a small proportion of the reads in shrews (**Figure 2.3**). There are 94 species, 87 genera, 51 families and 14 orders uniquely identified by Gillet. Note that not all MOTUs had a percentage identity strong enough to assign all the way to species. Zeale primers show issues identifying MOTUs to a higher resolution than genus (**Figure 2.1**), which is the reason there are more uniquely identified genera than species when using Zeale primers (**Figure 2.4B**). No vertebrate DNA, other than host and human, was detected in this trial study.

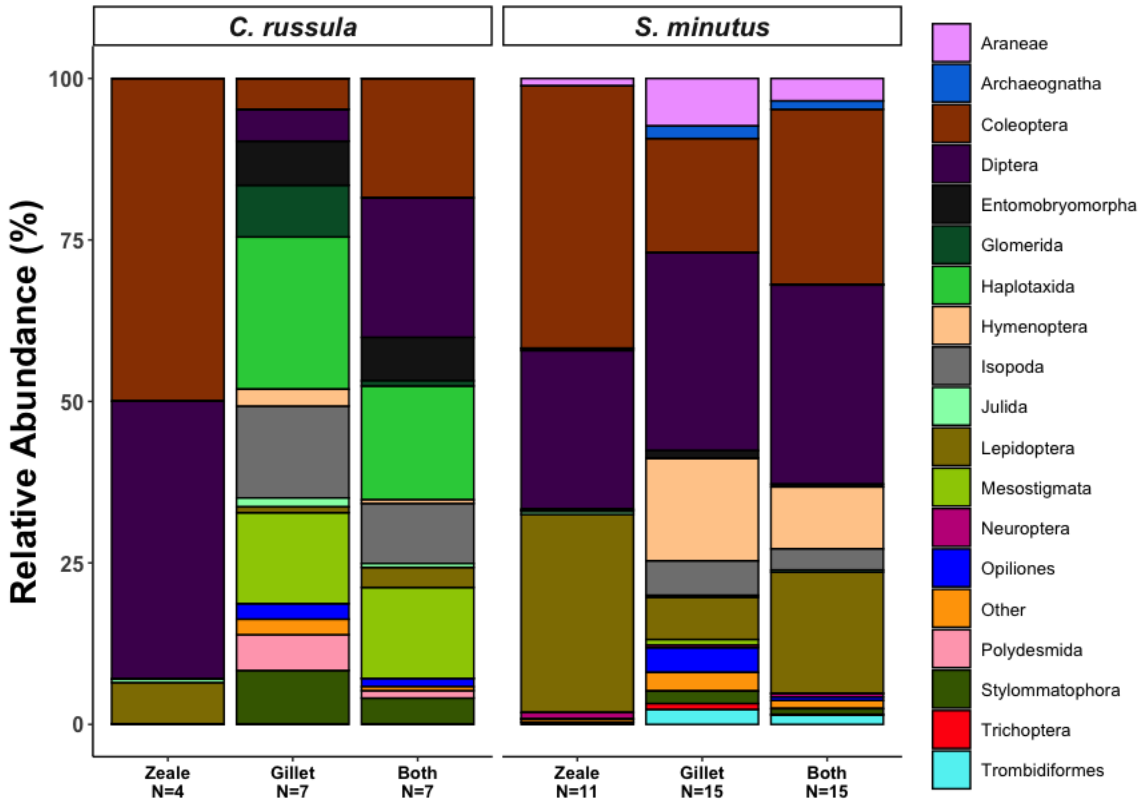



**Figure2.3.** Barplot showing the proportion of reads belonging to the most abundant prey taxa grouped to the order level. Samples are grouped according to species of shrew and the primer used. N values refer to the sample size after quality filtering.

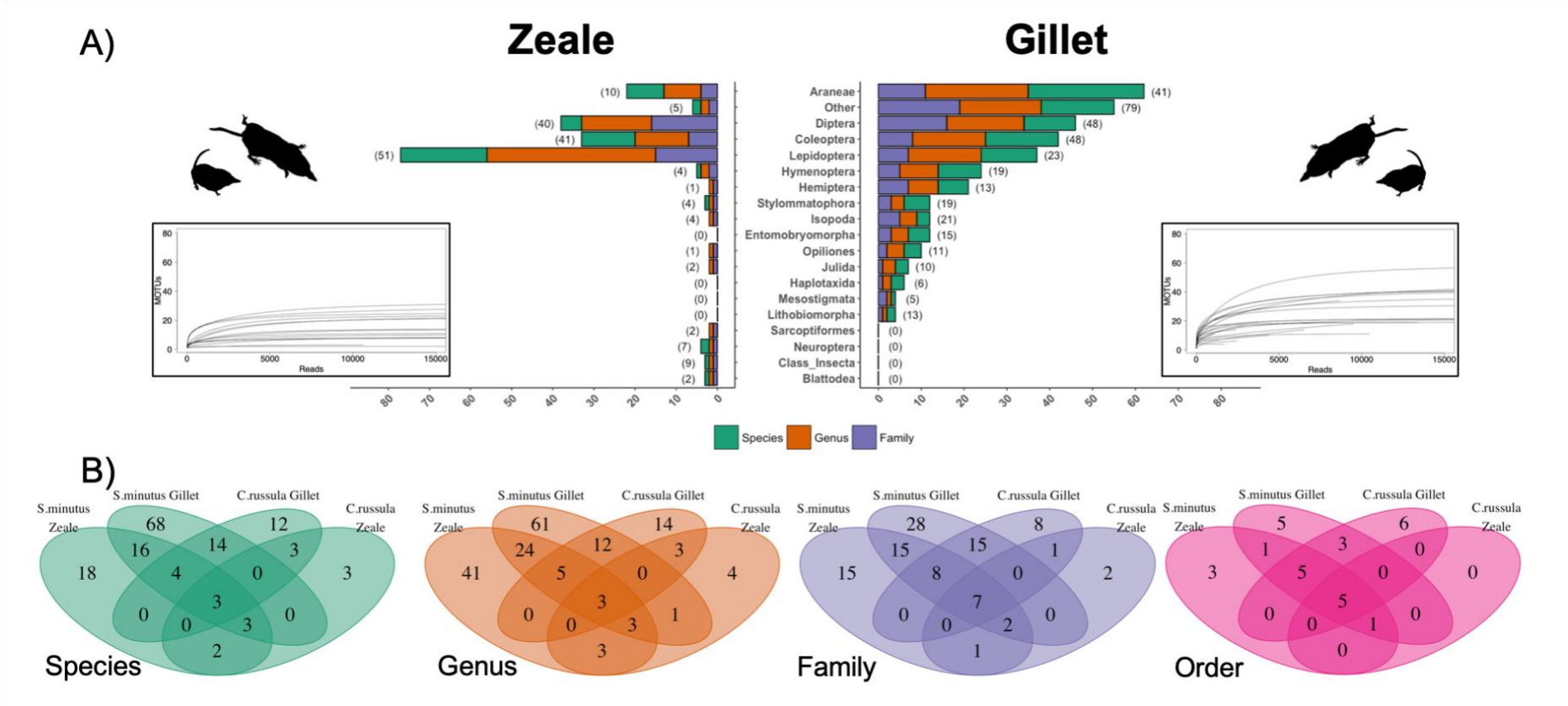



**Figure 2.4.** Taxonomic range of Zeale and Gillet primers in shrews. A) Barplots show the number of families, genera and species detected within the most abundant orders. The values in parentheses are the number of MOTUs identified within that order. The inset graphs are rarefaction curves for each individual using each primer. B) Venn diagrams show the number of unique and overlapping prey taxa detected between species of shrew and primer used. These are shown at species, genus, family and order level.

### Alpha Diversity

The primary differences seen in alpha diversity measurements between primers are higher diversity estimates when using the Gillet primers (**Figure 2.5**). Kruskal-Wallis analysis showed no significant differences when looking at raw species richness (effect size = 0.106, p-value = 0.060).

Kruskal-Wallis analysis shows there are significant differences in the Shannon diversity values between species and primer used (effect size = 0.273, p-value = 0.002; **Figure 2.5**). There are higher Shannon diversity measures for using Gillet primers in *S. minutus* (mean = 1.23, SD = 0.46) compared to using just the Zeale primers in either *S. minutus* (mean = 0.64, SD = 0.44; Dunn's test adjusted p-value = 0.009) or *C. russula* (mean = 0.39, SD = 0.40; Dunn's test adjusted p-value = 0.021). Combining both primers does not produce higher diversity measures compared to using the Gillet primers alone.

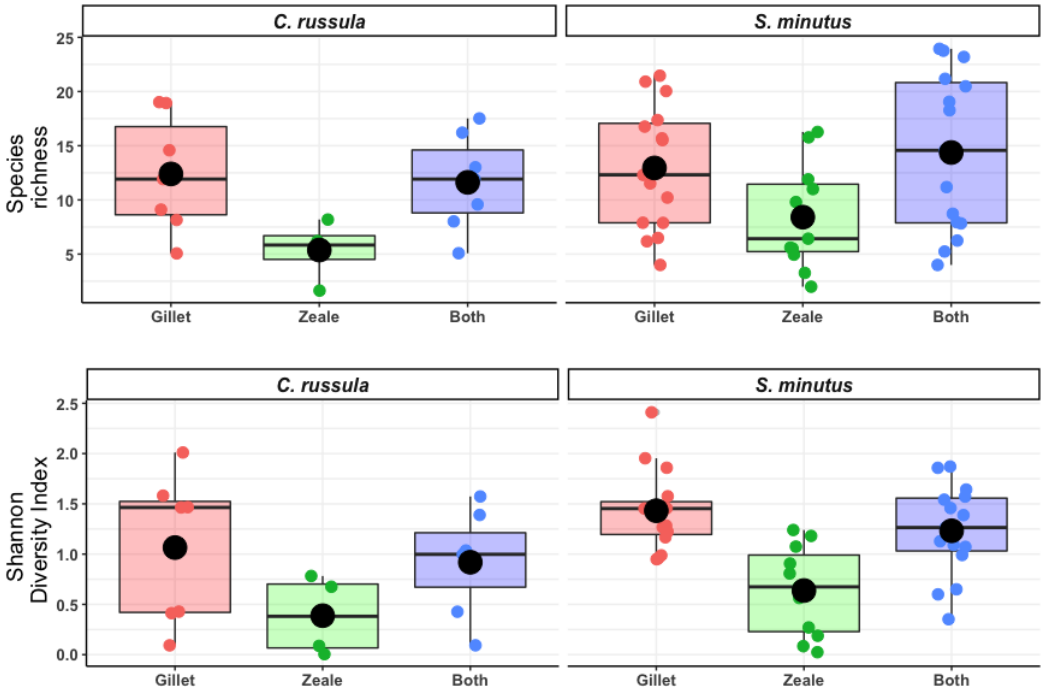



**Figure 2.5.** Alpha diversity measures for each sample. The top panel is the observed species richness. The bottom panel is the Shannon diversity index. The Zeale primers detect the lowest diversity in both species of shrews.

After obtaining the mean relative read abundance (RRA) of samples grouped by species and primer, the lowest mean Shannon diversity estimate were samples amplified with Zeale primers (*C. russula* = 1.86, *S. minutus* = 2.96), while Gillet (*C.*

*russula* = 2.95, *S. minutus* = 3.9) and Both (*C. russula* = 2.89, *S. minutus* = 3.89) were considerably higher.

**Beta Diversity**

When including only the Gillet and Zeale data, the PERMANOVA results show a significant difference in the prey composition detected between samples amplified with different primers ($F = 1.44$, $R^2 = 0.04$, $p = 0.016$). The significant difference in the prey composition detected between primers is consistent with MOTUs agglomerated up to order level. The orders that contribute most to the differences between the Gillet and Zeale datasets (i.e. have the strongest coefficient values from the PERMANOVA) are from Coleoptera, Diptera and Lepidoptera. These orders are preferentially amplified by the Zeale primers.

When including samples using Both primers, the PERMANOVA showed significant compositional differences between samples of different species of shrews ($F = 1.85$, $R^2 = 0.03$, $p = 0.005$), but not between samples amplified with different primers ($F = 0.76$, $R^2 = 0.03$, $p = 0.944$). Again, this pattern remained up to order level, with Haplotaxida and Lepidoptera largely contributing to this observed difference. This correlates with observed compositional patterns seen in **Figure 2.3**.

There was an equal level of homogeneity of dispersion between *C. russula* and *S. minutus* samples (permutest: $F = 1.722$, $p = 0.122$). This shows that difference levels of dispersion between samples are likely not influencing the difference shown in the PERMANOVA.

NMDS plots show that the difference between Zeale and Gillet primers are accentuated when grouping taxa to orders (**Figure 2.6**). When including the combination of both primers it becomes apparent that the Gillet primers have a much stronger influence on the beta diversity of the detected prey (**Figure 2.6**; top panels)

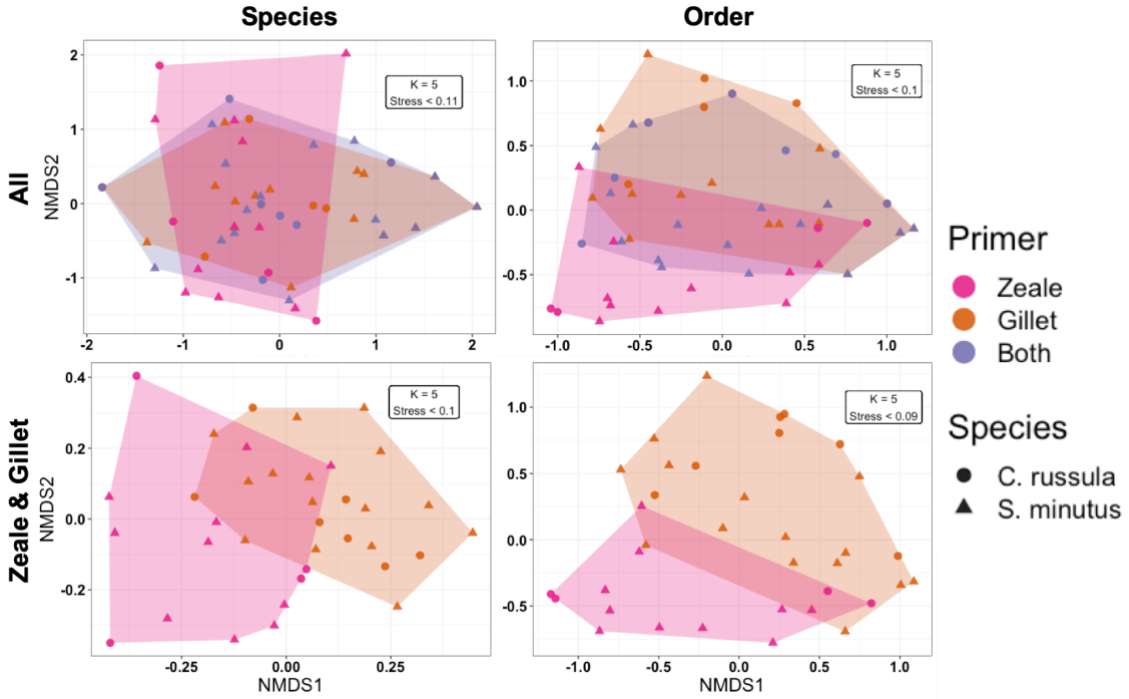



**Figure 2.6.** NMDS plots. The top two panels include samples using Zeale, Gillet and Both primers. The bottom panel only includes the Zeale and Gillet data. The left panels are species agglomerated together, while the right panels show the MOTUs agglomerated to order level. The Gillet and Zeale primers detect a different composition of prey taxa, while Gillet dominates the composition detected when using a combination of both primers.

### Random Forest Classifier

The accuracy was low for classifying samples to *C. russula* or *S. minutus* using Zeale (73.33%), Gillet (68.18%) or both (68.18%). The top 20 taxa for classifying species of shrew mainly consisted of taxa within Lepidoptera and Coleoptera when amplified using Zeale primers. Using Gillet, or both primers, the top 20 taxa are distributed more evenly amongst more orders such as Haplotaxida, Opiliones, Stylommatophora and Diptera.

Correctly determining if samples were amplified with Zeale or Gillet primers could be done with an accuracy of 83.78% based on the prey composition. The 20 most informative taxa for differentiating between primers belong to the orders Coleoptera (6/20), Diptera (4/20), Entomobryomorpha (1/20), Hemiptera (2/20), Isopoda (1/20), Julida (1/20), Lepidoptera (2/20), Opiliones (1/20) and the classes Arachnida (1/20) and Insecta (1/20). Accuracy decreased when classifying shrews between Zeale and Both primers (54.05%) or between Gillet and both primers (2.27%).

## 2.4. Discussion

The Zeale and Gillet primers perform very differently for detecting invertebrate prey taxa in the stomachs of shrews, meaning that primer choice will have a significant impact on ecological inferences from data generated about a shrew's diet. Previous literature shows that the Zeale primers are extensively used and have proved efficient in determining the diet of bats (Vesterinen *et al*., 2018), but this trial shows that in terrestrial insectivores they are still limited to the three orders Coleoptera, Diptera and Lepidoptera (**Figure 2.4**). These three orders are also detected by Gillet but previous studies have shown that these orders may only constitute a small proportion of the shrew's diet (Churchfield and Rychlik, 2006; Churchfield, 2008a, 2008b). The Gillet primers show that ~50% of the detected diet of *S. minutus* consist of these three orders, and a particularly low proportion seen in *C. russula* (**Figure 2.3**). This suggests that the success of Zeale primers is highly dependent on whether the animal has recently consumed prey from these three orders. Zeale has a higher rate of samples being filtered out due to low read counts and would under estimate the diversity of prey consumed by samples that do pass filtering criteria.

One major advantage of the Zeale primer is that there is practically no host amplification (33 reads in total), meaning that all information returned by the Zeale primer pair is potential prey. In contrast, the Gillet primers have a notable disadvantage in terms of host amplification in shrews. The proportion of reads attributed to host is not uniform across samples and the individuals with the highest proportion of host reads (up to 99% of reads) could be due to stomachs being empty at the time of trapping, leaving only shrew DNA to amplify. On average, the level of host amplification is higher in *C. russula* samples compared to *S. minutus* (**Figure 2.2**) which may be due to a higher probability to have empty stomachs, amplification bias between host species DNA and the primers or a combination of both. No vertebrate DNA, other than host and human, was detected in this trial study. This is likely due to the small sample size in this trial as previous studies have detected various species of birds, mammals and amphibians with the Gillet primers (Biffi, *et al*., 2017a; Esnaola *et al*., 2018; Galan *et al*., 2018). Host amplification is not desirable here, but the capability to amplify vertebrate DNA is beneficial to determine if *C. russula* are indeed consuming local vertebrate taxa in Ireland.

This level of host amplification means that the average amount of reads attributed to invertebrates in each sample is approximately three times lower in Gillet (64,034 reads)

compared to Zeale (182,832 reads), of which nearly all reads returned are attributed to invertebrates. An insufficient read depth will reduce the likelihood of detecting the entire prey community, but rarefaction estimates suggest that the majority of prey are detected in each shrew with a sequencing depth of between 1,000 and 5,000 reads (**Figure 2.4**). Despite the reduced read depth, more samples satisfied the filtering criteria when amplified with Gillet rather than Zeale. This is due to the Gillet primers ability to amplify a wider range of taxa, including an additional 14 orders (**Figure 2.4B**). Many of these additional orders constitute a large portion of a shrew's diet, such as slugs/snails (Stylommatophora), spiders (Araneae), woodlice (Isopoda), millipedes (Polydesmida) and worms (Haplotaxida) (**Figure 2.3**; Pernetta, 1976). These results show that after removing host sequences, Gillet primers can still provide more information on invertebrate and vertebrate prey than Zeale without using blocking primers, once sufficient sequencing depth is achieved.

The results here suggest that the Gillet primers alone can gather the majority of information of prey consumed by shrews. Their high degeneracy allows them to detect a wider range of invertebrate taxa (also shown by Esnaola *et al*., 2018) and will likely produce a more accurate representation of the proportions of prey taxa (Krehenwinkel *et al*., 2017). They also show a higher number of MOTUs being able to be assigned to species level (**Figure 2.1**), due to either the availability of reference sequences or the shorter amplicon length being easier to align to a reference sequence with >98% sequence identity. Recent studies suggest that using more than one marker will cover a wider range of taxa and give a more informative overview of the diet of these animals (Esnaola *et al*., 2018). This is true considering that Gillet and Zeale detect unique prey taxa in shrews (**Figure 2.4**), but the composition of the diet appears heavily influenced by the Gillet set (**Figure 2.6**) and the RFC analysis had a very low efficiency differentiating samples that had been amplified with Gillet primers or both. This shows that while adding more primers will increase taxonomic range, one primer may have a dominant influence on the prey composition detected.

If dietary studies employ multiple primers, sequencing depth must be sufficiently high to get the most information out of each primer. For example, consider that a sequencing run has a finite number of reads it can produce. The alpha diversity measures here indicate that at the same finite number of reads, Gillet primers can detect more taxa than Zeale. However, the combined effect of both primers at that same finite number of reads is not significantly higher than using Gillet primers alone

(**Figure 2.5**). If there is a large sample size, additional sequencing runs (or using a more powerful sequencer) may be required to get sufficient read depth to detect all prey taxa with both primers. In addition, a combined effect of primers will restrict dietary studies to frequency/occurrence-based analyses. Although many studies stick to a more conservative frequency-based interpretations of dietary data, the relative read abundance (RRA) can still accurately represent the proportions of prey in an animal's diet at the population level (Deagle *et al*., 2019). Combining both primers here will require the sequencing depth to be normalised between the primer datasets if RRA methods are to be used since the proportions of prey taxa becomes skewed in favour of Coleoptera, Diptera and Lepidoptera (**Figure 2.3**). This highlights the importance of considering budget, sample size and research goals of projects before making a decision on using one or multiple primers.

## 2.5. Conclusion

Dietary studies on novel species or systems should perform preliminary assessments of primers and make a decision based on their taxonomic assignment, taxonomic range, diversity and host amplification (Piñol, Senar and Symondson, 2019; Tournayre *et al*., 2020). The broad diet of shrews will benefit more from a highly degenerative primer set such as Gillet rather than Zeale. Host amplification is a draw back with the Gillet primers but can be compensated for with high sequencing depth (Piñol *et al*., 2014). Here the Gillet primers detect a wide range of invertebrate taxa that are common prey of *C. russula* and *S. minutus* (Churchfield and Rychlik, 2006; Churchfield, 2008a, 2008b) without using vertebrate blocking primers. In addition to achieving sufficient information on the diet, Gillet primers have repeatedly shown an ability to detect vertebrate prey DNA in dietary studies (Biffi *et al*., 2017a; Esnaola *et al*., 2018; Galan *et al*., 2018).

Taking everything into consideration, it was decided to use only the Gillet primers on the full dataset based on the following observations; i) A higher number of samples will pass filtering criteria ii) they will identify a much wider range of prey taxonomic groups to a higher taxonomic resolution iii) they detect higher diversity of prey iv) they have the potential to detect predation on native vertebrates in Ireland v) the relative read abundances will likely represent a closer version of the true diet vi) using a single primer will minimise costs.

# Chapter 3 – Metabarcoding meals: New competition for prey resources drives local replacement of shrews in Ireland

## 3.1. Introduction

The rate at which species are introduced into novel non-native ranges has been increasing over the last decades due to increased trade and transport across the globe (Hulme, 2009; Seebens et al., 2017). The detrimental effects that newly introduced species can have on local ecosystems are so severe that they are now deemed the most common cause of vertebrate extinctions (Bellard, Cassey and Blackburn, 2016). In response to these impacts, policies and legislations have been established to prevent or mitigate further introductions (Darling *et al*., 2017). However, introductions can still occur, and once populations have established they will require accurate impact assessments that can inform new management schemes and policies to mitigate further impacts (Browett, O'Meara and McDevitt, 2020). Negative impacts can arise through resource use and trophic interactions between native and invasive species (see Section 1.3 for full definition of 'invasive species'; David *et al*., 2017). For example, after the black rat (*R. rattus*) was introduced to New Zealand it has been predating on the endemic frog species *Leiopelma archeyi* and *L. hochstetteri*, the former being classified as Critically Endangered (Egeter *et al*., 2019). Another well-known mammalian invader, the American mink (*N. vison*), competes with native European mammalian carnivores for prey resources (Sidorovich, Polozov and Zalewski, 2010; Melero *et al*., 2012).

Accurately predicting the impact of invasive species will have on novel ecosystems is incredibly difficult, resulting in the majority of research and impact assessments occurring after the introduced species have already established a population (Griffen *et al*., 2020). Research into these trophic interactions are a necessity for prioritising management resources towards conservation practice. The Telfair's skink (*L. telfairii*) was introduced to Ile aux Aigrettes for conservation purposes but unexpectedly met potential threats from the invasive Asian musk shrew (*S. murinus*). A DNA metabarcoding study by Brown *et al*. (2014) identified significant interspecific dietary over-lap, concluding with a suggestion that controlling the shrew populations would

benefit the skink population. Alternative approaches to predict impacts of invasive species have been taken by examining effects of competition between invasive and native amphibians in laboratory settings (Richter-Boix *et al*., 2013).

The recent introduction of the greater white-toothed shrew (*C. russula*) into Ireland is an example of how unpredictable the impacts of species introductions can be on local fauna. *Crocidura russula* was accidently introduced into Ireland around the year 2000 via horticultural imports from mainland France (Tosh *et al*., 2008; Gargan *et al*., 2016). Before their arrival, the pygmy shrew (*S. minutus*) was the only species of shrew present in Ireland. It was initially suggested that the *C. russula* population could be beneficial to Ireland's ecosystem by providing an extra food source for locally threatened raptors such as the barn owl (*T. alba*; Tosh *et al*., 2008). Over time it became clear that the expanding range of *C. russula* in Ireland was associated with the local disappearance of *S. minutus* (Montgomery, Lundy and Reid, 2012; McDevitt *et al*., 2014; Montgomery, Montgomery and Reid, 2015). This can be considered an unexpected result considering that *S. minutus* is sympatric with multiple other species of shrews across Europe, including *C. russula* (Churchfield and Sheftel, 1994; Vega *et al*., 2016). On the small island of Belle Île (France), *S. minutus* and *C. russula* are the only species of shrews to co-exist in high abundance among a similar small mammal community as Ireland (McDevitt *et al*., 2014). Differential resource use and niche separation is suggested to be integral for facilitating multi-shrew communities (Rey, Noguerales and García-navas, 2019).

Resource competition is a possible driver for the invasion-extinction event between *C. russula* and *S. minutus* in Ireland (McDevitt *et al*., 2014). Niche separation facilitates the sympatric existence of *S. minutus* with larger species of shrews in mainland Europe (Churchfield and Rychlik, 2006), although *S. minutus* represents between 2.9% - 4% of total shrews in some areas (Yalden, Morris and Harper, 1973; Butet, Paillat and Delettre, 2006). By being the only shrew species to successfully colonise Ireland thousands of years ago (McDevitt *et al*., 2009, 2011), *S. minutus* has likely experienced a competitive release (reduced resource competition) opportunity and has changed physiologically and behaviourally compared to mainland populations. The shape of their skulls and mandibles are distinct from mainland populations (Vega *et al*., 2016). Controlled experiments have shown *S. minutus* to increase predation on larger invertebrates in the absence of larger competitors such as the common shrew (*S. araneus*; Dickman, 1988). The 'island rule' states that insular populations of small

mammals show a trend for increasing in size compared to their mainland counterparts (Lomolino *et al*., 2013). After their arrival to Ireland, *S. minutus* may have increased in size due to the island rule and become dependent on larger prey species in the absence of larger competitors. Considering *C. russula* also goes for larger prey taxa and both species are surface foragers (Ellenbroak, 1980; Brahmi *et al*., 2012), they may have a high niche overlap in Ireland. The considerable bite force and broad diet of *C. russula* gives them a competitive advantage that has already out-competed and displaced the smaller lesser white-toothed shrew (*C. suaveolus*) across Europe (Cornette *et al*., 2015; Biedma *et al*., 2018). The rapid arrival and dispersal of a superior competitor may leave *S. minutus* unable to alter their behaviour and diet in response to successfully co-exist in Ireland within such as short period of time.

This is a recent and ongoing invasion which presents an opportunity to examine the impact of resource competition between a native species and an invasive competitor before, during and after the invasion in a real-time setting. There is a brief region at the edge of the *C. russula* range in Ireland where both shrew species are still present (McDevitt *et al*., 2014). *Sorex minutus* is absent further inside the range of *C. russula* where the longer established invasive populations are. The goal is to identify what the shrews are eating in areas inside the invasive range (*C. russula* only), at the edge of the invasive range (where both species overlap) and outside the invasive range where *C. russula* has not yet reached (*S. minutus* only). Not only will this sampling design identify if there is a high level of niche overlap between the species but will also identify if either are adapting their diet in response to the presence of each other. In addition, the diet of both species will be identified in Belle Île where they are sympatric with each other. Belle Île is an ideal natural 'control' site as the habitat types are similar to Ireland with *C. russula*, *S. minutus*, *M. glareolus* (bank vole) and *A. sylvaticus* (wood mouse) being the most abundantly trapped mammals in both islands (McDevitt *et al*., 2014). This will identify if niche segregation is significantly higher in a region where they can successfully co-exist. To account for seasonal variation in a shrew's diet (Churchfield and Rychlik, 2006), sampling will occur from September to October (late Summer/early Autumn) and again from March to April (late Winter/early Spring).

Shrews are opportunistic predators with broad diets comprising of invertebrates with occasional consumption of small vertebrates by *C. russula* (Pernetta, 1976; Brahmi *et al*., 2012; Ware *et al*., 2020). DNA metabarcoding is a molecular technique that can simultaneously identify entire communities of taxa from a homogenised sample (such

as faeces or gut contents) by amplifying a standardised region of DNA and employing next generation sequencing (Pompanon *et al*., 2012). This technique can be used to simultaneously identify the diet of hundreds of insectivores such as bats (Galan *et al*., 2018), shrews (Brown *et al*., 2014; Biffi *et al*., 2017a) and Pyrenean Desman (*G. pyrenaicus*; Gillet *et al*., 2015; Biffi *et al*., 2017b). The ability of this molecular method to identify prey taxa to species level gives it a huge advantage over morphological methods, which are often restricted to higher levels of classification such as Order (Brahmi *et al*., 2012; Tournayre *et al*., 2020). DNA metabarcoding has some important considerations that must be addressed before taking on a full study for invasive species assessments (Alberdi *et al*., 2018; Browett, O'Meara and McDevitt, 2020). The metabarcoding protocol for *C. russula* and *S. minutus* has been optimised in Chapter 2 and was put to practice on this full-scale study.

To determine if resource competition is a contributing factor to the local displacement of *S. minutus* in response to *C. russula* in Ireland, DNA metabarcoding was applied to the gut contents of shrews to; i) characterise the diet of *S. minutus* and *C. russula* in both Ireland and Belle Île for the first time using a DNA metabarcoding approach. DNA metabarcoding can identify digested prey to species level and provide greater details on the diet of these shrews compared to previous studies using morphological methods; ii) determine if there is higher levels of interspecific competition between shrews in Ireland compared to Belle Île. We predicted that there is a higher level of dietary overlap in Ireland, which contributes to the displacement of *S. minutus* in the presence of *C. russula*; iii) Determine if interspecific competition between shrews at different stages of the invasion is causing shrews to rapidly adapt their diet. If the dietary overlap is high between *C. russula* and *S. minutus*, we predicted that changes in the diet would be observed between stages of the invasion in Ireland. Body measurements of shrews were also taken to identify any intraspecific differences in body size between shrews in Ireland and Belle Île.

## 3.2. Methods

### 3.2.1. Sample Collection

#### Sampling Design

This is an ongoing invasion, therefore there are areas in Ireland where *C. russula* has not yet reached. Sampling sites were chosen to capture information at various stages along their invasive route. The south of Ireland was sectioned into three 'zones' of the invasive range. There is the 'inside' zone, classified as the area in well-established *C. russula* territory where *S. minutus* is no longer present. The 'edge' zone is classified here as the outer perimeter of the invasive range. This is the only zone in Ireland where both *C. russula* and *S. minutus* are present. The 'outside' zone consists of the areas that *C. russula* has not yet reached. Only *S. minutus* are present in this zone. The invasive range has a radial distribution. To accommodate for geographical variation of available prey, shrews were sampled from eastern, western and southern transects of the invasive range (**Figure 3.1**).

Season has already been recorded to have an effect on *S. minutus*' diet in Ireland (Grainger and Fairley, 1978). To account for seasonal variation in diet, sampling was conducted over two seasonal time periods. The first seasonal sampling period took place from 19/08/2017 to 17/10/2017, referred to hereafter as summer sampling. The second sampling period took place from 16/02/2018 to 06/04/2018, referred to hereafter as winter sampling. These dates by-pass major breeding months and should target the same cohort of shrews across the year. Male and female adults were included.

Because Belle Île is such a small island (84 $km^2$), both species of shrews were sampled from 15 sites across the entire island during both seasons (**Figure 3.2**).

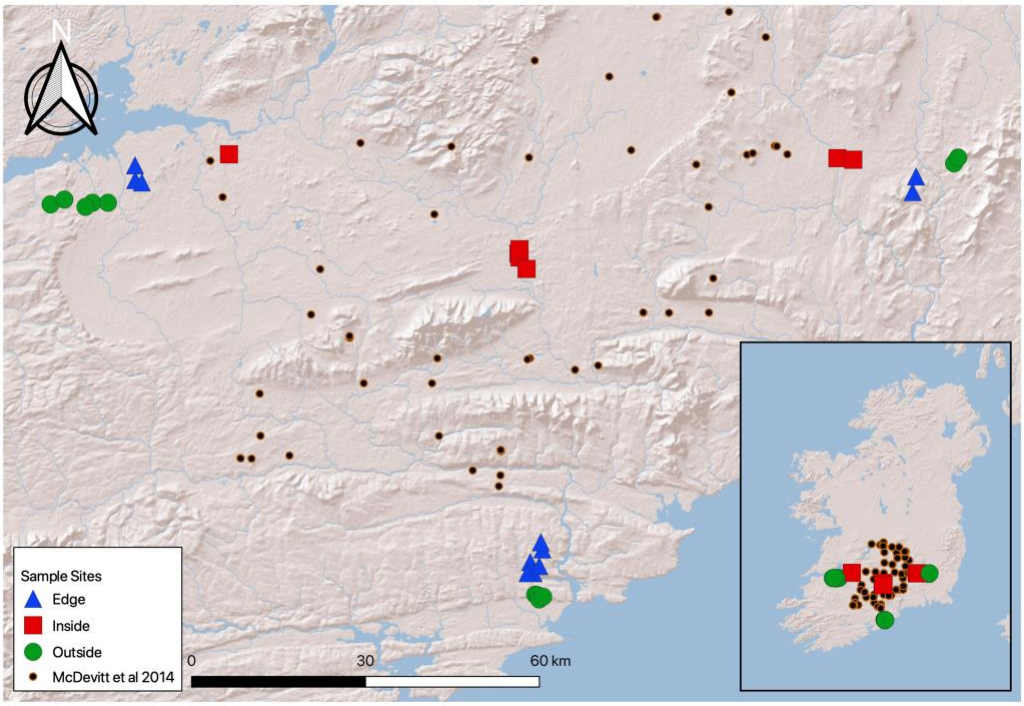



**Figure 3.1.** Trapping sites in Ireland. The sites are distributed to cover a western, southern and eastern transect of the *C. russula* range. Small black points represent sample sites from McDevitt *et al*. (2014) to highlight the previous range of *C. russula*.

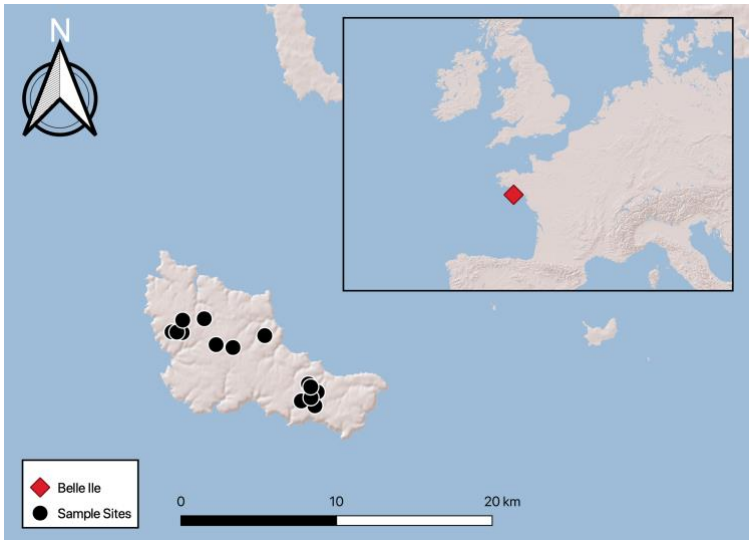



**Figure 3.2.** Trapping sites in Belle Île. Sample sites were distributed accordingly to cover the north western and south eastern regions of the island.

**Trapping**

Trap sites were chosen at hedgerows along secondary and tertiary roads adjacent to agricultural land (pasture or arable). Up to 100 Trip-traps (Proctor Bros. Ltd.) were placed, without bait, every 5m into long grass or mammal runs at the base of a single road side hedgerow (**Figure 3.3**). One hedgerow may not produce enough samples, therefore each of the 9 zone/transect sample sites may consist of 2 or more sub-sample sites within 10km of each other (**Figure 3.1**). To avoid hunger related stress, traps were checked every 30 – 60 minutes. Non-target animals were released immediately, while target animals were immediately killed by cervical dislocation following guidelines set out by (Sikes, 2016). Appropriate licenses and permits had been issued (see **Section 6.4**)

Each shrew was weighed using a 50g Pesola spring scale. The total length (tip of the nose to the end of the tail), head length, body length, fore limb length and rear limb length were measured for each sample. The sexual organs of *S. minutus* can be small and difficult to visually distinguish, therefore shrews were sexed whenever possible. All shrew carcasses were stored in separate disposable bags in a cooler until dissection later that day (Max 10 hrs).

The entire gut tract was removed and stored in absolute ethanol at a 1:4 (sample:ethanol) ratio (Egeter, Bishop and Robertson, 2015). To avoid cross-contamination, all dissections were performed on disposable bench covers and all tools were cleaned and flamed between samples. Gut contents were stored at -20$^{o}$C upon returning from the field to the lab (max 12 days).

A total of 99 *S. minutus* and 124 *C. russula* were caught from Ireland (see **Table 3.1** for breakdown of sample sizes). In Belle Île, 20 *S. minutus* and 20 *C. russula* were trapped here during each sampling period, giving a total of 40 *C. russula* and 40 *S. minutus*.

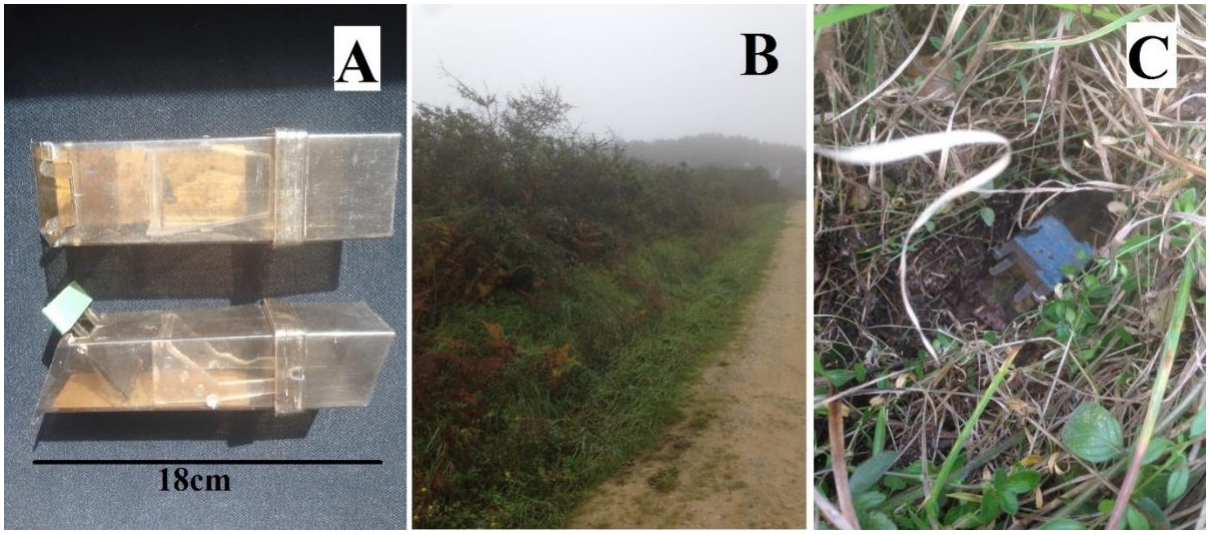



**Figure 3.3.** Trip Traps. A) Image of the trip traps used to capture samples. B) and C) are typical areas for placing traps.

**Table 3.1.** Sample size of shrews trapped in Ireland.

| *Season* | *Invasion Zone* | *Species* | *Sample Size* | | | |
|---|---|---|---|---|---|---|
| | | | West | South | East | **Total** |
| Summer | Inside | *C. russula* | 10 | 10 | 10 | **30** |
| | | *S. minutus* | - | - | - | **0** |
| | Edge | *C. russula* | 10 | 13 | 7 | **30** |
| | | *S. minutus* | 8 | 7 | 10 | **25** |
| | Outside | *C. russula* | - | - | - | **0** |
| | | *S. minutus* | 10 | 10 | 10 | **30** |
| Winter | Inside | *C. russula* | 11 | 10 | 12 | **33** |
| | | *S. minutus* | - | - | - | **0** |
| | Edge | *C. russula* | 11 | 10 | 10 | **31** |
| | | *S. minutus* | 9 | 8 | 5 | **22** |
| | Outside | *C. russula* | - | - | - | **0** |
| | | *S. minutus* | 5 | 10 | 7 | **22** |
| Total | Ireland | *C. russula* | 42 | 43 | 39 | **124** |
| | | *S. minutus* | 32 | 35 | 32 | **99** |

### 3.2.2. Lab Protocols

#### DNA Extraction

Gut tracts were defrosted on ice, removed from ethanol and air dried. Gut contents were removed from the intestines and stomach using sterile instruments (see **Figure 3.4**). Dissection was performed on disposable bench covers and tools were cleaned and flamed in between each sample to avoid cross-contamination. DNA was extracted from the gut contents using the Qiagen Power Soil Kit, with protocol altered according to Alberdi *et al*. (2018). This kit removes PCR inhibitors that are associated with the digestive tract. Entire gut contents were weighed and added to the bead tubes as gut contents rarely amounted to the full recommended weight for the kit (0.3g). Some *C. russula* samples had more than 0.3g worth of gut contents. In such cases, the entire contents were manually homogenised in a 2ml Eppendorf, and the required amount was used for DNA extraction. Five extraction blanks were included.

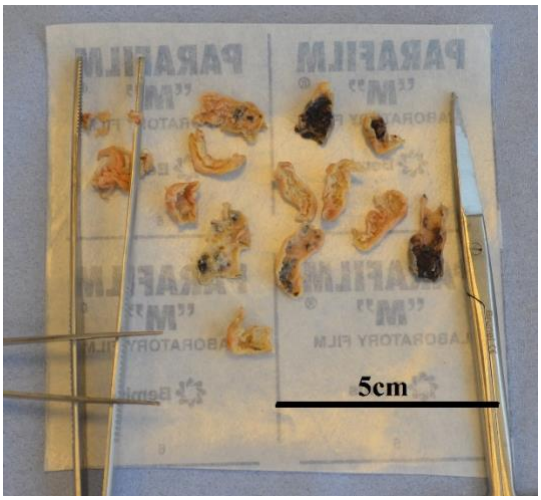



**Figure 3.4.** Example of gut contents during dissection for DNA extraction. Food content was a dark brown/black colour in comparison to the gut itself.

DNA extractions were quantified using the Qubit broad range (BR) kit (Thermo Fisher Scientifc). DNA concentrations ranged from 43.9 ng/µl to 540 ng/µl. The lower DNA concentrations of some samples are likely due to too much sample (under-estimated the weight) and/or residual ethanol that interfered with the extraction reagents. All DNA extractions were subsequently diluted in molecular grade water down to 10 – 15 ng/µl.

**PCR**

DNA from all 305 shrew's gut contents were amplified using the Gillet primers using the optimal conditions determined during the primer trials (see Chapter 2). The set of 24 primer pairs were arranged into 192 different combinations of forward and reverse MID tags. Gillet primers have issues with amplifying large amounts of host DNA (see results for details), but it was decided not to use vertebrate blocking primers because a) we want to detect if *C. russula* is predating on native vertebrates in Ireland and b) blocking primers could potentially block prey DNA to an extent (Vestheim, Deagle and Jarman, 2011).

Samples were randomly distributed amongst 4 PCR plates, randomising host species, season, country, zone and transects to mitigate artificial inflation of inter-species/samples effects. Within each plate, 5 PCR blanks were included, randomly distributed amongst the plate, along with extraction blanks (see **Figure 3.5**). Each one of these PCR plates constitutes a library of 77 to 86 samples, including negative controls. Each library was amplified in triplicate, but these PCR replicates were not individually barcoded (i.e. triplicates were pooled into a single representative sample). After PCR, triplicates were pooled and visually examined on a 1.2% agarose gel, stained with ethidium bromide. 2µl of sample were loaded into each well, with a 50bp ladder for reference. After amplification, each library was kept separate up to adapter ligation.

| | Library 1 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | FR01 | E01 | S73 | W01 | FR41 | E42 | S41 | W61 | E34 | S01 | E03 | |
| B | FR02 | E02 | S74 | W02 | FR42 | E43 | S42 | W62 | E35 | S02 | E44 | |
| C | FR03 | PCR BLNK | S75 | W03 | FR43 | PCR BLNK | S43 | W63 | E36 | S03 | W66 | |
| D | FR04 | E05 | S76 | W04 | FR44 | E45 | S44 | W64 | E37 | S04 | PCR BLNK | |
| E | FR05 | E06 | S77 | W05 | FR45 | E46 | S45 | W65 | E38 | S05 | | |
| F | FR06 | E07 | S78 | W06 | FR46 | E47 | S46 | PCR BLNK | E39 | S06 | | |
| G | FR07 | E08 | PCR Blank 1 | W07 | FR47 | E48 | S47 | W67 | E40 | S07 | | |
| H | FR08 | E09 | Extr Blank 1 | W08 | FR48 | E49 | S48 | W68 | E41 | S08 | | |

| | Library 2 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | FR09 | E10 | S09 | W09 | FR49 | E50 | S49 | PCR BLNK | FR73 | S33 | FR14 | |
| B | FR10 | E11 | S10 | PCR BLNK | FR50 | E51 | S50 | W70 | FR74 | S34 | W10 | |
| C | FR11 | E12 | S11 | W11 | FR51 | E52 | S51 | W71 | FR75 | S35 | E55 | |
| D | FR12 | E13 | S12 | W12 | FR52 | E53 | S52 | W72 | FR76 | S36 | W69 | |
| E | FR13 | E14 | S13 | W13 | FR53 | E54 | S53 | W73 | FR77 | S37 | PCR BLNK | |
| F | PCR BLNK | E15 | S14 | W14 | FR54 | PCR BLNK | S54 | W74 | FR78 | S38 | Extr BLNK | |
| G | FR15 | E16 | S15 | W15 | FR55 | E56 | S55 | W75 | FR79 | S39 | | |
| H | FR16 | E17 | S16 | W16 | FR56 | E57 | S56 | W76 | FR80 | S40 | | |

| | Library 3 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | FR17 | E18 | S17 | PCR BLNK | FR57 | E58 | S57 | W77 | E74 | FR81 | | |
| B | FR18 | E19 | S18 | W18 | FR58 | E59 | S58 | W78 | E75 | FR82 | | |
| C | FR19 | E20 | S19 | W19 | FR59 | E60 | S59 | W79 | E76 | W17 | | |
| D | FR20 | E21 | S20 | W20 | FR60 | E61 | S60 | W80 | E77 | E25 | | |
| E | FR21 | E22 | S21 | W21 | FR61 | E62 | S61 | PCR BLNK | E78 | PCR BLNK | | |
| F | FR22 | E23 | S22 | W22 | FR62 | E63 | S62 | Extra BLNK 3 | E79 | | | |
| G | FR23 | E24 | S23 | W23 | FR63 | E64 | S63 | EXTR BLNK 4 | E80 | | | |
| H | FR24 | PCR BLNK | S24 | W24 | FR64 | E65 | S64 | PCR BLNK | E81 | | | |

| | Library 4 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | PCR BLNK | E26 | S25 | W25 | FR65 | E66 | S65 | W33 | FR33 | E82 | FR40 | |
| B | FR26 | E27 | S26 | W26 | FR66 | E67 | S66 | W34 | FR34 | E83 | EXTR BLNK | |
| C | FR27 | E28 | S27 | W27 | FR67 | E68 | S67 | W35 | FR35 | E84 | PCR BLNK | |
| D | FR28 | E29 | PCR BLNK | W28 | FR68 | PCR BLNK | S68 | W36 | FR36 | E85 | | |
| E | FR29 | E30 | S29 | W29 | FR69 | E70 | S69 | W37 | FR37 | E86 | | |
| F | FR30 | E31 | S30 | W30 | FR70 | E71 | S70 | W38 | FR38 | FR25 | | |
| G | FR31 | E32 | S31 | W31 | FR71 | E72 | S71 | W59 | FR39 | S28 | | |
| H | FR32 | E33 | S32 | W32 | FR72 | E73 | S72 | W60 | PCR BLNK | E69 | | |

**Figure 3.5.** PCR plate layout. Orange squares are randomly distributed PCR blanks. Blue squares are extraction blanks.

## Quantifying and Normalising PCR products

To increase accuracy of normalising the full dataset, PCR products were quantified on a Fluorometer (FLUOstar OPTIMA), including between 6 and 7 standards. Numerous *S. minutus* samples showed amplification of regions larger than the 250bp Gillet region, causing complications directly comparing DNA concentrations with *C. russula* samples. Within each library, subsets of samples from the same species of shrews were normalised prior to DNA purification/bead clean.

## Bead Clean

Before library preparation (i.e. the ligation of sequencing adapters onto PCR products), a bead clean was performed to purify the PCR products. To remove

unwanted fragments smaller than 100bp, a left-side bead clean was performed using MAGBio HighPrep™ PCR Clean-up System beads at a 1.1 X ratio. To remove fragments larger than 300bp, a right-side bead clean was performed using a 0.8x ratio of beads to DNA template. Once the larger fragments have been removed from the S. minutus subsets, their concentrations are comparable to those of *C. russula* subsets. Each purified library subset was then quantified on the Fluorometer (FLUOstar OPTIMA), using 6 - 7 standards. Each subset was then pooled at equimolar concentration to form the 4 library pools. A second round of left side bead cleans were performed on each of the pools to remove any remaining primer dimer.

The success of each cleaning step was verified on an Agilent Tape Station using High Sensitivity screen tapes. Any fragments larger than the target amplicon (250bp) that remain at this point will not cause issues during the adapter ligation (library preparation) steps that follow. During library preparation, adapters will preferentially ligate to the smallest fragments available.

**Adapter Ligation**

Two sessions of adapter ligation were performed. The first session ligated adapters onto library 1 and 2, and the second session was to ligate adapters onto libraries 3 and 4. This was to keep each library with identical MID combinations separated from each other during library prep to mitigate tag-switching effects.

Adapters were ligated using the KAPA Hyper Prep Kit PCR-Free protocol with no modifications to the manufacturers protocol. The NEXTFlex single index sequencing adapters for Illumina platforms were ligated onto each library. These adapters have a single 6bp index. A unique adapter index was associated with each of the 4 libraries, allowing the 330 samples to be multiplexed into a sequencing run.

To verify if adapters have successfully ligated and no un-ligated adapters remain, each library was examined on the Tape Station using the High Sensitivity screen tapes.

**Sequencing**

The libraries were quantified by qPCR using the KAPA library quantification kit for Illumina sequencing with 6 standards included. Each library was then diluted to 50nM

and pooled at equal ratios to create a single equimolar pool at 50nM. This pool was then diluted to 8nM and clarified on another qPCR run using the same protocol. This pool was subsequently diluted to 4nM, containing all 305 samples and 25 blanks. This library was checked again on the Tape Station using the High Sensitivity screen tapes before sequencing. The 4nM library was sequenced on two Illumina MiSeq runs using V2 300 cycle kits, both loaded aiming for 9pM with a 5% PhiX spike.

### 3.2.3. Bioinformatics

#### Sequence Processing

Sequence quality was examined using FastQC (Andrews, 2010). No trimming of sequences was required for either of the two MiSeq runs. Processing of raw sequence reads was performed using Obitools metabarcoding software (Boyer *et al*., 2016). Paired-end sequences were aligned using *illuminapairedend*, discarding any reads below a phred quality score of 40. Sequences were demultiplexed using *ngsfilter* according to the unique dual MID tag combination for each sample and sequences between 128bp and 138bp in length were retained. Unique sequences were combined using *obiuniq*. Chimeras were detected using the *uchime denovo* method (Edgar *et al*., 2011) in Vsearch (Rognes *et al*., 2016). Any detected chimeras were subsequently removed. Sequences were clustered into Molecular Operational Taxonomic Units (MOTUs) using *sumaclust*. Based on the results from Chapter 2 and recommendations from literature (Clare *et al*., 2011; Razgour *et al*., 2011; Alberdi *et al*., 2018), the full dataset was processed using the 98% clustering threshold. Singletons are MOTUs represented by a total of one sequence read in the entire dataset. These were removed prior to taxonomic assignment to aid computation time.

#### Taxonomic assignment

Sequence MOTUs were taxonomically assigned using *blastn* against the NCBI Genbank database. Sequences required at least 80% identity and 90% alignment for a match. The top 25 matches were returned, and the most common taxid (taxonomy identifier) was assigned to that MOTU. MOTUs required at least 98% identity for species level assignment (Clare, Symondson and Fenton, 2014; Arrizabalaga-

Escudero *et al*., 2018). While some studies include species assignment at identity levels below 98% (Shutt *et al*., 2020), here taxonomic assignment of MOTUs are restricted to different levels depending on the percentage identity from blast search. MOTUs between 95% and 98% were restricted to genus level assignment. MOTUs between 93% and 95% were restricted to family level assignment. MOTUs between 90% and 93% were restricted to order level assignment.

MOTU sequences were blasted against the GenBank database because of its larger depository. In addition, a recent study shows that for insect taxa, GenBank performs on par for identification to the Barcode of Life Database (BOLD; Meiklejohn, Damaso and Robertson, 2019). However, MOTUs with low taxonomic resolution were manually blasted against the BOLD database to increase resolution of dataset.

**MOTU and Sample Filtering**

MOTUs of which more than 1% of the reads were found in the blanks were removed. All MOTUs belonging to non-prey taxa (such as vertebrates and parasites) were removed. Samples with less than 1000 reads were removed. To avoid the inclusion of false positive taxa, MOTUs in the trial dataset were removed from each sample if they were represented by less than 0.01% of the total reads of that individual sample (Alberdi *et al*., 2018). Because a higher number of samples were included in the full dataset, the read depth per sample would naturally be lower. The filtering threshold was thus increased from 0.01% to 0.1% for the full-scale shrew dataset to account for the lower read depth per sample (Deagle *et al*., 2019). Using a percentage threshold will account for variable sequencing depth of samples, rather than an absolute threshold such as 5 reads (Kemp *et al*., 2019) or 20 reads (Shutt *et al*., 2020).

To determine the coverage of samples, rarefaction curves and species accumulation curves were generated using the R package vegan (Oksanen *et al*., 2019). In addition, the *depth_cov()* function in the *hilldiv* R package (Alberdi and Gilbert, 2019) was used to clarify if sufficient read depth was obtained for each sample, using the qvalue = 1 (equivalent to Shannon diversity measure).

A second dataset containing a more ‘core’ diet was created by removing rare prey taxa found in a single sample (referred to as ‘core MOTUs’). This strategy is

recommended for dietary studies, particularly for calculating resource overlap values (Brown *et al*., 2014; Arrizabalaga-Escudero *et al*., 2018).

**Alpha Diversity**

To determine the niche width of populations, individuals were grouped according to shrew species and country by summing the reads for each detected MOTU in each individual. The diversity of each group was then measured using the Levin's index, standardised Levin's index and Shannon diversity measure (for details on measurements see Razgour *et al*., 2011) using the R package *spaa* (Zhang, 2016). These metrics are variations on measuring the diversity of prey consumed by each group of shrews.

**Beta Diversity**

The compositional variance within each of these tested groups was measured using the *betadisper()* function in the vegan package. This calculates the multivariate distances of samples to the group centroid. A permutation test for homogeneity of multivariate dispersions was then performed on the multivariate distances to see if there was a similar level of variance (average distance of samples to the group centroid) between each group (Oksanen *et al.*, 2019). This measure of variance is often referred to as the homogeneity of dispersion within groups.

Data was normalised by transforming sequence counts into relative read abundances per sample. A distance matrix was created for the dataset using the Bray-Curtis dissimilarity method. To determine the compositional difference in prey taxa diet identified between different experimental groups, PERMANOVA's were performed using the *adonis()* function in the vegan package in R. The PERMANOVA examines whether the centroid of one group's distances significantly differs from the centroid of other groups and are commonly used for this purpose (Knowles *et al.*, 2019). The explanatory variables were species of shrew, country, season, invasion zone, transect and trap site. The *adonis()* function treats variables sequentially (i.e. the effect of one

variable will rely on the variable stated before it). Therefore, the analysis was performed multiple times using variables in different orders to define the groups. To visualise differences (or similarities), an NMDS plot was generated using the Bray-Curtis dissimilarity method, allowing 5 dimensions (k = 5) to reduce stress on the plot.

**Diet Composition**

There are ongoing discussions and opinions about the best method to quantify the importance of different taxa to a population's diet. Therefore, it is recommended that multiple metrics are empirically tested (Deagle *et al*., 2019). The first method tested is relative read abundances (RRA) per population, which is the average proportion of each taxa found across individuals of a population. The second metric is percentage of occurrence (POO), which is the proportion of the population that has consumed a given taxa, but rescaled so that all taxa adds up to 100%. The third metric is weighted percentage of occurrence (wPOO), which is similar to POO but weights each taxa occurrence according to the number of prey taxa within each sample (i.e. samples with few prey taxa are weighted equally with samples with many prey taxa). See Deagle *et al*. (2019) for full details on these methods.

These three methods were tested at the order level of prey taxa, for each species in each country to determine how closely they agree with each other.

**Niche Overlap**

The Pianka (1973) niche overlap index (Ojk) can be used to determine dietary overlap between species. Its value ranges between 0 (no overlap) and 1 (total overlap). The Pianka's niche overlap index was calculated from the POO values in the R package *ecosimR* (Gotelli and Ellison, 2013) to identify overlap in diet between *S. minutus* and *C. russula* in different countries and invasion zones. To determine if resource overlap was significantly higher or lower than expected, a null model was created by running 10,000 resource utilization simulations using randomisation algorithm RA3. This null model was used to compare to the observed overlap values.

## 3.3. Results

### Body Measurements

*Sorex minutus* individuals are approx. 25% heavier in Belle Île (mean 4.43g, SD = 0.78) than Ireland (mean 3.34g, SD = 0.45) (ANOVA post hoc Tukey p-value < 0.001; **Figure 3.6**). In addition to this, the total length of *S. minutus* is longer in Belle Île (mean 96.47mm, SD = 5.35) than Ireland (mean 91.417mm, SD = 4.54) (Tukey p-value < 0.001). The mean body size of *S. minutus* is also significantly longer in Belle Île (mean 37.23mm, SD = 2.37) than Ireland (mean 34.62mm, SD = 2.84) (Tukey p-value < 0.001).

Conversely, *C. russula* are heavier in Ireland (mean of 11.38g, SD = 1.72) than Belle Île (mean of 9.73g, SD = 1.30) (Tukey p-value < 0.001). They are also longer in Ireland (mean 115.84mm, SD = 4.38) than Belle Île (mean 111.79mm, SD = 4.97) (Tukey p-value < 0.001).

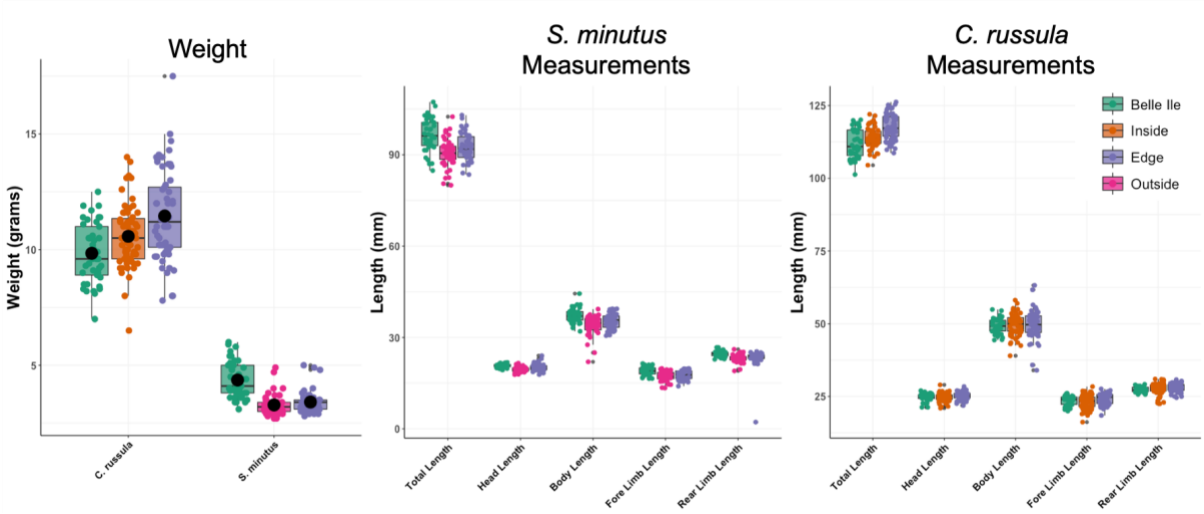



**Figure 3.6.** Body measurements of shrews. The left panel shows the weights of *C. russula* and *S. minutus* in Belle Île (purple) and Ireland (orange). The middle panel shows the length of different body parts of *S. minutus* in Belle Île and Ireland. The right panel shows the length of different body parts of *C. russula* in Belle Île and Ireland.

### Sequencing

The two sequencing runs generated a total of 30,172,418 reads. After quality filtering of sequences and chimera removal, there were 21,091,503 reads for the 305 samples and 25 negative controls. Full breakdown of retained sequences are in (**Table 3.2**).

**Table 3.2.** Breakdown of number of sequences retained through quality filtering steps. Sample size does not include controls. Last column is blank for individual libraries as libraries were combined prior to singleton removal.

| *Library* | *Sample Size* | *Sequencing Run* | *Reads Generated* | *Quality Filter* | *Correct Length* | *Chimera removal* | *Singletons Removal* |
|---|---|---|---|---|---|---|---|
| 1 | 78 | 1 | 3961614 | 3104266 | 2883535 | 2883535 | - |
| 1 | 78 | 2 | 4221401 | 3300256 | 3065428 | 3065428 | - |
| 2 | 80 | 1 | 3589389 | 2679527 | 2418157 | 2418157 | - |
| 2 | 80 | 2 | 3918315 | 2911989 | 2629218 | 2629218 | - |
| 3 | 70 | 1 | 2956215 | 2223848 | 2042631 | 2042631 | - |
| 3 | 70 | 2 | 3188761 | 2407114 | 2212002 | 2212002 | - |
| 4 | 77 | 1 | 4029211 | 3135357 | 2826117 | 2826117 | - |
| 4 | 77 | 2 | 4307512 | 3340485 | 3014415 | 3014415 | - |
| Total | 305 | - | 30172418 | 23102842 | 21091503 | 21091503 | 21045832 |

### MOTU and Sample Filtering

The dataset utilising the sequence clustering threshold at 98% similarity yielded 33,801 non-singleton MOTUs. There was a total of 38,535 reads (0.18% of total reads) from 394 MOTUs identified in the negative controls. The collective read count of MOTUs in the negative controls range from 1 to 10,061. The most prominent contaminants were from the family Soricidae (shrews) (**Figure 3.7**). This is due to strong host amplification using Gillet primers. Host amplification ranged between ~15.6% and ~99.95% in *C. russula* and between 0.14% and ~99% in *S. minutus*.

Two MOTUs found in the negative controls belong to Chilopoda and Diplopoda. These MOTUs were found in one extraction blank, but also found in a high read abundance from one sample extracted in the same session. In addition, these MOTUs are found

in high abundance in samples extracted on other sessions/days. This was likely a brief cross-contamination between one sample and extraction blank and thus were not removed from the dataset. The number of MOTUs removed due to high presence in blanks was 198.

The final dataset contained 977 MOTUs across 179 samples (60 *C. russula* and 119 *S. minutus*) with an average read depth of 34,964 reads per individual. Due to numerous factors such as host amplification and empty stomachs, the average read depth of *C. russula* is 12,957 reads with a total of 777,393 reads. The average read depth of *S. minutus* is 46,061 reads with a total of 5,481,302 reads. Host amplification affected *C. russula* samples more, and thus the sequencing depth of *S. minutus* was higher.

The sequencing depth showed sufficient coverage for each of the 179 samples, with richness (q=0) showing 100% coverage and eveness (q=1) showing values >98%. Species accumulation curves show that at the species/MOTU level, the plateau was not reached for either species in each country (see **Figure 3.8**). Sample coverage improves when agglomerating taxa to higher levels, with a plateau reached at order level. This is a common feature of metabarcoding for diets of insectivore species (Tournayre *et al*., 2020).

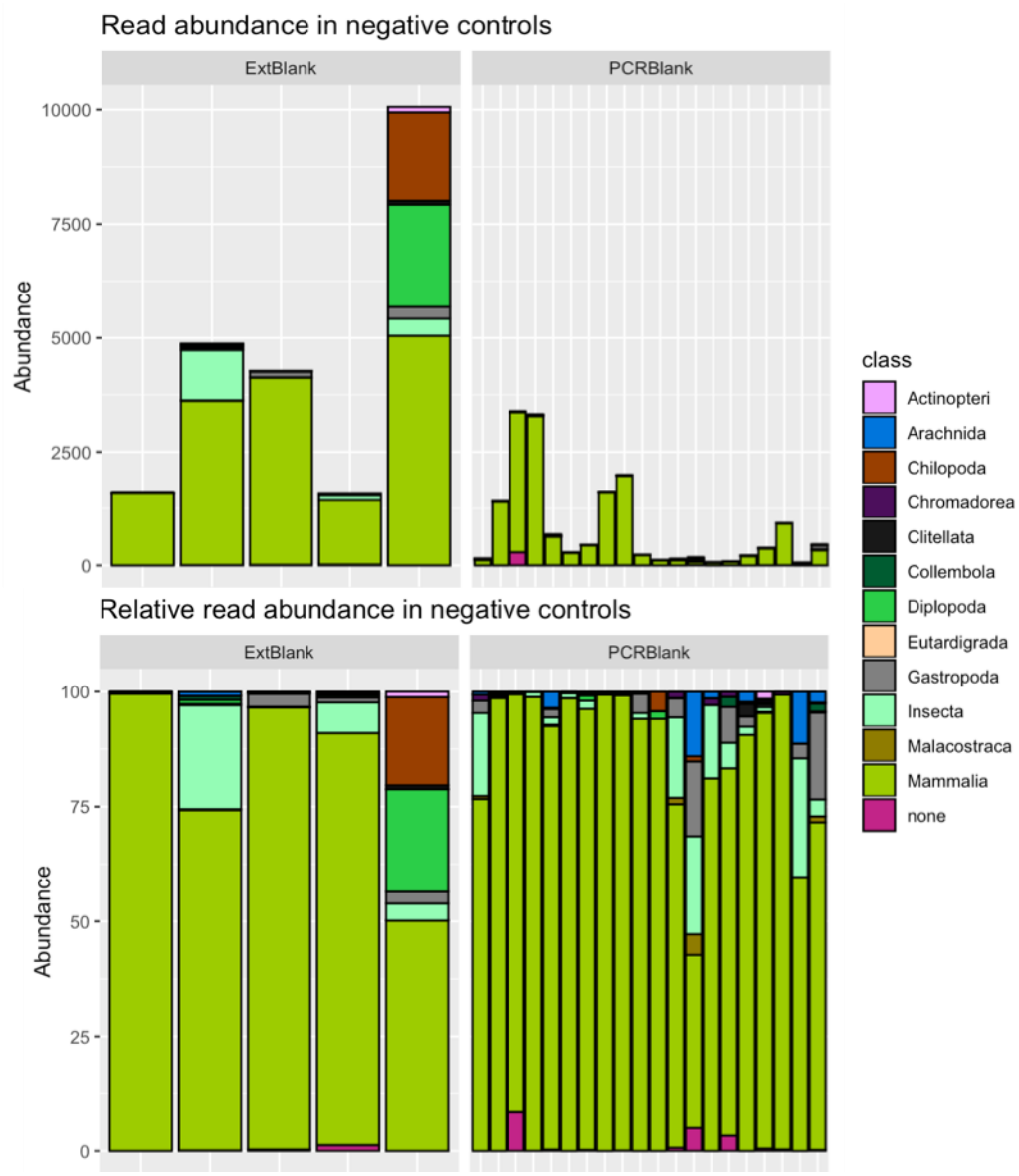



**Figure 3.7.** Negative controls. The top panel is total read count, while the bottom is the relative read abundance. ‘none’ represents reads that could not be taxonomically assigned.

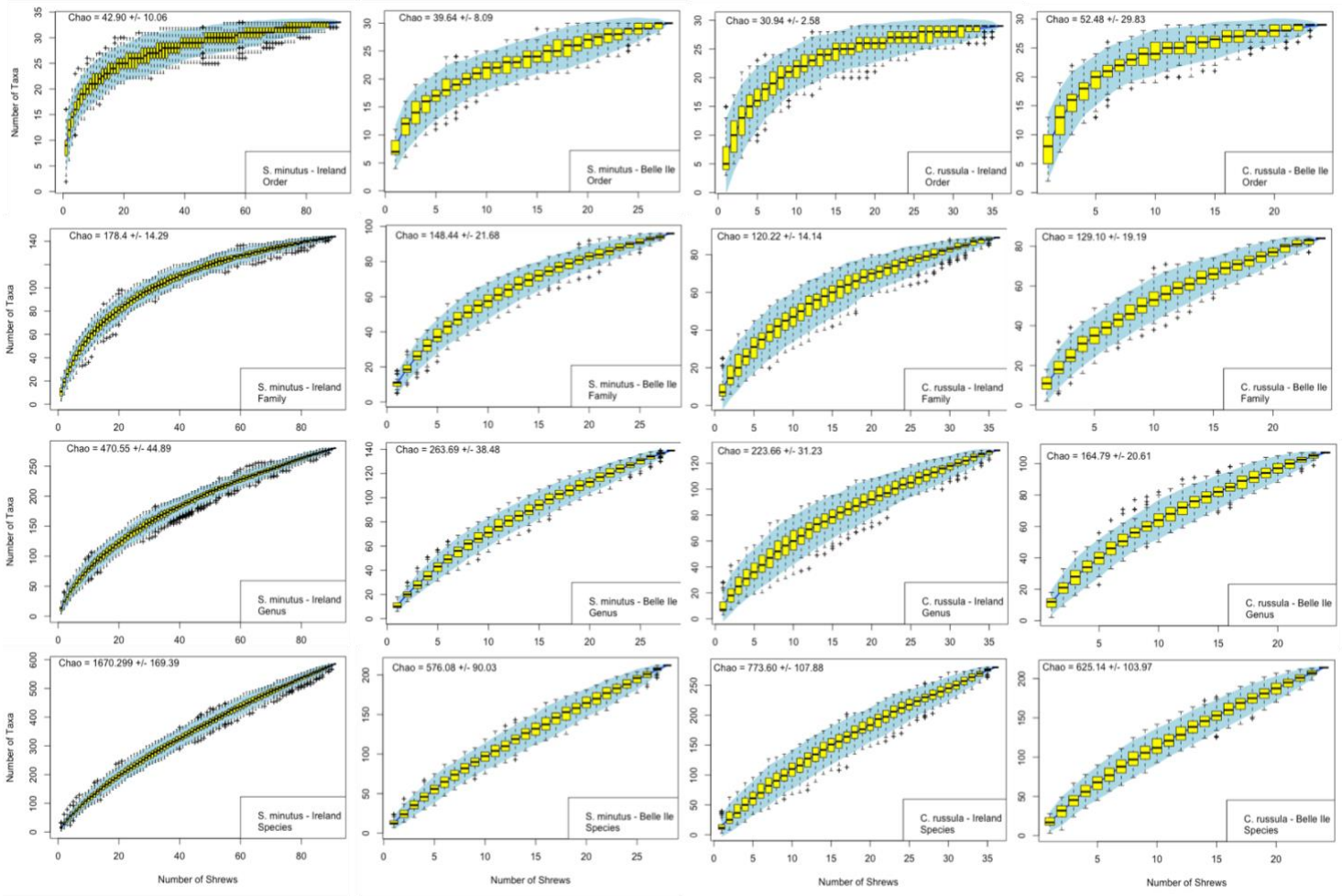



**Figure 3.8.** Species accumulation curves for (from left to right) *S. minutus* in Ireland, Belle Île and *C. russula* from Ireland and Belle Île. From bottom to the top, taxa have been kept at MOTU/species level and agglomerated to genus, family and order level.

### Alpha Diversity

The total number of species consumed by *S. minutus* (717) is higher than *C. russula* (442). This is possibly due to a correlation between the higher number of successfully sequenced *S. minutus* samples and detected MOTUs (**Table 3.3**). The population with the highest Shannon diversity estimates is *S. minutus* from Ireland (SM-Ire), again possibly due to their highest sample size. The standardised Levin's value (which is a measure of niche breadth between 0 and 1) indicates that *S. minutus* from Belle Île (SM-Bel) has the widest niche width, while SM-Ire have the narrowest niche width of all 4 populations. Both *C. russula* populations have similar niche widths. Values are shown in **Table 3.3**.

**Table 3.3.** Diversity and niche width measures for each species of shrews in Belle Île and Ireland.

| *Region* | *Species* | *Sample Size* | *Richness* | *Levins* | *Stnd. Levins* | *Shannon* |
|---|---|---|---|---|---|---|
| Belle Île | *C. russula* | 24 | 214 | 34.77 | 0.16 | 3.98 |
| | *S. minutus* | 28 | 212 | 63.60 | 0.30 | 4.44 |
| Ireland | *C. russula* | 36 | 280 | 50.50 | 0.18 | 4.32 |
| | *S. minutus* | 91 | 585 | 72.20 | 0.12 | 5.02 |

### Beta Diversity

When looking at samples grouped according to shrew species and country, the permutest showed a difference in dispersion/homogeneity between groups (permutest: F = 8.831, p-value < 0.001). Post-hoc pairwise permutest showed significant differences (pairwise permutest; p-value < 0.026) occurring with lower levels of dispersion in *C. russula* from Belle Île (CR-Bel; mean dispersion = 0.61, SD = 0.12) and other groups (mean dispersion = 0.66, SD = 0.04), and lower dispersal in *C. russula* in Ireland (CR-Ire; mean dispersion = 0.66, SD = 0.05) compared to SM-Ire (mean dispersion = 0.68, SD = 0.04; pairwise permutest; p-value = 0.042).

When grouping samples by species, country, season and zone, there was a difference between the homogeneity of group diet calculated using a permutest (F = 2.64, p-value < 0.004). Post-hoc pairwise permutest showed the differences occurring with lower

levels of dispersal in *C. russula* groups (mean 0.62, sd = 0.09) compared to *S. minutus* groups (mean = 0.66, SD = 0.05; permutest p-value < 0.047).

When grouping samples by species, country, season and transect, there was no significant difference between the dispersal calculated using the permutest method (F = 1.70, p-value = 0.061). This supports the sampling design that different transects may not influence differences between invasion zones in the PERMANOVA.

PERMANOVA shows there is a significant difference in the composition of the diet at the MOTU level between shrew species ($R^2$ = 0.02, p = 0.001), and between countries the shrews were sampled in ($R^2$ = 0.02, p = 0.001). Among the top 20 MOTUs contributing most to the differences between shrews and country, there are mostly MOTUs belonging to Gastropoda (slugs and snails), Clitellata (worms), Diplopoda (millipedes). There are notable differences in proportions of these orders in the diet of both shrews (**Figure 3.13**).

While PERMANOVAs showed no difference between CR-Ire according to the season, transect or trap site, there was a significant difference according to invasion zone ($R^2$ = 0.05, p = 0.029). This difference is seen with zone nested in transect and transect nested within zone. This difference is primarily caused by MOTUs from Insecta and Gastropoda. Season also showed no effect in CR-Bel, but there was an observed difference between trap sites within the island ($R^2$ = 0.45, p = 0.001). The core diet dataset was consistent with these results, except season showed a significant effect on the CR-Bel population.

The SM-Ire population shows a significant change in dietary composition according to season ($R^2$ = 0.02, p = 0.001), transect ($R^2$ = 0.04, p = 0.001) and trap site ($R^2$ = 0.23, p = 0.001). The majority of MOTUs contributing to the differences in transect and sites are the same, primarily belonging to Coleoptera and Lepidoptera. The difference occurring between seasons is primarily driven by MOTUs from the Insecta class, which is a likely result of seasonal life stages/cycles affecting their availability to shrews. The SM-Bel population also show differences in the composition of their diet between seasons ($R^2$ = 0.08, p = 0.001), but not trap sites. This shift in seasonal diet is primarily influenced by Insecta and Arachnida, which is noticeable in compositional change using POO measures (see **Figure 3.14**).

The NMDS plot shows that while all 4 populations may be significantly different in their centroid/core diet, there is still considerable overlap between samples (**Figure 3.9**).

These patterns complement the PERMONOVA results, showing significant differences between groups but with low $R^2$ values that indicate that the tested variables explain less than 10% of the variation, except trap sites explaining up to 23% of the variation in SM-Ire and 45% in CR-Bel.

When considering only Irish samples trapped at different invasion zones, there is still considerable overlap. However, as MOTUs are agglomerated in genus, family (family not shown) and order, the *C. russula* samples captured at the edge of the invasive range appear the most different, complementing PERMANOVA results that zone explains variation in the diet of CR-Ire. These plots suggest a higher similarity in diet between *C. russula* captured inside the invasive range and *S. minutus*, particularly when prey species are grouped to higher taxonomic levels. The core diet dataset showed similar NMDS patterns and is therefore not shown here.

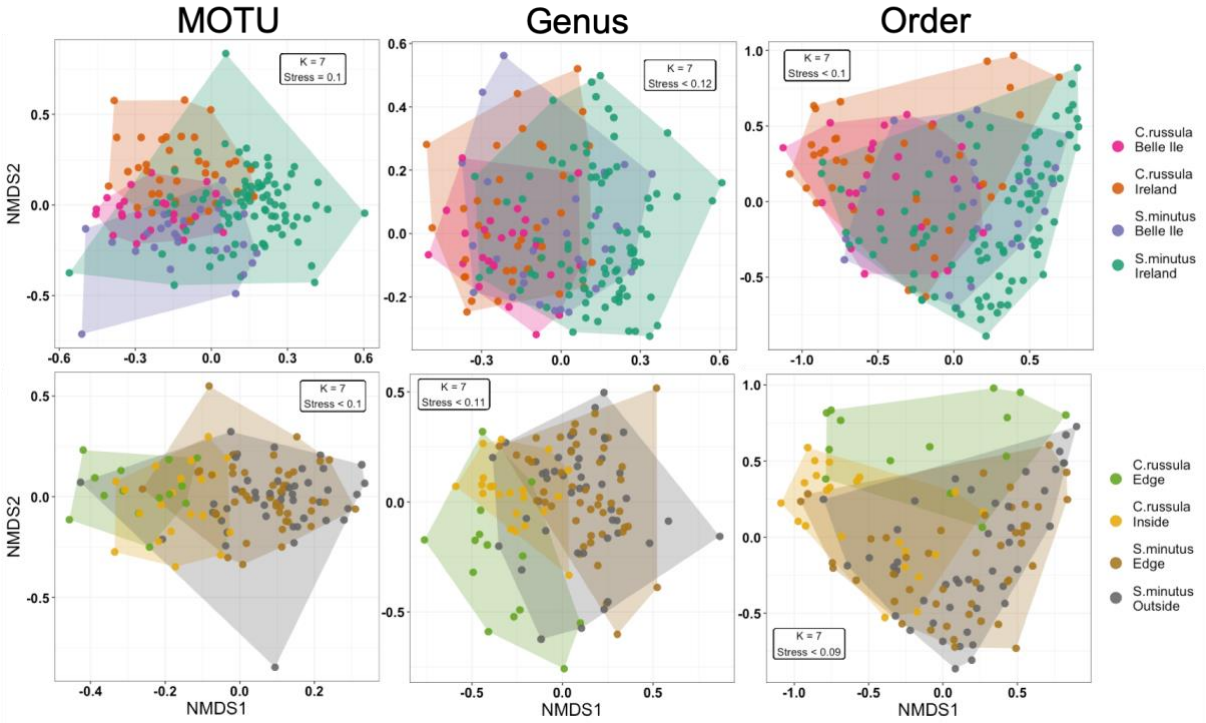



**Figure 3.9.** NMDS plot (stress < 1.3). Top three panels show all samples, grouped according to species and country. The bottom three panels show samples trapped in Ireland, grouped according to invasion zone. From left to right, plots were generated using prey at MOTU/species level, genus level and order level. Family level is not shown but resembles NMDS grouped at order level.

**Table 3.4.** PERMANOVA results. Variables are treated sequentially in a PERMANOVA, therefore each variable is affected by the variable before it. Changing the order of variables in the Irish populations didn't change which variables became significant.

| *Country* | *Species* | *Variable* | *Df* | *F.Model* | *R2* | *Pr(>F)* |
|---|---|---|---|---|---|---|
| Both | Both | Country | 1 | 4.05 | 0.02 | **0.001** |
| | | Shrew | 1 | 4.30 | 0.02 | **0.001** |
| | | Country : Shrew | 1 | 1.78 | 0.01 | **0.004** |
| Belle Île | *C.russula* | Season | 1 | 1.58 | 0.06 | 0.07 |
| | | Trap Site | 8 | 1.58 | 0.45 | **0.001** |
| | *S.minutus* | Season | 1 | 2.41 | 0.08 | **0.000** |
| | | Trap Site | 8 | 1.14 | 0.31 | 0.06 |
| Ireland | *C.russula* | Season | 1 | 0.73 | 0.02 | 0.82 |
| | | Zone | 1 | 1.78 | 0.05 | **0.022** |
| | | Transect | 2 | 0.74 | 0.04 | 0.91 |
| | | Trap site | 11 | 0.92 | 0.30 | 0.83 |
| | *S.minutus* | Season | 1 | 1.94 | 0.02 | **0.000** |
| | | Zone | 1 | 1.22 | 0.01 | 0.09 |
| | | Transect | 2 | 1.72 | 0.04 | **0.000** |
| | | Trap site | 19 | 1.13 | 0.23 | **0.002** |

## Taxonomic Range

The 977 MOTUs detected belonged to 8 classes, 31 orders, 158 families, 288 genera and 313 correctly identified species. There are 275, 139, 63 and 179 MOTUs restricted to genus, family, order and class levels respectively. There were 207 MOTUs retained in the 'core diet' dataset. These core taxa belong to 8 classes, 23 orders, 75 families, 103 genera and 110 identified species.

A proportion of the diet in both shrew species could only be identified to class level (Arachnida, Insecta and Malacostraca). These classes comprise of tens to hundreds of thousands of species. These MOTUs may represent a gap in the reference database for invertebrate species

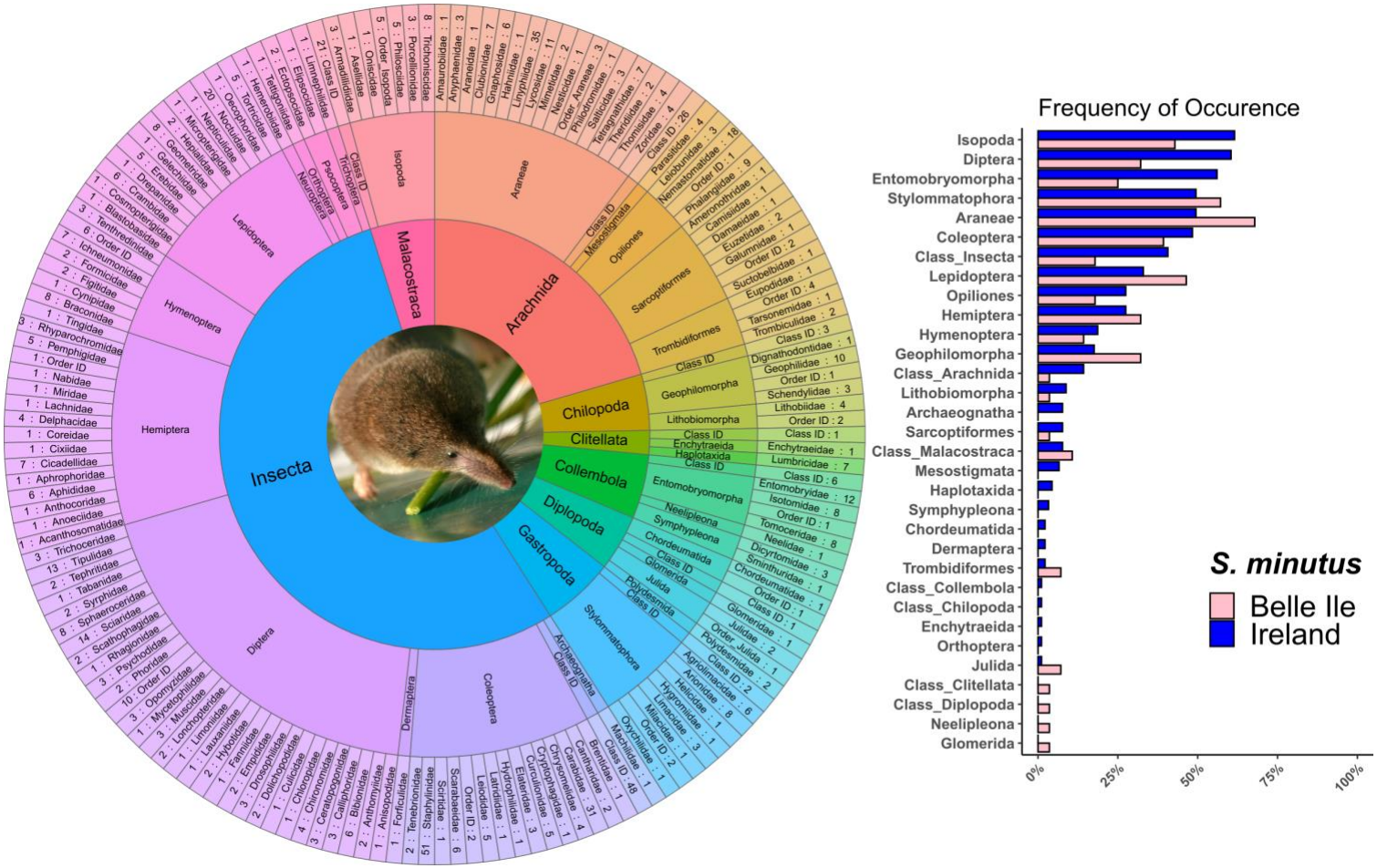



**Figure 3.10.** Range of taxa identified in *S. minutus*. The layers of the taxonomic wheel move from inside to the outside showing class, order and family level. The numbers represent the number of MOTUs found in each group. 'Class ID' and 'Order ID' indicate the number of MOTUs only identifiable to class and order level, respectively. The bar plot shows the frequency of occurrence for each taxonomic order, in order of highest prevalence in the Irish population

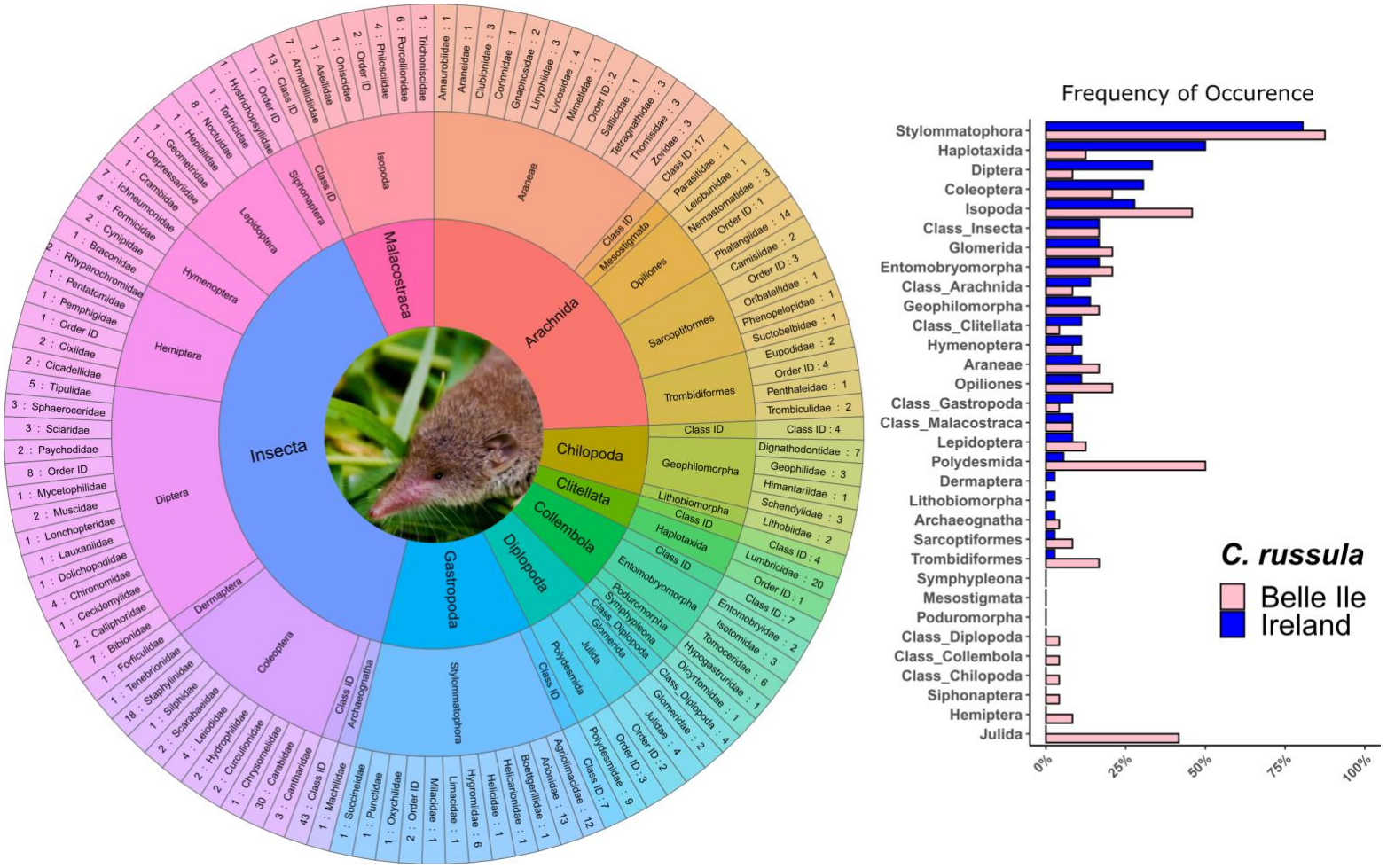



**Figure 3.11.** Range of taxa identified in *C. russula*. The layers of the taxonomic wheel move from inside to the outside showing class, order and family level. The numbers represent the number of MOTUs found in each group. 'Class ID' and 'Order ID' indicate the number of MOTUs only identifiable to class and order level, respectively. The bar plot shows the frequency of occurrence for each taxonomic order, in order of highest prevalence in the Irish population.

### Comparisons of Composition Metrics

RRA, POO and wPOO performed very similarly for groups of samples (**Figure 3.12**). However, there were discrepancies between the methods when quantifying the proportion of certain orders in the shrew diets. RRA returns higher proportion values for physically large orders of prey such as Stylommatophora (10mm – 70mm) in all populations and Coleoptera (5mm – 17mm) in SM-Ire. In the SM-Bel population, RRA returns high proportions of Araneae (2mm – 11mm) of which comprise species of larger bodied wolf spiders. Lepidoptera also returns high RRA values in SM-Bel, which could be a result of high predation of *Xestia xanthographa* larvae (up to 35mm) over the winter sampling period (see **Figure 3.14**). Note that these orders are consumed by a large proportion of the populations (**Figure 3.10**).

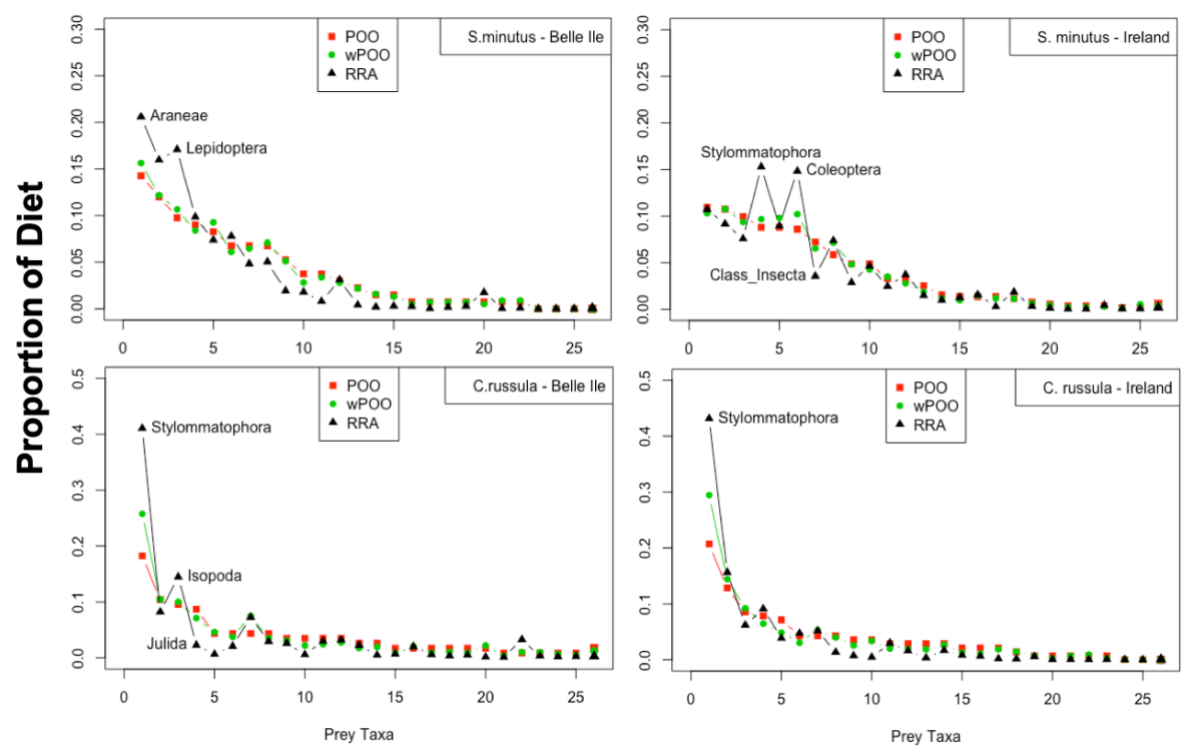



**Figure 3.12.** Comparison of methods to estimate proportion of diets from each taxa group (at order level). Certain groups where relative read abundance (RRA) disagrees with POO and wPOO estimates are labelled. For example, RRA (black) potentially over-estimates the proportion of the diet consisting of Stylommatophora compared to POO (red) and wPOO (green).

### Composition of Shrew's Diet and Niche Overlap

When using RRA of order level taxa for each individual shrew (**Figure 3.13A**), it is evident that there is a wide variety between individuals. This complements the high level of variation between samples seen in the beta diversity measures.

POO reveals a similar diet composition between SM-Ire and SM-Bel. The SM-Ire population has a higher proportion of Diptera, Enterobryomorpha and Isopoda, while SM-Bel show a higher rate of predation on Araneae (see **Figure 3.13** and **Figure 3.14**). Visually the composition of the *C. russula* diet differs at the order level between Belle Île and the two invasion zones within Ireland, complementing the PERMANOVA results. The composition of *C. russula* from inside the invasive range has the closest resemblance to the *S. minutus* diet.

Many prey orders appear to remain consistent throughout the year, such as Stylommatophora and Haplotaxida in CR-Ire (**Figure 3.14**). *Sorex minutus* show the most notable seasonal shifts in prey orders (also shown by PERMANOVA results). SM-Bel show a decrease in predation on Hemiptera in the winter season with a dramatic increase in predation on Araneae and Lepidoptera. Spiders may become more lethargic and easier to catch during this season, while the spike of Lepidoptera could be the result of predation on the nocturnal, grass feeding, caterpillars. A large proportion of the Lepidoptera order are *Xestia xanthographa*. Similar spikes in larvae during these months are recorded in earlier studies of *Sorex* shrews (Pernetta, 1976).

Among the entire dataset, trace amounts of DNA was detected from the mammals *Myodes glareolus* (bank vole; 268 reads), *Microtus agrestis* (field vole; 33 reads), *Mus musculus* (house mouse; 14 reads), *Bos taurus* (cattle; 103 reads), *Sus scrofa* (pig; 84 reads), *Ovis aries* (sheep; 19 reads) and *Cervus nippon* (sika deer; 4 reads) and the avian *Turdus merula* (blackbird; 4 reads).

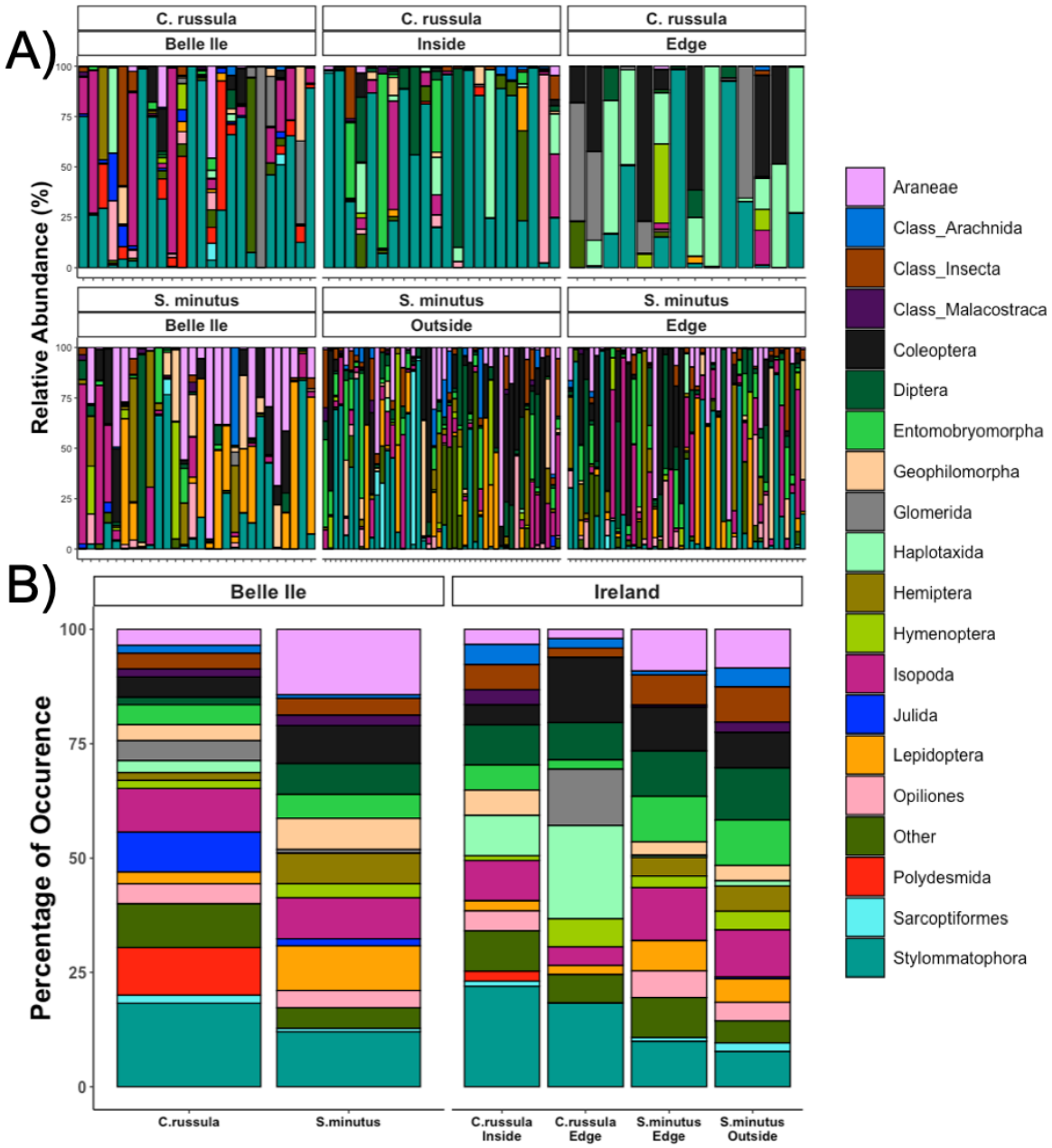



**Figure 3.13.** Composition of shrew diet grouped to order level. **A)** The RRA of prey order in each sample, sectioned according to species, country and invasion zone. Each vertical bar represents an individual's diet before capture **B)** The POO of each prey order according to consumer, country and invasion zone.

The overlap of prey resources (measured using the Pianka's index; Ojk) between *C. russula* and *S. minutus* is generally high at ~38% to ~49% overlap, depending on using all MOTUs or core MOTUs (**Table 3.5**). When compared to simulations, these are significantly higher values than would be expected. *Crocidura russula* and *S. minutus* show a higher dietary overlap in France (All MOTUs Ojk = 0.45641, $P < 0.05$; Core MOTUs Ojk = 0.49945, $P < 0.001$) than Ireland (All MOTUs Ojk = 0.38273, $P < 0.01$; Core MOTUs Ojk = 0.42697, $P < 0.01$). When splitting the samples in Ireland according

to invasion zone, *S. minutus* have a much higher resource overlap with *C. russula* trapped inside the invasive range (39% - 47%) compared to *C. russula* trapped at the edge of the invasive range (13% to 23%). In addition, when accounting for all MOTUs, the level of overlap between *C. russula* at the edge and *S. minutus* was significantly lower than expected compared to simulated data (Ojk = 0.13594 and 0.14956, P < 0.05). *S. minutus* experiences higher levels of competition for prey resources in Ireland from the inside population of *C. russula*.

**Table 3.5.** Dietary overlap (Pianka index) values using POO and ecosimR. Index values range between 0 and 1. A value of 0 means there is no overlap, while 1 means there is complete overlap. *0.05; **0.01; ***0.001

| *Interspecific Comparisons* | | | *All.MOTUs* | *Core.MOTUs* |
|---|---|---|---|---|
| *C.russula* - Belle Île | vs | *S.minutus* - Belle Île | 0.45641* | 0.49945*** |
| *C.russula* - Ireland | vs | *S.minutus* - Ireland | 0.38273** | 0.42697** |
| *C.russula* - Edge | vs | *S.minutus* - Edge | 0.13594 | 0.21747 |
| *C.russula* - Edge | vs | *S.minutus* - Outside | 0.14956 | 0.23664 |
| *C.russula* - Inside | vs | *S.minutus* - Edge | 0.446** | 0.46032* |
| *C.russula* - Inside | vs | *S.minutus* - Outside | 0.39381* | 0.43729* |

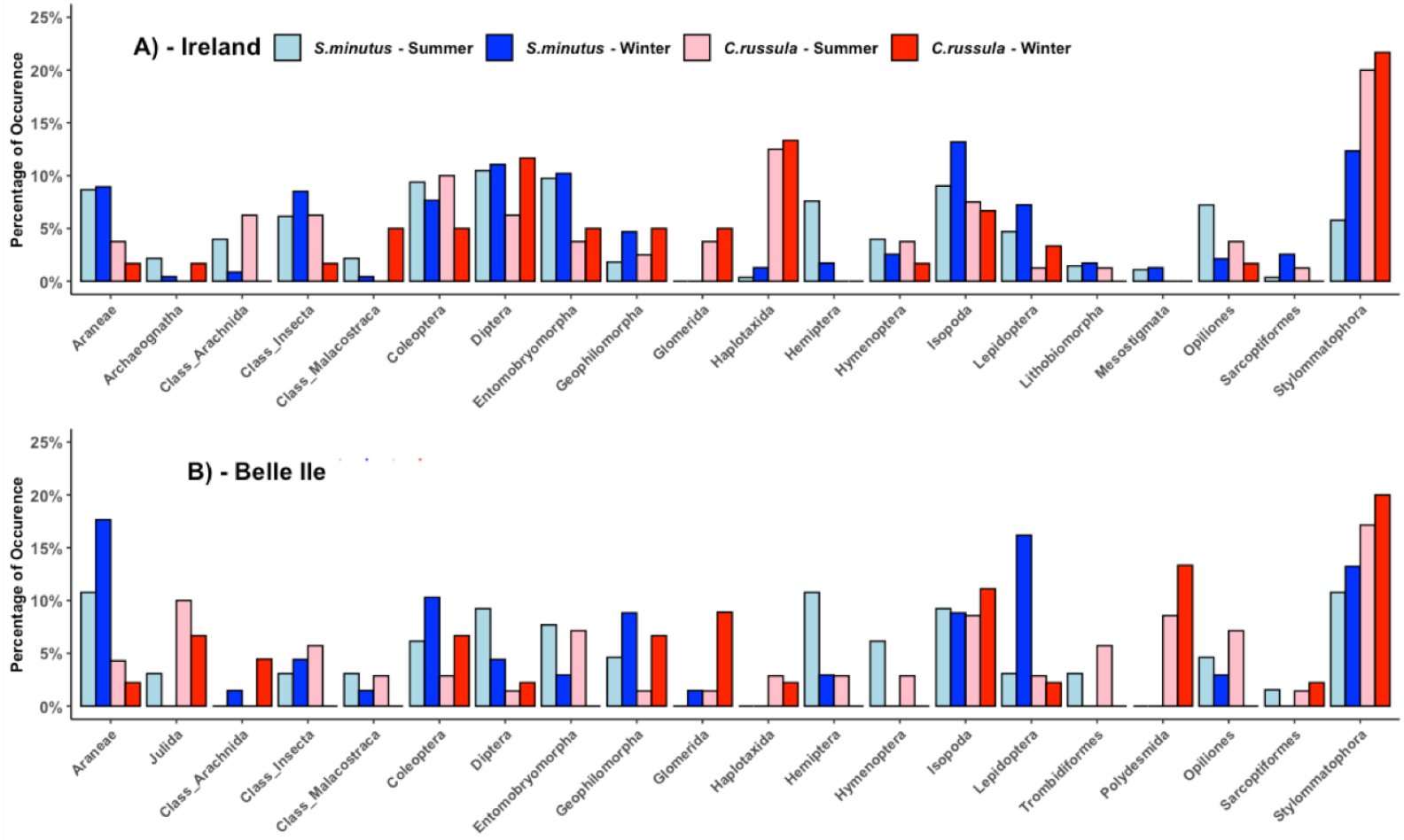



**Figure 3.14.** Percentage of occurrence (POO) of top 20 orders (some only identified to class level). This acts to show the proportion of the diet each order contributes to. Groups are split into species and season in Ireland (top) and Belle Île (bottom).

## 3.4. Discussion

DNA metabarcoding has shown considerable power in determining the important components of the diet of small elusive shrews. Although there is a large overlap in the diet between *C. russula* and *S. minutus* in Belle Île, certain prey groups such as Araneae, Lepidoptera, Julida and Polydesmida may provide key resources that allows their co-existence (**Figure 3.14**). This resource partitioning in Belle Île could be a result of behavioural differences and habitat use of the shrews, or sufficiently high resource availability to allow co-existence. In Ireland however, there is only an initial period during the invasion where both species can co-exist (**Figure 3.1**). This is right at the edge of the invasive range, where *C. russula* has only recently colonised and predates heavily on invertebrates that are likely too large for *S. minutus* to hunt. This scenario changes over time as *C. russula* potentially exhausts local resources of these large prey taxa and begin shifting their diet towards the smaller prey taxa that are essential for the survival of *S. minutus*, increasing interspecific competition (**Table 3.5**). Depending on the stage of the invasion (i.e. how recently they have invaded the area), *C. russula* can have varying impacts on local *S. minutus* populations through trophic interactions. Competition for prey plays a key role in the disappearance of *S. minutus* in response to the *C. russula* invasion in Ireland.

Following recommendations by Deagle *et al*. (2019) to fully interpret the data, different methods were used and compared to determine the composition of the shrew's diets. The different metrics used here (RRA and POO) showed discrepancies between the importance of different food groups (**Figure 3.12**). When using RRA to determine the composition of a consumer's diet, there are biological and technical biases to consider. Tissue from different prey species is digested by the consumer at different rates (Piñol, Senar and Symondson, 2019). Prey of smaller size have less DNA to be detected. RRA estimated lower levels of MOTUs classified under the Insecta class, which could be due to consumption of small species that results in fewer sequence reads. Some taxa groups and species are amplified better than others, resulting in a mis-match between proportions of input prey DNA before and after PCR amplification (Krehenwinkel *et al*., 2017; Bista *et al*., 2018). Feeding trials have shown a discrepancy of 3 times more or less the actual biomass reflected by relative read abundances (Thomas *et al*., 2016). Because of these biases, the diet reported at the population level will be using the POO metric here. The wPOO metric performed very similarly to

POO and was thus not used. RRA was used in tandem to these analyses as a comparison.

Other than this study, only one other known study has used molecular techniques to determine the diet of *Crocidura* or *Sorex* shrews, which used only 5 samples of *S. minutus* in the UK (Ware *et al*., 2020). The majority of dietary analysis of *Crocidura* and *Sorex* species have relied on morphological analysis of gut contents (Pernetta, 1976; Churchfield and Sheftel, 1994; Churchfield and Rychlik, 2006; Brahmi *et al*., 2012) which are often restricted to classifying prey to order level. This is the largest molecular study of the diet of a *Sorex* and *Crocidura* species to date and offers new insights into the diets of these elusive mammals. DNA metabarcoding has shown that both shrews predate on a similar diversity of prey (**Table 3.3**), contradicting morphological methods that led to a consensus that *C. russula* predates on a wider variety of taxa than *S. minutus* (Churchfield, 2008a). However, the larger size of *C. russula* means that while they can readily predate on smaller prey taxa such as springtails (Entomobryomorpha), a large proportion of the detectable diet here consists of relatively large prey groups such as worms (Haplotaxida) and tough shelled taxa such as snails (Stylommatophora) and millipedes (Julida, Glomerida, Polydesmida). There is huge variability between what individual shrews are eating, highlighting the importance of large sample sizes to characterise a shrew's diet (**Figure 3.13**). A small number of reads originating from large livestock such as cattle were detected in the gut contents of multiple shrews. As the predation of cattle by shrews is highly unlikely, these reads likely occurred through secondary detection after shrews or prey invertebrates moved around mammalian dung. The low number of reads detected from other small mammals, such as *M. glareolus*, do not definitively suggest predation of small mammals occurred. As a large number of *M. glareolus* were caught using the same traps during fieldwork, these reads are more likely a form of field contamination during trapping. This shows the primers ability to detect vertebrate DNA, but there was no clear evidence that *C. russula* has predated on any small vertebrates in Ireland or Belle Île as has been proposed in other regions (Brahmi *et al*., 2012).

Morphological identification methods have not typically suggested that *S. minutus* consume Stylommatophora (slugs and snails), likely due to their size (Pernetta, 1976; Churchfield and Rychlik, 2006). It should be acknowledged that soft bodied animals such as slugs may not be identifiable from morphological analysis (Deagle, Kirkwood and Jarman, 2009). DNA metabarcoding may provide the first evidence that slugs

contribute to a significant portion of *S. minutus* diet. They are detected in over 50% of samples in both Ireland and Belle Île (**Figure 3.10**) and have a POO of around 10% (**Figure 3.13**). The Stylommatophora species majorly contributing to *S. minutus* diet are relatively small (approx. 15 – 20mm long) and do not have any shells (e.g. *Deroceras laeve* and *Arion intermedius)* which would make predation easier for small shrews. Secondary detection (i.e. detecting the food of the shrew's food) could possibly explain the detection of Stylommatophora if a shrew consumes an invertebrate that has come into contact with slug mucous or ingested slug tissue. The high number of reads coupled with the frequency of detection suggests that secondary detection is an unlikely reason for this result and *S. minutus* can actively predate on slugs. A small proportion of *S. minutus* samples in Ireland also contained reads from Haplotaxida (worms). This finding is similar to previous dietary assessments of *S. minutus* that concluded this to be opportunistic or scavenging behaviour due to the large size of worms and epigeal foraging behaviour of *S. minutus* (Churchfield and Rychlik, 2006).

The Pianka index identified considerable overlap in diet between these two shrews in both Ireland (up to 46%) and Belle Île (up to 50%) (**Table 3.5**). This is supported by PERMANOVA showing significant, but minimal, differences in the composition of prey between shrew species and country ($R^2$ = 0.01, p = 0.004; **Figure 3.9**). This level of dietary overlap has been seen between sympatric populations of *N. fodiens* and *S. minutus* in Poland (44% overlap), which was considered low for shrews (Churchfield and Rychlik, 2006). Overlap between the diet of sympatric shrews is considered high in general, and multi-species communities likely function as a result of subtle differences between habitat use and resource utilisation (Churchfield and Sheftel, 1994). Therefore, the level of dietary overlap alone may not be enough to explain coexistence in Belle Île, but not Ireland. The POO values indicate that the majority of prey orders in Ireland are consumed by both predators (**Figure 3.14A**). In contrast, there are key taxa that are consumed in Belle Île by one predator but not the other. There is an increased consumption of the orders Araneae, Hemiptera and Lepidoptera by SM-Bel compared to SM-Ire, but not utilised by CR-Bel (**Figure 3.14**). Instead, CR-Bel have approx. 30% of their diet consisting of millipedes (Glomerida, Julida and Polydesmida), of which SM-Bel does not predate on. These prey orders may be key to providing competitive release between the shrews.

It still remains uncertain why the segregation of these key prey orders is possible in Belle Île but not Ireland. The inability to co-exist in Ireland could be a result of resource

limits rather than the competition itself (Biedma *et al*., 2019). For example, resource availability in Argentinean habitats will determine if native river otters (*Lontra provocax*) can co-exist with invasive North American mink (*N. vison*) (Fasola et al., 2009). While invertebrate community data is not currently available for the trapping sites in this study, there are patterns in the metabarcoding data that suggest limited resources may be playing a role. In Belle Île, a large proportion of the detected diet of *C. russula* consists of Julida (**Figure 3.13** and **Figure 3.14**), predominantly the species *Cylindroiulus latestriatus*. This species is mainly found on coastal regions in Ireland (National Biodiversity Data Centre, Ireland) rather than the inland sites where trapping took place. The small size of Belle Île may have more suitable habitats to accommodate a higher abundance of this prey species for *C. russula* and relieve competitive pressure from *S. minutus*. SM-Bel have a drastically increased consumption of Lepidoptera during the Winter (**Figure 3.14**), similar to previous observations of winter spikes of consuming Lepidopteran larvae using morphological approaches (Pernetta, 1976; Butterfield, Coulson and Wanless, 1981). While DNA metabarcoding cannot identify life stage, it has identified a large proportion of this winter spike to be *Xestia xanthographa*. This moth species over-winters as nocturnal larvae (up to 35mm in size), feeding on various grasses (Skinner and Wilson, 2009). The nocturnal behaviour of *S. minutus* means they can take advantage of this slow moving and substantial food source during the less favourable winter conditions free from competition from *C. russula*. Another study in the Netherlands has also shown partial niche segregation between *S. minutus* and the larger *S. araneus* over seasons may reduce interspecific competition (Ellenbroak, 1980). The small difference in prey taxa consumed by SM-Bel between seasons (PERMANOVA; $R^2 = 0.06$, $p = 0.000$) suggests that they are predating on more readily available taxa between seasons, such as the apparent switch from Hemiptera in the Summer to Lepidoptera in the Winter (**Figure 3.14**).

Another factor affecting resource use could be the morphology of the shrews. Bite force and mechanical leverage of a shrew's mandibles can determine the limits of prey size they can capture and consume (Cornette *et al*., 2015). Vega *et al*. (2016) examined the variation of shape and size of mandibles and skulls from *S. minutus* samples from various European regions including Ireland, Belle Île and multiple other islands. They showed that *S. minutus* can exhibit morphological variability between different regions and islands in response to various environmental factors such as food availability and presence of competitors. They also showed that the mandible size and shape of SM-

Ire is distinct from other populations while SM-Bel is more similar to continental populations where they co-exist with other species of shrews. The larger size of SM-Bel determined by this study (**Figure 3.6**) and the distinct mandible structure inferred from Vega et al. (2016) may allow them to avail of a wider range of sizes of prey, which could explain the wider niche breadth measured by the Standardised Levin's index (**Table 3.3**). For example, species of Araneae consumed by SM-Bel are larger wolf spiders from the genera *Pardosa* and *Alopecosa* that can be up to 11mm in size, providing a substantial energy resource. SM-Ire shows a reliance on smaller spiders such as *Pachygnatha clercki* measuring between 4.5mm and 6mm (Nentwig *et al*., 2020).

In Ireland, the composition of the *C. russula* diet is changing between invasion zones (PERMANOVA; $R^2$ = 0.05, p = 0.022). Larger invertebrates such as worms (Haplotaxida), beetles (Coleoptera) and tough shelled millipedes (Glomerida) comprise a large portion of the *C. russula* diet at the edge of the population, but is greatly reduced at the inside zone (**Figure 3.13**). The combination of high abundance, small territories, and wide diet means that *C. russula* are known to exhaust local resources (Genoud, 1985; Brahmi *et al*., 2012). This invasive shrew could potentially be exhausting preferable prey resources and forcing themselves to switch towards smaller prey taxa. As a result, the level of interspecific dietary overlap increases from between 14% - 24% at the edge zone to between 39% - 46% at the inside zone (**Table 3.5**), and the NMDS plot shows a higher overlap between *S. minutus* and *C. russula* from the inside zone (**Figure 3.9**). In other words, they only begin consuming large volumes of small prey taxa that are more essential to the *S. minutus* diet in the well-established range (i.e. the inside zone). This is why there's a brief area of overlap, which turns into a point where they cannot co-exist.

The invasion is occurring with a 'layered' effect. The first layer of *C. russula* (samples trapped at the edge zone) are 20% larger in body mass (**Figure 3.6**) which may be a result of dispersal abilities (Phillips *et al*., 2006; Burton, Phillips and Travis, 2010). Their larger size may aid in their ability to predate on invertebrates that are too large for *S. minutus*, thus reducing competitive pressure. Previous studies have shown that shrews that differ greatly in size tend to have reduced niche overlap compared to shrew species closer in size (Churchfield and Rychlik, 2006). This would explain why both *C. russula* and *S. minutus* co-exist in high abundance at a restricted area where their ranges first meet (McDevitt *et al*., 2014). The relatively narrow niche width of *C. russula*

(**Table 3.3**) provides some contradiction to previous claims that predation in shrews is likely opportunistic with little selection of prey (Castien and Gosalbez, 1999). The second layer of *C. russula* (samples trapped at the inside zone) are altering their diet to smaller prey taxa as a result of either exhausted resources, decrease in size (**Figure 3.6**) or both. Alternatively, the decrease in body mass in the second layer could suggest reduced energy intake from reduced food resources after the first layer (Seymour *et al*., 2005). This second layer of *C. russula* is what likely out-competes the *S. minutus* for small prey resources that are key for their survival (This study; Pernetta, 1976; Churchfield and Rychlik, 2006; Ware *et al*., 2020). After the long-term establishment of *C. russula* in Ireland, *S. minutus* cannot compete and disappears from the area.

## 3.5 Conclusion

This layered effect of the expanding range of *C. russula* has huge implications for Ireland specifically and broader scale invasive species management. Firstly, the results here indicate the high possibility that *C. russula* is changing the invertebrate community in the hedgerows. This can have further negative downstream effects on ecosystem services. Secondly, this study shows that the initial impacts of an introduced species appear to be minimal. But over a relatively short period of time, the introduced species may adapt and begin having negative effects not previously predicted. This highlights that introduced species must be carefully and continuously monitored, regardless of initial risk assessments.

Future assessments of this shrew invasion should include different habitat types (e.g. woodland and peatland) to see if co-existence is possible through habitat use. Certain habitat types support co-existence between *C. russula* and the lesser white-toothed shrew (*C. suaveolus*) in Europe (Biedma *et al*., 2018), while Keckel, Ansorge and Stefen (2014) have shown that choice of microhabitat between *N. fodiens* and *N. anomalus* explains co-existence more than diet itself. After being isolated over a long period in Ireland with the absence of larger competitors such as the common shrew (*S. araneus*), it is suggested that *S. minutus* has altered their habitat use compared to their mainland counter-parts (Michielsen, 1966). Woodland and peatland habitat types are potentially favoured by SM-Ire populations, which could support interspecific niche separation and act as potential refuge for *S. minutus* despite the presence of *C. russula*

(McDevitt *et al*., 2014). However, Ireland has a relatively homogenous landscape and these potential refuge habitat types are sparse. Eradication is not feasible at this point since the CR-Ire population is already too large, but *S. minutus* are still present on Ireland's offshore islands (McDevitt *et al*., 2014). These islands are important refuge sites for Irish *S. minutus* populations, so efforts must be put in place to ensure that *C. russula* do not reach these islands in fear they will have the same effect as the mainland.

# Chapter 4 – Microbiome characteristics of wild shrews from native and invaded territories

## 4.1. Introduction

Vertebrates naturally house tens to thousands of species of bacteria within their guts (Shapira, 2016). In return for a 'home', symbiotic microbes can benefit the host through various functional/phenotypic traits such as nutrient production, metabolism and protection from pathogens and viruses (Moeller, Sanders and Moeller, 2020). The core microbiome structure is often correlated to host phylogeny, with phylogenies of similar ecological and dietary niches known to converge on host-microbiome functions (Ley *et al*., 2008a; Colston and Jackson, 2016). For example, herbivores host microbes that are more functionally adapted for synthesising amino acids, whilst carnivores host microbes involved in degrading amino acids (Muegge *et al*., 2011). Even with a change of diet, there can still be remnants of microbiota reflecting the host's evolutionary history such as the giant panda (*Ailuropoda melanoleuca*); even though this species now feeds exclusively on bamboo, it still hosts a carnivore-like gut microbiota (Xue *et al*., 2015).

As well as host-microbiome relationships over evolutionary time scales, the microbiome composition can be highly plastic and evolve rapidly in response to short-term environmental change (Alberdi *et al*., 2016; Hauffe and Barelli, 2019). For example, populations of house mice that live at higher altitudes show enriched microbiome function for the renin-angiotensin system, which plays a role in blood pressure regulation, than their lower altitude counter-parts (Suzuki, Martins and Nachman, 2019). Habitat degradation, and subsequent plant/food diversity loss, has led to changes in functional diversity of the microbiome of endangered red colobus monkey (*Procolobus gordonorum*) in Africa (Barelli *et al*., 2015). Seasonal changes in diets can explain up to 25% of a compositional change in microbiome in various mammalian species (Kartzinel *et al*., 2019). Even social interactions can lead to convergence of microbiome structure between individuals (Archie and Tung, 2015; Antwis *et al*., 2018; Raulo *et al*., 2020).

As the functional role of host-associated microbiomes can act as host phenotypes that evolve quickly in response to the environment, Alberdi *et al.* (2016) argue that the

microbiota may play an important role in adaptation. Until the more recent surge in microbiome studies in wild vertebrates, much of our understanding of rapidly adapting microbiomes in non-human subjects has largely relied on lab settings using murine models (Colston and Jackson, 2016) or using wild vs captive/zoo systems, such as bats (Mckenzie *et al*., 2017; Lechner *et al*., 2020). There has recently been a call to characterise more wild population microbiomes to increase our understanding of their adaptability to an ever-changing world (Hird, 2017). One aspect of changing environments are biological invasions, in which the invading organisms must adapt quickly to new surroundings, whilst the microbiomes of native hosts potentially have to adapt to the presence of new competition. Biological invasions are increasing rapidly (with increasing global trade), with significant effects on biodiversity across ecosystems (Young *et al*., 2017), which means they require scientific attention for further understanding and have subsequent implications for species/landscape management. Although the microbiome is extensively researched in invasive plant hosts (Kowalski *et al*., 2015; Li *et al*., 2019), recent studies have documented the microbiomes of notoriously invasive vertebrates (e.g. tilapia fish, *Oreochromis mossambicus*; Gaikwad, Shouche, and Gade, 2017). However, there is still limited information on the microbiome of invasive vertebrates in both “native” and newly invaded regions.

The recent invasion of the greater white-toothed shrew (*C. russula*) into Ireland and their negative impact on the local population of pygmy shrews (*S. minutus*) (McDevitt *et al*., 2014) provides an excellent model to study the microbiome of invasive vertebrates and their impacts. *Sorex minutus* is present across Europe but remained the only species of shrew found in Ireland for over 5000 years (McDevitt *et al*., 2011). *Crocidura russula* is a rapidly spreading species that, since crossing the Gibraltan strait from northern Africa before the last glacial maximum, has been expanding their range across Europe and has colonised several Mediterranean islands (Cosson *et al*., 2005). A small number of *C. russula* that likely originated from France established a population within Ireland around the year 2000 (Tosh *et al*., 2008; Gargan *et al*., 2016). The expansion of *C. russula* across southern Ireland has now led to the displacement of the local populations of *S. minutus* (McDevitt *et al*., 2014). Both species of shrews are insectivores and successfully co-inhabit in other regions such as the island of Belle Île, France. Considering that Ireland and Belle Île are ecologically similar islands, with similar small mammal communities, it has been puzzling why both species can co-inhabit Belle Île but not Ireland. Chapter 3 has shown that *C. russula* in Ireland are

changing their dietary preferences to smaller invertebrate species that overlaps with the *S. minutus* diet in Ireland without an alternative food source for *S. minutus* to take advantage of. In Belle Île, *S. minutus* likely relies on locally abundant prey taxa which aren't a substantial part of the *C. russula* diet, such as Araneae (spiders) and Lepidopteran (moths) larvae.

This invasion scenario gives us multiple opportunities to expand our knowledge of adapting microbiomes between species, regions and invasions. The first opportunity focuses on *C. russula*. We have sampled *C. russula* from populations that have arrived in the area from one year ago (Ireland; edge of the invasion), ~10-20 years ago (Ireland; inside the invasion), and thousands of years ago (Belle Île). As such, we can essentially compare the microbiome structure of a rapidly spreading species in a 'native' range and a newly established range. Different traits can benefit individuals from different environments (Ley *et al*., 2008a), therefore understanding the diversity and composition of microbial taxa hosted by *C. russula* at different invasion stages may provide insight into their adaptive potential (Fietz *et al*., 2018). Invading a new area can be strenuous as the animals may have to adapt when coming from an environmentally different origin. A diverse and structurally stable microbiome may be one of the *C. russula* attributes to their high dispersal abilities.

The second opportunity focuses on *S. minutus*. They have been sampled in two geographically separate, yet ecologically similar, 'native' ranges, meaning we can detect if these populations' microbiomes have diverged to adapt to local conditions over thousands of years. Studies have been performed on bats (Presley *et al*., 2020) and small terrestrial mammals (Knowles, Eccles and Baltrūnaitė, 2019) to show that while host phylogenies are strongly correlated to microbiome structure, geographic location can also have an effect. However, these studies lacked detailed dietary data to determine if differences are correlated to environmental differences or the food availability of different habitats. Here we have already characterised the diet of *S. minutus* in Belle Île and Ireland using DNA metabarcoding, which can help us differentiate if any microbiome change is diet related or due to another adaptive pressure. As these populations have been isolated for thousands of years, this gives us an idea of how variable their microbiome can be over a long time-frame. In addition, *S. minutus* has been sampled from areas in Ireland before and during the *C. russula* invasion. Resource competition, or potential introduction of novel pathogens (Nally *et al*., 2016), may have a downstream effect on the *S. minutus* microbiome composition,

thus affecting their fitness. Indeed, high microbiome diversity has shown to aid mitigation of disease infections (Antwis and Harrison, 2017), so we can identify the potential susceptibility of *S. minutus* to any emerging disease. This will allow us to determine any long time-frame divergent changes between two distant populations and short time-frame changes in microbiome in response to a newly introduced competitive predator.

The third opportunity will focus on any direct effects of *C. russula* on *S. minutus* microbiomes in Ireland. *Crocidura russula* have already been shown to introduce a novel strain of Leptospirosis into Ireland (Nally *et al*., 2016). By characterising the microbiome of this invasive shrew, we can detect any potentially pathogenic bacteria that may be residing within their guts that may be passed onto the local wildlife. If any pathogens are detected, we can determine if any such pathogen is spreading to *S. minutus* in Ireland, which could have detrimental effects to this protected species.

To characterise the microbial community structures within the guts of *C. russula* and *S. minutus* at different stages of the invasion, 16S rRNA gene metabarcoding was applied to the DNA extractions of the gut contents described in Chapter 3 (Kozich *et al*., 2013). However, this technique has potentially detected the microbiome of the prey (Knight *et al*., 2018) and so it is integral to interpret any changes of gut microbial community structures in conjunction with key results on the shrew's diet (obtained from Chapter 3). The sampling design allowed us to control for smaller scale geographic variation by using transects and seasonal variation by using two sampling periods, in addition to accounting for diet.

In summary, the research goals here are to use 16S rRNA metabarcoding to; **1)** determine how structurally different the *C. russula* microbiomes are between a long-time native range, Belle Île, and regions in Ireland invaded between 1-20 years ago. We predicted that the microbiome of the invasive population of *C. russula* is different to the Belle Île population. This could be a result of the small size of the founding population, subsequent sub sampling of the population through range expansion and stresses associated with adaptating to novel environments in Ireland; **2)** determine to what degree *S. minutus* microbiomes have diverged from each other between two long term established native ranges (Belle Île and Ireland). We expected to see differences as even small geographic distances can have an effect on the microbiome structure of small mammals (Knowles, Eccles and Baltrūnaitė, 2019); **3)** determine if *C. russula* harbour any potentially hazardous pathogens that could be introduced into the Irish

population of small mammals. The invasive population of *C. russula* harbour novel strains of the hazardous pathogen *Leptospira* (Nally *et al.*, 2016), therefore it is likely that *C. russula* also hosts hazardous bacterial species in their gastrointestinal system; **4)** determine if the recent arrival of *C. russula* has disrupted the Irish *S. minutus* microbiome. Chapter 3 shows high levels of resource competition between *C. russula* and *S. minutus* in Ireland. We predicted that this would have downstream effects on the microbiome of *S. minutus* in Ireland.

## 4.2. Methods

### 4.2.1. Molecular Work

#### DNA extraction

See **Section 3.2.1**. for details on sample collection. Gut tracts of samples were opened and the gut contents were scraped out of the full intestinal tract and stomach using sterile equipment and fresh disposable work bench covers for each sample. DNA was extracted from full gut contents using the Qiagen Power Soil Kit, including five extraction blanks. Full DNA extraction details can be found in **Section 3.2.2.**

#### PCR

To identify bacteria, the primers F515 and R806 were used to target V4 region of the 16S rRNA gene according to (Kozich *et al*., 2013) and (Antwis *et al*., 2018). This consisted of primers already containing the adapter sequence required for Illumina sequencing platforms, and dual-index multiplex identifiers (MID tags) that are 8bp in length. This primer set contains 24 unique i7 indexes and 16 unique i5 indexes that are arranged in different combinations to allow the multiplexing of more than 384 samples onto a single sequencing run.

Samples were randomly distributed amongst four PCR plates, randomising host species, season, country, zone and transects to mitigate artificial inflation of inter-species/samples effects. Each plate included a PCR blank along with extraction blanks. A bacterial mock community was also included. This was the microbial mock community B (HM-783D) from BEI Resources (Human Microbiome project) that contained 20 bacterial strains.

The PCR reaction composition contained 3 µl Solis BioDyne 5x HOT FIREPol® Blend PCR Mastermix, 6 µl of molecular grade water, 1.5 µl of each primer (at 2 µm), and 3 µl of DNA template. PCR conditions included an initial denaturation at 95˚C for 15 minutes to activate the enzymes, followed by 25 cycles of 95˚C for 20 seconds, 55˚C for 60 seconds and 72˚C for 60 seconds, then a final extension at 72˚C for 10 minutes. All PCRs were performed in duplicate. Success of reactions were determined by checking a random subset of PCR samples on the Agilent 2200 Tape Station using High Sensitivity screen tapes.

### Bead Clean and Sequencing

PCR products of sample duplicates were combined. Each sample was purified using MAGBio HighPrep™ PCR Clean-up System beads to remove unwanted primer dimer and PCR reaction reagents. A left side bead clean was performed using a 1.5x bead to sample ratio to remove fragments smaller than 400bp.

To accurately quantify the amount of reads per 1 µl of each sample, a smaller sequencing run (referred to hereafter as a titration run) was performed (Kozich *et al*., 2013). 1µl of each sample was pooled into a single sequencing library. This library was quality assessed on the tape station using high sensitivity screen tapes and quantified using the QubitTM 3.0 Fluorometer high sensitivity kit according to the manufacturers protocol. The library was then sequenced on the MiSeq using a V2 300 cycle nano kit. The library was loaded at 4pM with a 10% PhiX spike.

The number of reads returned for each sample in this titration sequencing run was used to determine the volume of each sample to pool at an equal concentration for the full sequencing run. This was calculated by the following:

$$Volume\ of\ sample\ for\ library\ pool = \frac{(\%\ reads\ identified\ /\ Number\ of\ samples\ loaded)}{\%\ reads\ identified\ for\ sample}$$

The final normalised library was quality assessed on the Tapestation as previously described. It was then quantified using the NEBio library quantification kit for Illumina (i.e. quantitative PCR). To retrieve sufficient sequencing depth of samples, the library was sequenced twice on V2 500 cycle MiSeq kits. Libraries were loaded at 9pM with a 10% PhiX spike.

## 4.2.2. Data Analysis

### Bioinformatic Processing

Raw sequences were processed in R using the DADA2 v1.4 pipeline (Callahan *et al.*, 2016). Sequence quality was assessed, and forward reads were subsequently truncated to 240bp and reverse reads were truncated to 200bp based of the quality of a random subset of 10 samples. Reads were filtered using default settings but with the

maximum number of expected errors allowed in a read set to 2. Forward read error rates were determined from a subset of 423,037 reads from 54 samples, while the reverse error rates were determined from a subset of 505,678 reads from 67 samples. Samples were then dereplicated using these error rates, and subsequently identified into amplicon sequence variants (ASVs). Forward and reverse reads were then merged, and putative chimeras were removed. Sequences that were outside the expected size range (240bp to 260bp) were removed. ASVs were then assigned taxonomy using the Silva reference database (Quast *et al.*, 2013). A phyloseq object (McMurdie and Holmes, 2013) was created for further analyses. Samples from runs one and two were included together in the DADA2 pipeline and then merged by summing the read counts using phyloseq.

The mock community was assessed to make sure all 20 species/strains were detected during sequencing.

### Sample and ASV Filtering

ASVs with more than 0.1% of total reads in negative controls were removed. Non-bacteria were also removed. Samples with less than 9985 reads were removed from further analyses, determined by rarefaction curves created using the *vegan* (version 2.5-6) package in R (Oksanen *et al*., 2019).

### Alpha Diversity

Samples were rarefied to the lowest sampling depth (9985 reads) before alpha diversity measures were taken. The observed species richness and inverse Simpson index were calculated for each rarefied sample. To account for any stochastic results from rarefying samples (McMurdie and Holmes, 2014), each sample was rarefied and the diversity measurement was conducted using 100 permutations and the average diversity was taken for each sample across these. Differences in alpha diversity according to species, season and zone were tested in R using the non-parametric Kruskal-Wallis test and Dunn's post hoc test using the Benjamini-Hochberg method to correct for multiple testing (Lavrinienko *et al.*, 2018). Spearman rank correlations were calculated between the weight (grams) of shrews and each alpha diversity measure using the *cor.test()* function in R.

Samples were also merged according to species, zone and season by summing reads and then rarefying to the lowest read count. The observed species richness, inverse Simpson index, Shannon index and Pielou's eveness was calculated for each group.

**Beta Diversity**

Extra filtering criteria were set to look at beta diversity between samples. Only core ASVs with more than one read in at least 5% of individuals were kept (Knowles, Eccles and Baltrūnaitė, 2019). The phylogenetic relationship between ASVs was created by constructing a neighbour-joining tree, then fitting a GTR+G+I (Generalised time-reversible with Gamma rate variation) maximum likelihood tree using the neighbour-joining tree as the starting point (Callahan *et al*., 2019).

Due to the compositional nature of amplicon sequencing data, there are still many conflictions on the best normalisation method to accurately interpret beta diversity measures of microbiome data (Gloor *et al*., 2017). Therefore, two methods were applied for comparison. Firstly, samples were normalised using the centred log ratio transformation (clr-transformation) method (Gloor *et al.*, 2017). This transforms the read counts into ratios, or dominance, for each taxon relative to the geometric mean of all taxa. All distance matrices were created for the dataset using the Euclidean dissimilarity method. The Euclidean dissimilarity method of clr-transformed data is also known as the Aitchison distance method. The second method employed was rarefying the samples to the same number of reads as the sample with the lowest sequencing depth, and creating distance matrices using the weighted unifrac (w-unifrac) method (Weiss *et al*., 2017). This method takes into account ASV abundance and phylogenetic information, which reflect functional differences in the microbiome rather than taxonomic differences alone (Stothart, Palme and Newman, 2019).

To determine the compositional difference in bacteria identified between different experimental groups, PERMANOVA's were performed using the *adonis2()* function in the *vegan* package in R. The PERMANOVA examines whether the centroid of one group significantly differs from the centroid of other groups based on the variation or spread of the data. The explanatory variables were species of shrew, country, season, invasion zone, transect and trap site. The *adonis2()* function treats variables sequentially (i.e. the effect of one variable will rely on the variable stated before it). Therefore, the analysis was performed multiple times using variables in different orders

to define the groups. To make sure any difference in composition were not due to the dispersal of samples, the *betadisperser()* function was used to test the levels of homogeneity among samples.

NMDS plots were used to visualise any compositional differences between each species of shrew, their country of origin and which invasion zone they were sampled. The number of dimensions (K) was altered to keep plot stress levels close to 0.1. A NMDS was used to directly compare the two distance methods, but a PCA is more typically used for clr-transformed data because they require Euclidean distance methods (Gloor *et al*., 2017). A PCA was also used to visualise compositional differences between the same groups using the clr-transformed data.

**Random Forest Classifier**

The random forest classification (RFC) is a supervised learning method that classifies samples (such as prey composition) to their source, estimates the level of importance of each prey item to that classification and determines the accuracy of that classification (Knights, Costello and Knight, 2011). Here, RFC models were run to determine if samples could be accurately classified to shrew species, country of origin, season of sampling and invasion zone in Ireland based on the microbiome composition in the guts.

RFCs were performed on samples using the *randomForest* R package (Liaw and Wiener, 2002) using 10,000 trees. The out-of-bag (OOB) error was used to measure the accuracy of classification of samples to their correct group. The most important microbial taxa contributing to classification of samples were established using the 'Mean Decrease Mini' values.

**Differential Abundance**

Differential abundance measures were taken using the ANOVA-like differential expression (ALDEx2) analysis (Gloor, Macklaim and Fernandes, 2016). This method generates 128 posterior probabilities of observing each taxon by Monte-Carlo sampling from a Dirichlet distribution. The data is then clr-transformed and the Wilcoxin test is applied to test the simulated instance of each taxa. The effect size is the proportion difference between sample types divided by the maximum difference within

sample types averaging over all taxa instances. An expected p-value is calculated for each taxon by averaging over all taxa observation/proportions and applying the Benjamini-Hochberg correction.

The differential abundance was measured with taxa agglomerated to species level. This was assessed for the host species separately based on country of origin and invasion zone. Taxa were determined as differentially abundant if they had a p-value below 0.05 and an effect size appropriate for the sample size (in this case, and effect size of ~0.5) (Xia, Sun and Chen, 2018; Fernandes *et al*., 2019).

**Diet vs Microbiome Distance Measure**

The distance matrix for 16S dataset was done by clr-transformation and Euclidean distance. The filtered dataset with COI diet information was imported from Chapter 3. Two beta diversity distance matrices for COI (diet) were generated. The first matrix used taxa agglomerated to order level, transformed to RRA and used the Bray-Curtis distance method. The second matrix used taxa agglomerated to order level, raw read count with the Jaccard distance method. A geographic distance matrix was constructed between radiation distance and samples using longitude and latitude coordinates in Microsoft Excel (Antwis *et al*., 2020). Partial Mantel tests were used to determine if there was a correlation between beta diversity in microbiome and the diet of shrews using 10,000 iterations and Spearman's rank correlation between diet distance and microbiome distance. The geographic distance matrix was used as a covariate to mitigate effects of sampling location.

## 4.3. Results

### Sequencing

The two MiSeq runs generated a total of 11,792,270 reads. After filtering, a total of 10,389,696 reads were retained. See **Table 4.1** for a breakdown of sequences retained from each run through the dada2 filtering steps.

All 20 species from the mock community were detected. A total of 15,902 ASVs were identified before filtering ASVs and samples. 32 ASVs were detected in blanks, 22 of which were removed (i.e. those ASVs had >0.1% of the total reads found in the blanks). A total of 279 samples remained after removing samples with low sequencing depth and a breakdown of those samples can be seen in **Table 4.2**.

**Table 4.1.** The number of sequences from each MiSeq run retained through the filtering steps applied in the dada2 pipeline.

| | *Input* | *Filtered* | *Denoised* | *Merged* | *Non-chimera* |
|---|---|---|---|---|---|
| Run 1 | 3207293 | 3098991 | 3026781 | 2911347 | 2872199 |
| Run 2 | 8584977 | 7960520 | 7868673 | 7623341 | 7517497 |
| Total | 11792270 | 11059511 | 10895454 | 10534688 | 10389696 |

**Table 4.2.** Breakdown of samples retained after quality control and filtering. Values in parentheses represent the number of samples retained after using the extra filtering criteria for beta diversity analyses.

| *Season* | *Invasion Zone* | *Species* | *Sample Size* | | | |
|---|---|---|---|---|---|---|
| | | | West | South | East | **Total** |
| Summer | Inside | *C. russula* | 8(7) | 9 | 9 | **26(25)** |
| | | *S. minutus* | - | - | - | **0** |
| | Edge | *C. russula* | 9 | 12(11) | 6 | **27(26)** |
| | | *S. minutus* | 8 | 7 | 10 | **25** |
| | Outside | *C. russula* | - | - | - | **0** |
| | | *S. minutus* | 9 | 10 | 10 | **29** |
| | Belle Île | *C. russula* | NA | NA | NA | **20(19)** |
| | | *S. minutus* | NA | NA | NA | **20(19)** |
| Winter | Inside | *C. russula* | 10 | 9 | 8(6) | **27(25)** |
| | | *S. minutus* | - | - | - | **0** |
| | Edge | *C. russula* | 9 | 9 | 6 | **24** |
| | | *S. minutus* | 9 | 7 | 6 | **22** |
| | Outside | *C. russula* | - | - | - | **0** |
| | | *S. minutus* | 5 | 10 | 7 | **22** |
| | Belle Île | *C. russula* | NA | NA | NA | **17** |
| | | *S. minutus* | NA | NA | NA | **20(19)** |
| Total | | *C. russula* | 36(35) | 39(38) | 29(27) | **141(136)** |
| | | *S. minutus* | 31 | 34 | 33 | **138(136)** |

## Alpha Diversity and Composition

Alpha diversity measures were taken using 14,708 ASVs. For diversity measured within individual shrews, the Kruskal-Wallis test showed a significant difference in species richness (effect size = 0.32, p-value = 0.000), Shannon diversity (effect size = 0.24, p-value = 0.000), Inverse Simpson (effect size = 0.19, p-value = 0.000) and Pielou's Eveness (effect size = 0.19, p-value = 0.000) between shrews grouped according to species, invasion zone and season. Diversity measures were higher in *C. russula* compared to *S. minutus* (**Figure 4.1**), but Dunn's post-hoc tests have only shown significant differences between certain groups (**Tables S4.1 - S4.4**). The Dunn's post-hoc test showed no significant difference in diversity between groups within a shrew species, even across zones or seasons. All measures were significantly higher in *C. russula* from Belle Île (CR-Bel) during the winter than *S. minutus* in the outside zone during the summer. Although not significant, the mean diversity values in *S.*

*minutus* samples are notably higher during the winter than the summer (**Figure 4.1; Tables S4.1 – S4.4**).

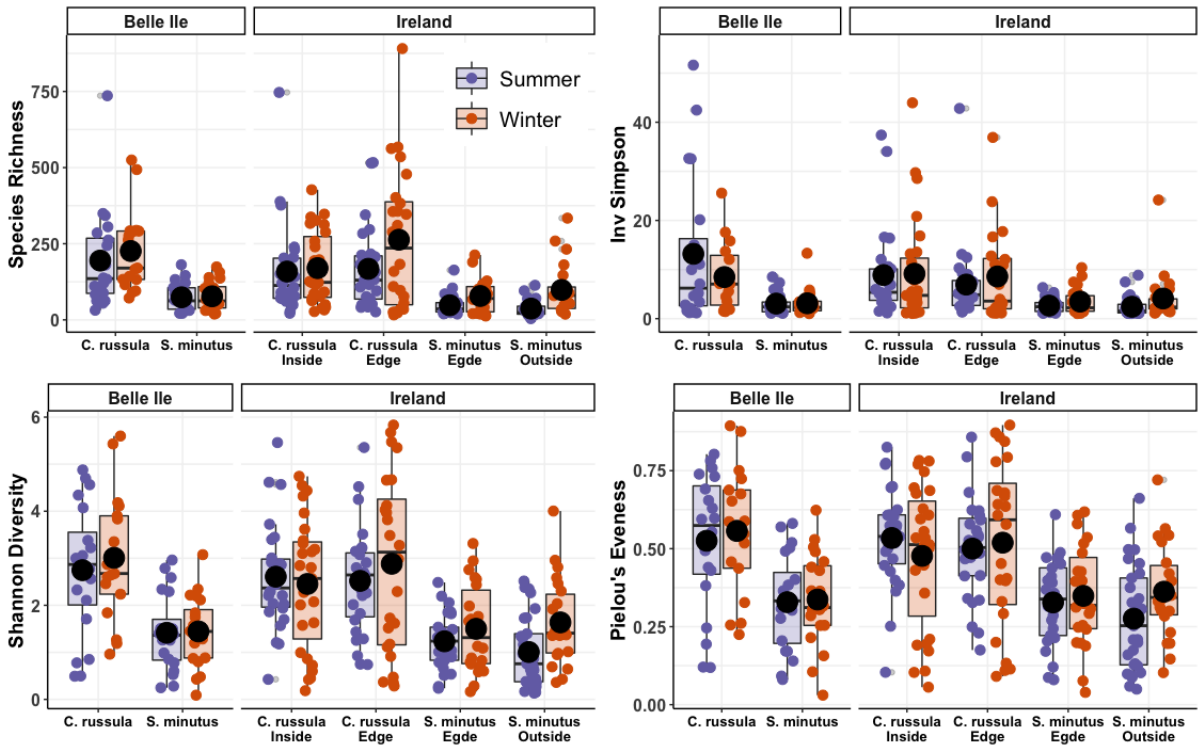



**Figure 4.1.** Box and jitterplot of alpha diversity measures for individual shrews grouped by species, zone and season. Large black circles are the mean value. Top left = observed species richness; top right = Inverse Simpson; bottom left = Shannon Diversity; bottom right = Pielou's eveness.

After merging samples according to shrew, zone and season and rarefying to the lowest read depth (415,513 reads), *C. russula* still has higher diversity measures than *S. minutus* with up to 3,189 more ASVs detected in *C. russula* than *S. minutus* (**Table 4.3**). *Crocidura russula* remain similar in terms of species richness at different zones, however there was a large increase of ~1,000 species from summer to winter in samples from the edge. In addition, the inverse Simpson (which takes into account abundance of taxa, not just presence/absence) value decreased from 29.60 to 11.31. The Shannon diversity and Pielou's metric remain similar, however. CR-Bel sampled during the summer has the highest diversity values for Inverse Simpson, Shannon diversity and Pielou's eveness.

*S. minutus* diversity remains similar for each season in Belle Île, with slightly higher values from the Inverse Simpson, Shannon diversity and Pielou's eveness in the summer. In Ireland, however, the microbiome diversity of *S. minutus* increased in observed species richness and all diversity measures during the winter in the outside zone. In contrast, at the edge of the invasion, the observed species richness increased in the winter, but the diversity measures all decreased (**Table 4.3**)

**Table 4.3.** Alpha diversity measures for shrews that have been merged according to sampling zone and season.

| Shrew | Zone | Season | Sample Size | Observed Richness | Inverse Simpson | Shannon Diversity | Pielou's Eveness |
|---|---|---|---|---|---|---|---|
| *C. russula* | Belle Île | Summer | 20 | 2792 | 51.565489 | 5.034744 | 0.6345372 |
| | | Winter | 17 | 2343 | 23.855883 | 4.464358 | 0.5753641 |
| | Inside | Summer | 26 | 2454 | 32.00538 | 4.495866 | 0.5759888 |
| | | Winter | 27 | 2612 | 20.2117 | 4.272531 | 0.5430352 |
| | Edge | Summer | 27 | 2744 | 29.63494 | 4.386869 | 0.5540954 |
| | | Winter | 24 | 3753 | 11.314713 | 4.434383 | 0.5387868 |
| *S. minutus* | Belle Île | Summer | 20 | 990 | 4.122169 | 2.611467 | 0.3785994 |
| | | Winter | 20 | 1044 | 3.357007 | 2.401414 | 0.3454866 |
| | Outside | Summer | 29 | 564 | 6.685118 | 2.576584 | 0.4067185 |
| | | Winter | 22 | 1639 | 12.88233 | 3.370845 | 0.4554062 |
| | Edge | Summer | 25 | 700 | 6.274398 | 2.572469 | 0.3926786 |
| | | Winter | 22 | 1070 | 2.580421 | 2.0822 | 0.2985056 |

There was no correlation between the weight of *S. minutus* and alpha diversity of bacteria in the gut (plots not shown). However, there were weak but significant correlations between weight of *C. russula* samples and species richness (rho = 0.3, p-value < 0.001), Shannon diversity (rho = 0.21, p-value = 0.01) and Pielou's eveness (rho = 0.17, p-value = 0.04) (**Figure 4.2**).

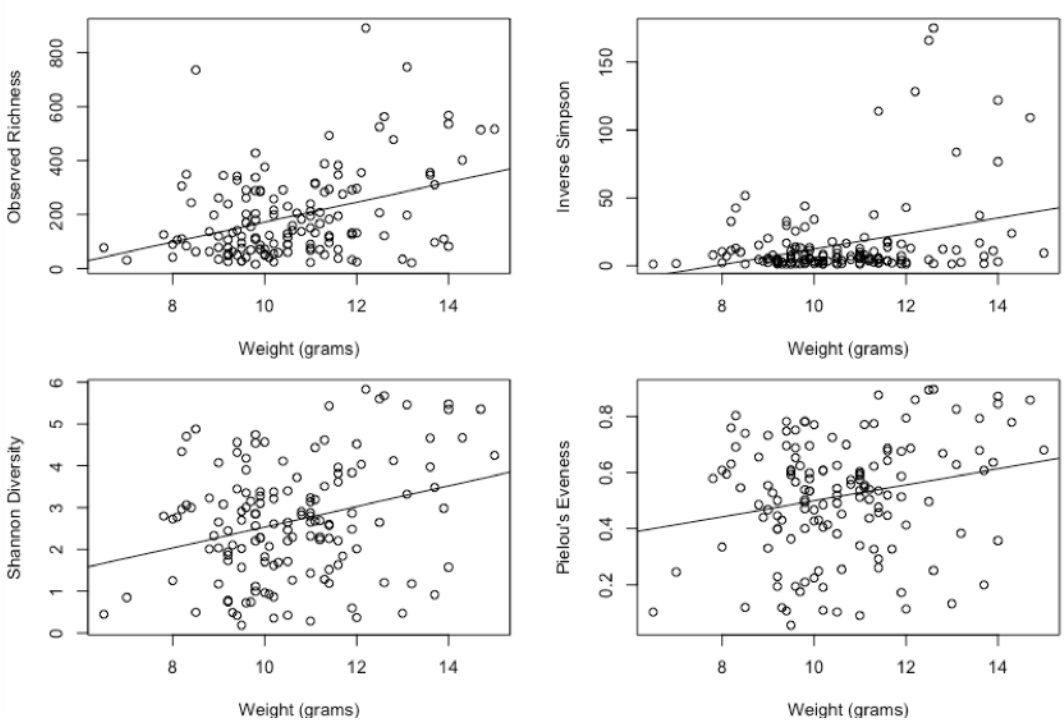



**Figure 4.2.** Spearman rank correlations between the weight of *C. russula* samples (in grams) and species richness (top left; rho = 0.3, p-value < 0.001), Inverse Simpson (top right; rho = 0.15, p-value = 0.07), Shannon diversity (bottom left; rho = 0.21, p-value = 0.01) and Pielou's eveness (bottom right; rho = 0.17, p-value = 0.04) of gut microbiomes.

The majority of the microbiome was made up of Firmicutes (RRA was 11.4% and 27% in *C. russula* and *S. minutus* respectively), Proteobacteria (RRA was 43.6% and 41.8% in *C. russula* and *S. minutus* respectively), Actinobacteria (RRA was 11.1% and 1% in *C. russula* and *S. minutus* respectively), Chlamidiae (RRA was 1.8% and 7.9% in *C. russula* and *S. minutus* respectively) and Tenericutes (RRA was 20.5% and 6.9% in *C. russula* and *S. minutus* respectively) (see **Figure 4.3**). The most diverse of these phyla were Actinobacteria and Proteobacteria.

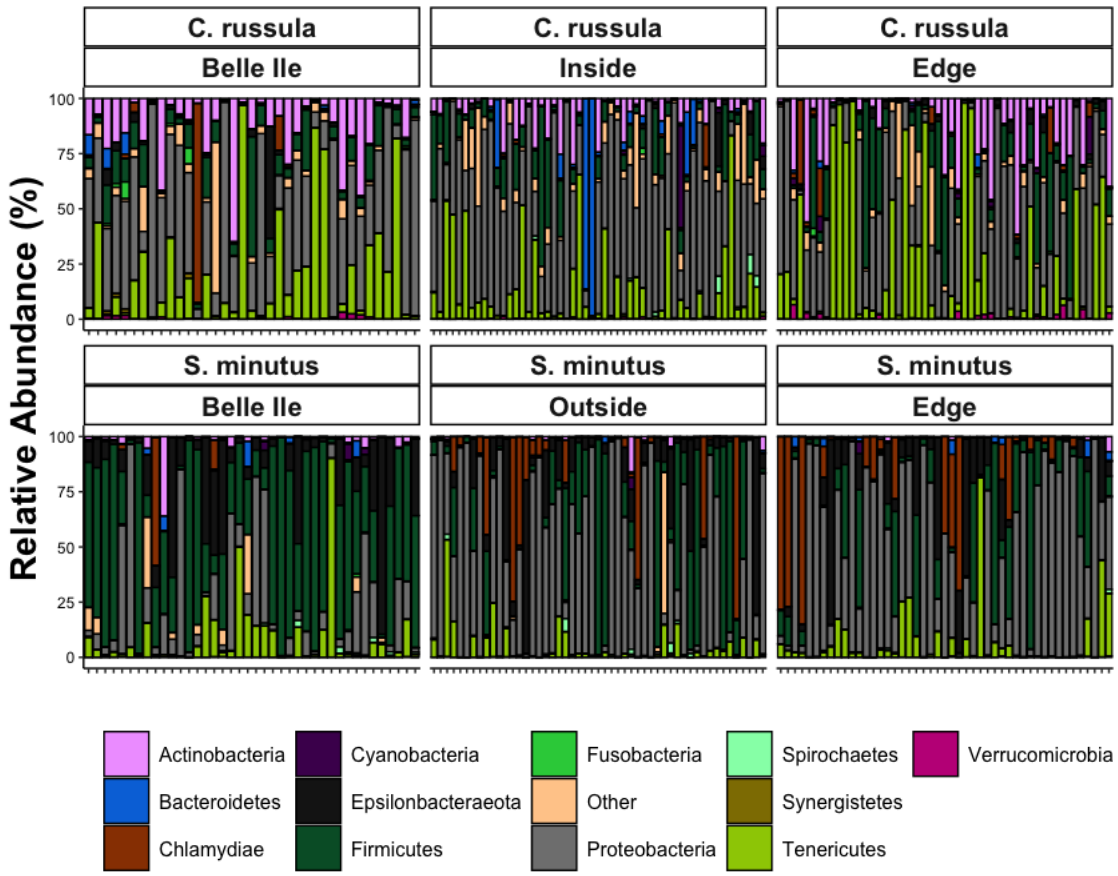



**Figure 4.3.** Proportion of reads belonging to the 12 most abundant phyla in all individual *S. minutus* and *C. russula* samples. Samples are grouped according to species and invasion zone.

## Beta Diversity

The beta diversity filter retained 469 ASVs, with an additional five *C. russula* and two *S. minutus* samples removed because their read count dropped below 7,500 reads due to hosting an abundance of outlier bacterial species.

PERMANOVAs using both Euclidean and w-unifrac methods showed a compositional difference between species of shrews, country and the interaction between shrew and country (all p values < 0.001). Although the Euclidean distance method showed shrew species to have a larger effect ($R^2$ = 0.08) than country ($R^2$ = 0.02), w-unifrac showed country of origin to explain more of the variation ($R^2$ = 0.20) compared to shrew species ($R^2$ = 0.02) (see **Table 4.4**)

When examining each species of shrew separately, all variables measured (season, zone, transect and trap site) showed a significant compositional difference except trap site for *C. russula* in Ireland (CR-Ire), and *S. minutus* in both Ireland and Belle Île when

using Euclidean distances. The w-unifrac method showed that *S. minutus* is compositionally different between Belle Île and Ireland ($R^2 = 0.10$, $p < 0.001$), but not for *C. russula*. Season may affect microbiome composition of CR-Bel ($R^2 = 0.05$, $p < 0.003$). In Ireland, the w-unifrac method shows that season and zone affect *C. russula*, ($R^2 = 0.05$, $p < 0.001$ and $R^2 = 0.05$, $p < 0.001$, respectively) while season, transect and trap site had a significant effect on *S. minutus* microbiomes ($R^2 = 0.03$, $p < 0.020$; $R^2 = 0.04$, $p < 0.050$ and $R^2 = 0.25$, $p < 0.030$, respectively).

The only variables considered significantly different by both normalisation and distance methods was species of shrew, country for *S. minutus* only, season for all *C. russula* samples and Irish *S. minutus* samples, invasion zone for *C. russula* and transect for *S. minutus*. See **Table 4.4** for full PERMANOVA results.

When using the Euclidean distance method to measure the dispersion of samples, there were no significant differences (permutest, F = 0.874, p-value = 0.474) in the homogeneity of samples between groups of *C. russula* samples (grouped according to zone and season). Although not significant, the rates of dispersion were still higher in winter samples compared to summer samples. There was a significant difference between the homogeneity of *S. minutus* samples (permutest, F = 14.95, p-value = 0.001). There were higher levels of dispersion of *S. minutus* samples (pairwise permutest, p-value = 0.338) at the invasion edge during the winter (mean distance to centroid = 31.86, SD = 9.68) than the summer (mean distance to centroid = 26.65, SD = 6.50). There were lower levels of dispersion in summer *S. minutus* samples from the outside zone (mean distance to centroid = 24.56, SD = 7.82) compared to *S. minutus* from Belle Île during the winter (mean distance to centroid = 31.24, SD = 9.46, pairwise permutest p-value = 0.010), *S. minutus* from Belle Ile during the summer mean distance to centroid = 30.85, SD = 8.44, pairwise permutest p-value = 0.011), *S. minutus* from the outside zone during the winter (mean distance to centroid = 33.15, SD = 9.84, pairwise permutest p-value = 0.001) and *S. minutus* from the edge zone during the winter (mean distance to centroid = 31.86, SD = 9.68, pairwise permutest p-value = 0.005). There is an observable increase in dispersion during the winter for almost all species/zone combinations (**Figure 4.4**)

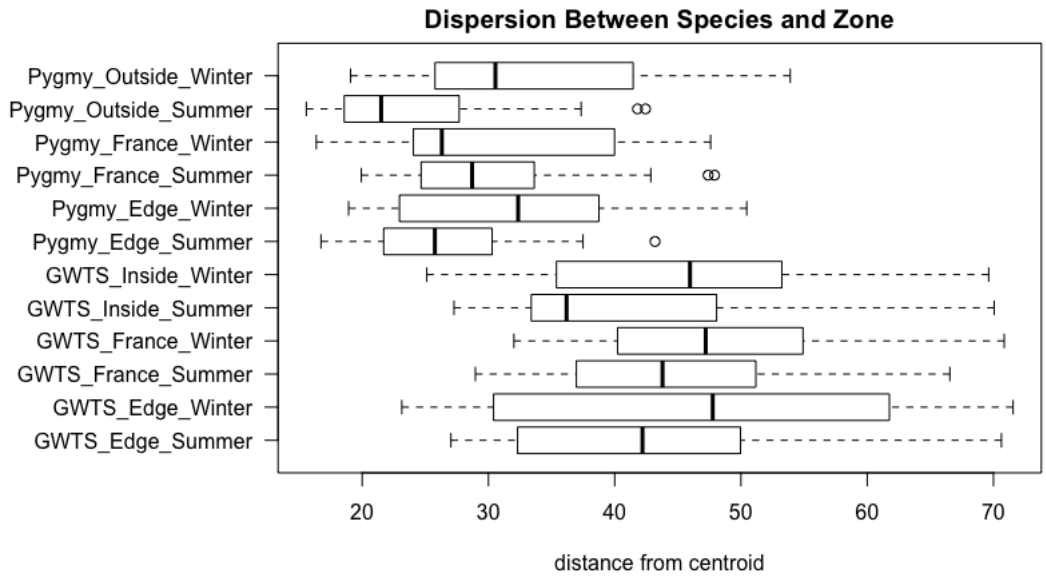



**Figure 4.4.** Levels of homogeneity between shrews grouped according to species, zone and season when using the clr transformed data and Euclidean distance method. Pygmy = *S. minutus*; GWTS = *C. russula.*

When using the weighted-unifrac distance method to measure the dispersion of samples, there were no significant differences (permutest, F = 0.955, p-value = 0.443) in the homogeneity of samples between groups of *C. russula* (grouped according to zone and season). There is a significant difference in homogeneity between groups (zone and sampling season) of *S. minutus* samples (permutest, F = 3.574, p-value = 0.007). There is higher dispersion of *S. minutus* samples in the outside zone during the summer (mean distance to centroid = 0.26, SD = 0.39) compared to *S. minutus* samples from Belle Île during the winter (mean distance to centroid = 0.17, SD = 0.13, pairwise permutest p-value = 0.001; **Figure 4.5**).

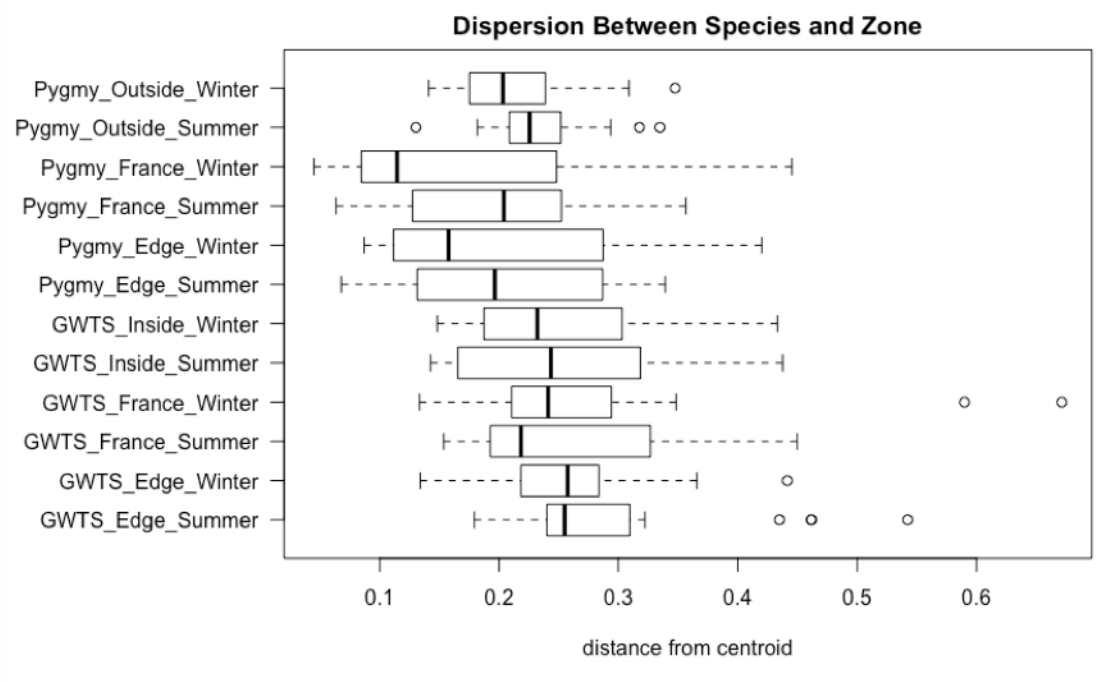



**Figure 4.5.** Levels of homogeneity between shrews grouped according to species, zone and season when using the rarefied and weighted-unifrac distance method. Pygmy = *S. minutus*; GWTS = *C. russula.*

These differences in homogeneity will possibly influence the differences found in the PERMANOVAs. Visualising the differences using NMDS (**Figure 4.6**) and PCA (**Figure S4.2**) show that *S. minutus* sample cluster closer together compared to *C. russula* overall, with some overlap (particularly when using the Euclidean distance). Slight shifts in the composition between countries for each species of shrew is more evident using Euclidean distances. The shift in composition between zones for *C. russula* is also more noticeable in NMDS plots using Euclidean distance methods.

**Table 4.4.** Results from each PERMANOVA analysis. Values are shown for both the clr-transformed data using Euclidean distance metric and the rarefied dataset using the w-unifrac distance metric. Values in bold are p-values <0.05.

| | | | | CLR Transformed | | | Rarified | | |
|---|---|---|---|---|---|---|---|---|---|
| **Country** | **Species** | **Variable** | **Df** | **R2** | **F** | **Pr(>F)** | **R2** | **F** | **Pr(>F)** |
| Both | Both | Country | 1 | 0.0222 | 6.7026 | **0.0001** | 0.1965 | 69.4731 | **0.0001** |
| | | Shrew | 1 | 0.0788 | 23.7704 | **0.0001** | 0.0241 | 8.5175 | **0.0001** |
| | | Country x shrew | 1 | 0.0107 | 3.2259 | **0.0001** | 0.0213 | 7.5478 | **0.0001** |
| | *C.russula* | Country | 1 | 0.0345 | 4.8968 | **0.0001** | 0.0121 | 1.6953 | 0.1254 |
| | *S.minutus* | Country | 1 | 0.0406 | 5.8178 | **0.0001** | 0.1038 | 15.6676 | **0.0001** |
| Belle Île | *C.russula* | Season | 1 | 0.0449 | 1.7105 | **0.0060** | 0.0487 | 1.7903 | **0.0028** |
| | | Trap Site | 8 | 0.2724 | 1.2967 | **0.0136** | 0.2383 | 1.0944 | 0.1473 |
| | *S.minutus* | Season | 1 | 0.0385 | 1.3785 | **0.0234** | 0.0208 | 0.7169 | 0.6305 |
| | | Trap Site | 11 | 0.2629 | 0.8551 | 0.8723 | 0.2331 | 0.7289 | 0.8844 |
| Ireland | *C.russula* | Season | 1 | 0.0336 | 3.6187 | **0.0001** | 0.0450 | 4.8296 | **0.0006** |
| | | Zone | 1 | 0.0353 | 3.8015 | **0.0001** | 0.0517 | 5.5478 | **0.0003** |
| | | Transect | 2 | 0.0314 | 1.6920 | **0.0005** | 0.0228 | 1.2252 | 0.2630 |
| | | Trapsite | 15 | 0.1567 | 1.1252 | 0.0641 | 0.1356 | 0.9706 | 0.5398 |
| | *S.minutus* | Season | 1 | 0.0414 | 4.3280 | **0.0001** | 0.0323 | 3.5167 | **0.0103** |
| | | Zone | 1 | 0.0348 | 1.8161 | **0.0001** | 0.0096 | 1.0475 | 0.3619 |
| | | Transect | 2 | 0.0124 | 1.2998 | **0.0499** | 0.0362 | 1.9676 | **0.0464** |
| | | Trap site | 20 | 0.2123 | 1.1082 | 0.1410 | 0.2509 | 1.3647 | **0.0201** |

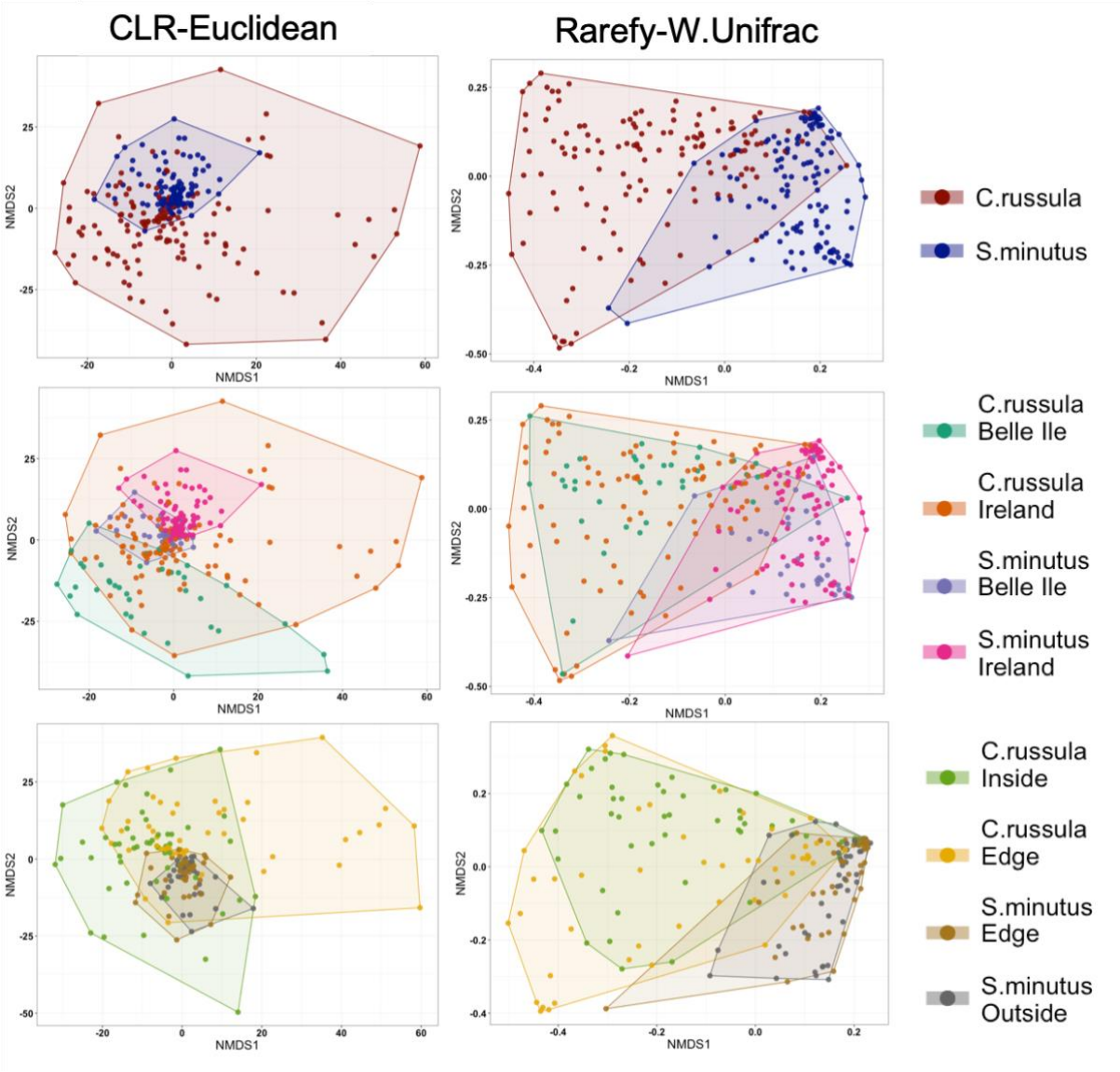



**Figure 4.6.** NMDS plots using clr-transformed data with Euclidean distance measures (left column) and rarefied with weighted-unifrac measures (right column). Top plot shows samples coloured according to species of shrew. Middle plots are the same plots coloured according to both species of shrew and country of origin. Bottom plots are only Irish samples coloured according to species of shrew and invasion zone.

### Random Forest Classifier

The random forest classifier algorithm identified samples from each shrew species with an error rate of 1.43%. The top 20 taxa with the strongest 'Mean Decrease Mini' values (i.e. the taxa contributing the most to being able to classify the samples) comprised of ASVs from the families Clostridiaceae (1/20), Mycoplasmataceae (9/20), Helicobacteraceae (2/20), Brevinemataceae (2/20), Rhodobacteraceae (1/20), the phylum Proteobacteria (4/20) and unknown bacteria (1/20).

Samples were classified to country with an error of 8.96% with the top ASVs coming from Diplorickettsiaceae, Campylobacteraceae, Beijerinckiaceae and Mycoplasmataceae. Samples were classified to the correct shrew species and country of origin with an error rate of 14.70% with the top ASVs coming from Clostridiaceae, Mycoplasmataceae, Diplorickettsiaceae and unknown bacteria.

Classifying *C. russula* and *S. minutus* samples to the correct season (within a country of origin) had an error rate of 44.09%. The most ambiguous samples were those from Belle Île, as the error rates for classifying those shrews into the correct season were 75-95%.

Classifying *S. minutus* in Ireland (SM-Ire) according to the invasion zone had an out of bag error rate of 60.2% while classifying CR-Ire according to the invasion zone had an out of bag error rate of 18.27%. The top taxa with the strongest 'Mean Decrease Mini' values (i.e. the taxa contributing the most to being able to classify the samples) comprised of the families Brevinemataceae, Aeromonadaceae, Demequinaceae and an unknown bacterial strain.

**Differential Abundance**

Taxa belonging to the families Diplorickettsiaceae, Simkaniaceae and Rhizobiaceae were determined to be significantly more abundant in SM-Ire samples compared to *S. minutus* in Belle Île (SM-Bel), while taxa belonging to the families Clostridiaceae and Moraxellaceae were determined to be more abundant (**Table 4.5** and **Figure 4.7**). The effect sizes estimated for these taxa ranged from -0.42 and 0.64, which are acceptable values to imply likely biological significance from a sample size this large (N = 98) (Fernandes *et al*., 2019). No taxa were identified as differentially abundant between *S. minutus* samples in each invasion zone.

Taxa belonging to the families Pasteurellaceae, Aeromonadaceae and Bacillaceae were returned significant p-values (<0.05) for higher abundance in CR-Ire compared to CR-Bel. However, the effect sizes of these taxa were 0.39 – 0.41 which can be considered low but may be low due to the larger sample size (N = 141) and are thus still noteworthy here. A larger diversity of families was identified to be higher in abundance in CR-Bel samples (**Table 4.5**). Only three taxa were found to be differentially abundant between *C. russula* samples at different invasion zones. Of these three taxa, the Brevinemataceae family (higher at the inside zone) and the

Demequinaceae family (higher in the edge zone) both had corrected p-values before 0.05 for both tests (Wilcoxin and Welch), and effect sizes exceeding the 0.5 cut-off.

Season was not considered for differential abundance testing since season did not give strong results in the random forest classifier analysis.

**Table 4.5.** Taxa that are differentially more abundant between populations of shrews, tested in ALDEX2 using the Welch and Wilcoxin test corrected and uncorrected p-values. For example, *Rickettsiella isopodorum* is significantly more abundant in *S. minutus* from Ireland compared to Belle Ile. Some species of bacteria could only be taxonomically assigned to genus or family level.

| Shrew Species | Group | Family | Species | Effect Size | P-value (Welch) | P-value corrected (Welch) | P-value (Wilcoxin) | P-value corrected (Wilcoxin) |
|---|---|---|---|---|---|---|---|---|
| ***S. minutus*** | **Ireland** | Diplorickettsiaceae | Rickettsiella isopodorum | 0.6398851 | 0.00000 | 0.00006 | 0.00000 | 0.00003 |
| | | Simkaniaceae | Genus_Candidatus_Rhabdochlamydia | 0.6215813 | 0.00000 | 0.00014 | 0.00000 | 0.00033 |
| | | Rhizobiaceae | Family_Rhizobiaceae | 0.5818209 | 0.00002 | 0.00115 | 0.00002 | 0.00137 |
| | **Belle Île** | Clostridiaceae_1 | Genus_Candidatus_Arthromitus | -0.4207868 | 0.00006 | 0.00254 | 0.00007 | 0.00402 |
| | | Moraxellaceae | Genus_Psychrobacter | -0.4941578 | 0.00079 | 0.01623 | 0.00013 | 0.00611 |
| ***C. russula*** | **Ireland** | Pasteurellaceae | Genus_Bibersteinia | 0.3909233 | 0.00273 | 0.03304 | 0.00366 | 0.03497 |
| | | Aeromonadaceae | Genus_Aeromonas | 0.4150177 | 0.00068 | 0.01299 | 0.00328 | 0.03223 |
| | | Bacillaceae | Family_Bacillaceae | 0.4187779 | 0.00516 | 0.05474 | 0.00258 | 0.03080 |
| | **Belle Île** | Mycoplasmataceae | Family_Mycoplasmataceae | -0.5483197 | 0.00067 | 0.01250 | 0.00066 | 0.01124 |
| | | Beijerinckiaceae | Genus_Microvirga | -0.5006978 | 0.00208 | 0.02394 | 0.00036 | 0.00820 |
| | | Mycoplasmataceae | Genus_Mycoplasma | -0.4974401 | 0.00011 | 0.00196 | 0.00011 | 0.00538 |
| | | Micrococcales_Ince | Genus_Luteimicrobium | -0.4699871 | 0.00559 | 0.03630 | 0.00159 | 0.01718 |
| | | Beijerinckiaceae | Genus_Methylobacterium | -0.458008 | 0.00248 | 0.02547 | 0.00015 | 0.00531 |
| | | Staphylococcaceae | Genus_Jeotgalicoccus | -0.4498898 | 0.00007 | 0.00260 | 0.00047 | 0.01143 |
| | | Nocardioidaceae | Genus_Marmoricola | -0.4497109 | 0.01034 | 0.07100 | 0.00236 | 0.02545 |
| | | Microbacteriaceae | Genus_Curtobacterium | -0.4079903 | 0.00818 | 0.06141 | 0.00225 | 0.02780 |
| | | Rhizobiaceae | Genus_Aminobacter | -0.4031223 | 0.00919 | 0.05829 | 0.00084 | 0.01485 |
| | | Cellulomonadaceae | Genus_Cellulomonas | -0.3971716 | 0.00229 | 0.02626 | 0.00003 | 0.00261 |
| | | Micrococcaceae | Genus_Glutamicibacter | -0.3865917 | 0.00765 | 0.06575 | 0.00244 | 0.03181 |
| | | Rhizobiaceae | Genus_Allorhizobium/ Neorhizobium/ Pararhizobium/ Rhizobium | -0.2482077 | 0.03995 | 0.17535 | 0.00336 | 0.03828 |
| ***C. russula*** | **Inside** | Kingdom_Bacteria | Kingdom_Bacteria | 0.4229723 | 0.00018 | 0.01450 | 0.00047 | 0.03236 |
| | | Brevinemataceae | Genus_Brevinema | 0.6953776 | 0.00001 | 0.00138 | 0.00001 | 0.00191 |
| | **Edge** | Demequinaceae | Genus_Demequina | -0.5298566 | 0.00430 | 0.04904 | 0.00101 | 0.02817 |

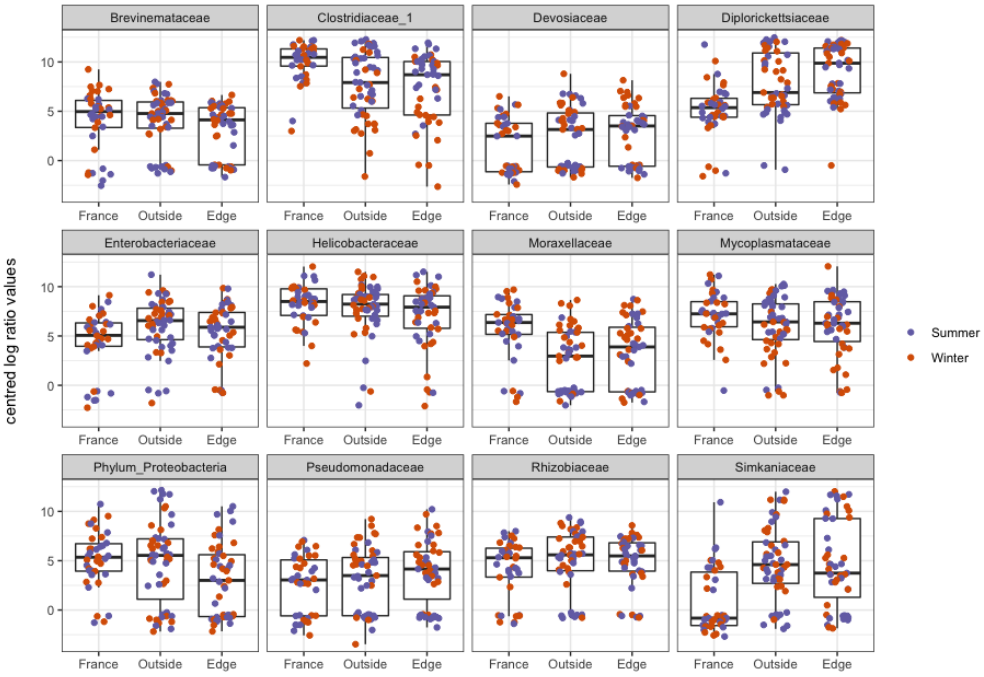



**Figure 4.7.** The abundance of the 12 most abundant families in *S. minutus* from different sampling areas, including those that showed significant differences in abundance. Points are coloured according to sampling season. Abundance is measured in clr values. France is Belle Île.

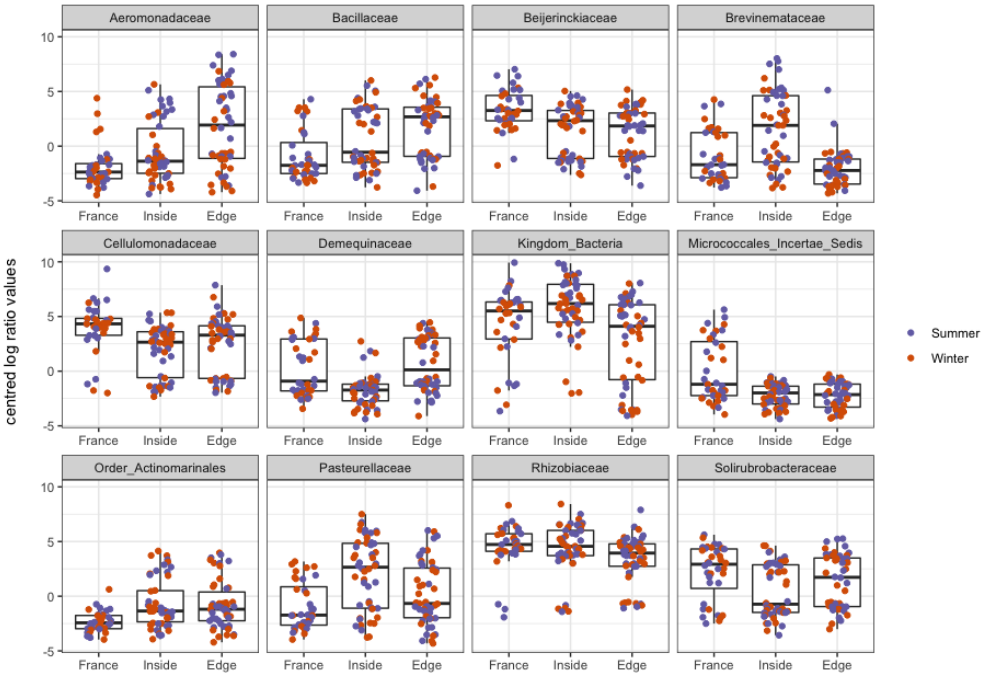



**Figure 4.8.** The abundance of the 12 most abundant families in *C. russula* from different sampling areas. Points are coloured according to sampling season. Abundance is measured in clr values. France is Belle Île.

**Partial Mantel Test**

The only population of shrews that showed a correlation between dissimilarity of samples according to diet compared to microbiome were SM-Ire using the Bray-Curtis method. Although it is a significant correlation, the relationship is weak (R = 0.11) (**Table 4.6**).

**Table 4.6.** Partial mantel test output. All tests were performed using 16S data clr-transformed and Euclidean distance measures compared against diet transformed to relative read abundances with Bray-Curtis and Jaccard distance metrics used. *<0.05

| Shrew | Country | R | p-value | R | p-value |
|---|---|---|---|---|---|
| | | Bray-Curtis | | Jaccard | |
| *S. minutus* | Ireland | 0.1104 | 0.011999* | 0.07831 | 0.077792 |
| | Belle Île | -0.09016 | 0.78842 | -0.1004 | 0.84232 |
| *C. russula* | Ireland | -0.03516 | 0.61784 | -0.1105 | 0.84652 |
| | Belle Île | 0.01028 | 0.44826 | 0.06885 | 0.30417 |

## 4.4. Discussion

Here we successfully characterised the microbiome of ~280 *S. minutus* and *C. russula* samples collectively. Although both shrews occupy similar niches and habitats, there are key differences in the microbiome between both species (shown by composition, alpha diversity and beta diversity measures). As well as interspecific differences, there are intraspecific patterns seen here that provide insight into long-term and short-term co-evolution of host population and microbiome. *Crocidura russula* shows the ability to retain a stable microbiome structure while colonising new areas while key differences of the SM-Ire microbiome may contribute to their displacement by an invasive shrew.

Although a large number of ASVs were detected in this data, both species have relatively low diversity for mammals. Carnivores tend to have a lower diversity of microbes in their gut system compared to omnivores and herbivores (Ley *et al*., 2008a). Nishida & Ochman (2018) highlight that gut physiology and capacity will restrict the composition of the microbiome, and that mammals <100g tend to host low levels of bacteria in terms of diversity, regardless of dietary capacity. In other words, the small volume of a shrew's digestive tract has a restricted capacity for microbes. *Crocidura russula* has a much more diverse microbiome compared to the smaller *S. minutus*, which may be explained by the positive association between body mass and gut microbiome diversity in insectivores shown by Nishida and Ochman (2018).

Bacteroidetes are recorded to be abundant in many vertebrate species (Ley *et al*., 2008b) including insectivorous birds (Cho and Lee 2020). They are linked to the degradation of complex molecules in the intestines, allowing optimal nutrient uptake, with an association with obesity in mammals (Turnbaugh *et al*., 2006). They are low in both prevalence and abundance in *C. russula* (average of 2.57% of reads) and *S. minutus* (average of 0.57% of reads). The majority of individuals that did host a relatively high abundance of these bacteria were restricted to *C. russula* samples from the inside zone. This is a similar pattern to some insectivorous bats (Lechner *et al*., 2020) and another study examining *S. minutus* and the common shrew (*S. araneus*) (Knowles, Eccles and Baltrūnaitė, 2019).

The two most diverse phyla in these samples were Actinobacteria and Proteobacteria. Actinobacteria are a common group among vertebrate guts (Colston and Jackson, 2016), and form a large part of the *C. russula* gut microbiome, but not in *S. minutus*. Both shrews have an abundance of Proteobacteria in their guts (contributing to 43.6%

and 41.8% of reads found in *C. russula* and *S. minutus*, respectively), which are a group highly associated with both vertebrates and invertebrates/arthropods (Esposti and Romero, 2017). Within the Proteobacteria phylum, *C. russula* harbour a range of the classes Gammaproteobacteria, Alphaproteobacteria and Deltaproteobacteria, with the dominant being Gammaproteobacteria. *Sorex minutus* on the other hand primarily house only the class Gammaproteobacteria (other classes are below 1% of reads). Gammaproteobacteria typically break down complex sugars and can aid in nutrient production for the host (Colston and Jackson, 2016). However, the dominant member of Gammaproteobacteria detected in SM-Ire (~25% of reads) is the *Candidatus Rickettsiella isopodorum*, a known pathogen of isopods (Kleespies, Federici and Leclerque, 2020) which are a large constituent of the *S. minutus* diet (Chapter 3). There is the possibility that there is a strain of this bacterial species associated with vertebrate guts, but with the information currently available, it is likely detecting the bacteria of ingested isopods.

Bacteria within the phylum Tenericutes ideally require a host to survive and grow, therefore they are difficult to culture and study. For this reason, little is known about them but are suspected to play a role in nutrient processing for their host (Colston and Jackson, 2016). They appear more abundant in *C. russula* (20.5% of reads) compared to *S. minutus* (6.9% of reads) (**Figure 4.3**). The abundance of Tenericutes and Proteobacteria may give *C. russula* an advantage in utilising resources in new environments if these phyla are indeed involved with nutrient processing and uptake.

### *Crocidura russula* in a Native and Non-native Range

*Crocidura russula* has a more diverse microbiome compared to *S. minutus* (**Figure 4.1**, **Table 4.3**). This diversity could provide *C. russula* with sufficient phenotypic plasticity for expanding their range into new environments, as microbiome diversity (and its corresponding functional role) has been suggested to provide the potential for rapid adaption (Alberdi *et al*., 2016). That being said, any changes in the microbiome structure of *C. russula* found to be significant in this study are subtle. Although the PERMANOVAs using the clr-transformed data showed a minor but significant difference between the Belle Île and Irish population, no difference was detected when phylogenetic information was included for the ASVs (**Table 4.4**). This could imply that

although strains differ between the islands, the functional roles of the detected ASVs may be similar (Stothart, Palme and Newman, 2019).

There are more taxa considered differentially abundant in CR-Bel (**Table 4.5**), which is considered here as this shrew's 'native' range. This invasion will result in a population bottleneck which can lose genetic variants (Colautti and Lau, 2015). The reduction of these microbial taxa in Ireland may be a result of either a small founding population of *C. russula* (Gargan *et al*., 2016) causing the loss of genetic variants associated with these microbes (Davenport *et al*., 2015). An alternative possibility is some form of the 'enemy release hypothesis', in which microbial groups have been constantly sub sampled during the range expansion and reduced (Colautti *et al*., 2004). This is a plausible explanation given that many of the bacterial taxa that are more abundant in Belle Île are reported in soil-plant interactions (*Microvirga*; Wang *et al*., 2017) or may be pathogenic (*Mycoplasma*; Waites *et al*.,2013) and may not have selective pressure acting on them. This is dependent on the assumption that the CR-Bel population closely resemble the origin population of the Irish population. Although CR-Ire likely originated from mainland France (Gargan *et al*., 2016), the similarity between the Ireland and Belle Île population shows that the microbiome of an invasive small mammal can remain stable when expanding into new habitats.

We already know that *C. russula* are adapting their diet along the invasion route in Ireland (see Chapter 3), potentially in response to resource availability. The partial Mantel tests performed here have shown that dissimilarity between samples according to diet and microbiome are not correlated. Although the microbiome of *C. russula* has shown significant differences along the invasion route (PERMANOVA; **Table 4.4**), they are minor ($R^2$ = 0.03 – 0.05) and are likely influenced by some outliers (**Figure 4.6**).

There is another likely reason for this change between invasion zones. *Crocidura russula* are larger at the edge of the invasion and appear to go for larger prey taxa (see Chapter 3, **Figure 3.6**). Small insectivores have been recorded to have positive relationship between size and alpha diversity of their microbiome (Nishida and Ochman, 2018). We have shown that microbial diversity increases slightly in larger *C. russula* individuals (**Figure 4.2**). Rather than random subsampling, the change of microbiome shown between the inside zone and the edge of the range in Ireland could be more simply explained by the shift in diet (not picked up by partial mantel tests) and/or the increase in body size and microbial diversity.

### *Sorex minutus* in Two Native Ranges

There is strong evidence here that there are differences in the gut microbiome between the Irish and Belle Île population of *S. minutus*, despite both islands being ecologically similar (McDevitt *et al*., 2014). PERMANOVAs showed compositional differences with both normalisation and distance methods, with random forest classifier analysis being able to differentiate samples to the correct island with a high accuracy (~90%) and multiple taxa showing differential abundances between the countries. Both the random forest classifier and differential abundance analyses identified taxa within the families Closteridiales, Ricketsiella and Simkaniaceae to be key in differentiating *S. minutus* samples from Belle Île and Ireland.

The significant increase of Closteridiales in the SM-Bel population is primarily due to ASVs identified to the genus *Candidatus Arthromitus*. This is a bacterium highly associated with the guts of arthropods. However, Thompson *et al*. (2012, 2013) found that a morphologically similar bacteria, *Candidatus Savagella*, is associated with the intestines of vertebrates and many studies have mistaken *Candidatus Savagella* for *Candidatus Arthromitus* (Stanley *et al*., 2014, 2015; Ericsson *et al*., 2018). Because *S. minutus* is an insectivore, it is possible that the prey microbiome has been detected. The maximum likelihood tree created with the 469 ASVs detected in this study shows that this ASV sits in the Clostridiaceae family (subset of phylogenetic tree in **Figure S4.1**), meaning it is *Candidatus Savagella* and was mis-identified during the DADA2 pipeline. The SM-Bel population thus have a higher abundance of *Candidatus Savagella* compared to the SM-Ire population.

*Candidatus Savagella* are segmented filamentous bacteria (SFB) that are host specific and influence the host's immune homeostasis such as activating T-cells and epithelial lymphocytes (Stanley *et al*., 2015). They can also promote Immunoglobin A (IgA) production (Farkas *et al*., 2015) which can bind to pathogens to prevent their attachment to the host mucosa, with the additional benefit of bound pathogens now being able to be recognised by phagocytes (Tao and Xu, 2016). The SM-Ire population have a much higher abundance of *Rickettsiella* species, which are associated as invertebrate pathogens (Esposti and Romero, 2017), as well as potentially parasitic species of the Simkaniaceae family (within the order Chlamydiales) (Pawlikowska-Warych and Deptuła, 2019).

The key microbial taxa showing strong changes between the SM-Ire and SM-Bel populations have contrasting functions inferred from the literature. There isn't a strong correlation with diet shown here, and the gut content samples collected during this study are more likely reflective of evolutionary time scale change (Ingala *et al*., 2018). These changes indicate that *S. minutus* may have adapted their microbial community over a long period of time in Ireland and Belle Île. Differences in microbiome structure is seen between isolated populations of other vertebrates, such as various species of carp fish (Eichmiller *et al*., 2016). Although these two populations of *S. minutus* belong to the same 'Western' mitochondrial DNA lineage (McDevitt *et al*., 2011), genetic differences accumulated over thousands of years of isolation between the populations may be sufficient to influence the bacterial communities seen here (Davenport *et al*., 2015). Alternatively, the microbial composition may have adapted slowly over time to suit environmental differences between Ireland and Belle Île. Although the advantage of lacking *Candidatus Savagella* (which is involved in host immunity) in Ireland isn't yet clear, this may have led to a predisposed disadvantage of SM-Ire to unexpected stresses, such as an invader.

### The Effects of *C. russula* on *S. minutus*

We found limited evidence of rapid changes in the *S. minutus* microbiome in response to *C. russula* invasion. Only the clr-transformed dataset showed any difference between *S. minutus* samples in the outside and edge sampling zones in the PERMANOVA analysis. The RFC could not differentiate *S. minutus* samples between invasion zones, while no taxa showed significant differences in abundance between these populations. It should be noted here that the gut microbiome of bats can change depending on if the samples are from guano or gut tissue lining (Ingala *et al*., 2018). The guano samples seem to reflect more recent changes (such as diet) compared to gut lining (more reflective of evolutionary change). Gut content scrapings were used for these shrew samples and may be less likely to show rapid changes to the microbiome structure.

In terms of the invasive *C. russula* potentially introducing novel pathogens (Nally *et al*., 2016), there is a higher abundance of *Aeromonas spp.* in the CR-Ire population, particularly at the edge zone shown by the differential abundance analysis (**Figure 4.8**; **Table 4.5**). This genus is known for pathogenic species in vertebrates, including

humans (Ceylan, Berktas and Ağaoğlu, 2009) and accounts for an average of ~1.5% of reads in *C. russula* samples at the edge zone. This will require further work to determine what species of *Aeromonas* this is and what risk this may pose for small mammals in Ireland. Currently, the *S. minutus* samples from this study show no signs of possessing *Aeromonas spp.* in Belle Île or Ireland.

## 4.5. Conclusion

Both *C. russula* and *S. minutus* inhabit similar hedgerow systems and predate on a wide variety of invertebrates with moderate levels of over-lap (see Chapter 3) and yet they have highly distinguishable microbiomes. *Sorex minutus* shows evidence of diverging microbiomes between Ireland and their Belle Île counter parts, likely due to a combination of genetic isolation and local adaptation over long periods of time (thousands of years; McDevitt *et al*., 2011). While no rapid changes are occurring in *S. minutus* gut microbiota during the invasion of *C. russula*, the reduced abundance of immunity-activating microbes in the SM-Ire population may leave them susceptible to newly introduced stresses.

# Chapter 5 – General Discussion

## 5.1. Summary of Research Goals

This main overall objective of this project was to understand the mechanisms behind the disappearance of the native pygmy shrew (*S. minutus*) in the face of the invasion of the greater white-toothed shrew (*C. russula*) in Ireland. This is an ongoing invasion (i.e. the invader is still expanding its range), which afforded us an opportunity to examine various factors such as prey resource competition, pathogen prevalence and microbiome structure at various stages of the invasion. In addition, shrews were examined from Belle Île, an island off the west coast of France where they both co-exist in relatively high numbers. These two species are the only species of shrews here, forming a similar small mammal community to Ireland. This shows that *C. russula* and *S. minutus* can co-inhabit on this island but cannot co-inhabit in a similar system on Ireland once the invasive *C. russula* have an established population.

This project covers empirical trials that determined the optimal protocol and primers for detecting a wide range of invertebrate prey taxa consumed by *C. russula* and *S. minutus* (Chapter 2). Applying this optimised protocol to shrews at various invasion stages in Ireland highlights that established populations of invasive *C. russula* switch to smaller prey that increases resource competition with *S. minutus*, causing the native shrew to disappear from the area. This study also highlights the importance of key prey groups to facilitate co-existence of these shrews in Belle Île (Chapter 3). The application of rRNA gene metabarcoding to gut content samples from these shrews highlights the potential role of microbiome structure in providing invasive shrews adaptive potential and the susceptibility of native shrews to invaders (Chapter 4).

## 5.2. Main Findings

### 5.2.1. Chapter 2

The use of DNA metabarcoding is a common tool for dietary studies (Pompanon *et al*., 2012), but requires empirical trials to optimise the protocol before being applied to unique study systems. Two degenerative sets of primers were chosen to trial on shrew gut contents based off their success in previous studies (Aizpurua *et al*., 2017; Biffi *et al*., 2017a; Esnaola *et al*., 2018; Aldasoro *et al*., 2019). Neither set of primers have

previously been used to determine the diet of either *C. russula* or *S. minutus* and ground-dwelling insectivores are generally under-represented in DNA-based dietary studies (relative to bats for example). The Zeale primers (Zeale *et al*., 2011) have been extensively used to detect hundreds of prey taxa of insectivorous bats that primarily predate on flying Diptera, Coleoptera and Lepidoptera (Aizpurua *et al*., 2017; Baroja *et al*., 2019). The empirical trials performed in Chapter 2 have shown that this primer set is still primarily restricted to these orders in terrestrial insectivores (**Figure 2.4**). The more degenerative Gillet primers (Gillet *et al*., 2015) show that these three orders only account for a portion of the shrew diet, supported with previous studies on shrew diets using morphological analyses (Churchfield and Rychlik, 2006; Brahmi *et al*., 2012). As a result, many individual shrews that had not recently consumed any Diptera, Coleoptera or Lepidoptera before trapping will provide no reads using the Zeale primers and will be subsequently filtered out to reduce the sample size of the dataset.

Not only can the Gillet primers detect taxa from the orders Diptera, Coleoptera and Lepidoptera, but also an additional 14 orders that include key components of a shrew's diet. These included slugs/snails (Stylommatophora), spiders (Araneae), woodlice (Isopoda), millipedes (Polydesmida) and worms (Haplotaxida) (**Figure 2.3**; Pernetta, 1976). The Gillet primers amplify a large amount of host reads from these shrew samples (**Figure 2.2**) and have detected various other birds, mammals and amphibians in other studies (Biffi, Laffaille, *et al*., 2017; Esnaola *et al*., 2018; Galan *et al*., 2018). Although host reads are not desirable as they reduce the read depth of prey consumed, vertebrate detection plays as an advantage for clarifying if *C. russula* are consuming any small native vertebrates in Ireland. The results from the empirical primer trials suggest that using the Gillet primers alone will provide a detailed description of the prey consumed by *C. russula* and *S. minutus*, with the added benefit of vertebrate detection. Considering the high number of samples filtered out using Zeale primers, including these primers as well would increase cost and likely provide little more information to the study.

DNA metabarcoding is still a relatively new technique (Yu *et al*., 2012), that has been applied to detect the food consumed by a range of animal species (De Barba *et al*., 2017; Shutt *et al*., 2020). There are still a lot of animal species that have not had their diets determined via metabarcoding. Although there is already a wide variety of primers capable of detecting various organisms, this study has shown the importance of empirically testing primers and protocols before committing to a full-scale analysis on

a novel species or system. Both these primers performed differently in terms of host amplification levels between *C. russula*, *S. minutus* and other terrestrial insectivores (Esnaola *et al*., 2018). This trial shows that Gillet primers are likely suitable for other terrestrial insectivores such as moles (*Talpa europaea*) or hedgehogs (*Erinaceus europaeus*). All previous studies looking at the diet of *C. russula* and *S. minutus* have relied on morphological identification from stomach contents and faeces (Pernetta, 1976; Churchfield and Sheftel, 1994; Churchfield and Rychlik, 2006; Brahmi *et al*., 2012), apart from one study that used a metagenomic approach to determine the diet of five *S. minutus* individuals (Ware *et al*., 2020). The DNA metabarcoding protocol optimised in Chapter 2 was efficient in detecting a wide range of prey consumed by these shrews (see Chapter 3), providing novel insight into their core diets and food resource competition.

### 5.2.2. Chapter 3

The detection of Stylommatophora in *S. minutus* diet is a novel finding that has not yet been reported to our knowledge (Pernetta, 1976; Churchfield and Rychlik, 2006; Ware *et al*., 2020). This study detects DNA from small Stylommatophora species (approx. 15 – 20mm long with no shells) in over half of *S. minutus* individuals and contributes to ~10% of their detectable diet (**Figures 3.10** and **3.13**). These invertebrates were generally considered too large for *S. minutus*, but whether or not the consumption of slugs is due to predation or scavenging behaviour, they are a key component to the diet of these shrews.

The general level of interspecific dietary overlap between *S. minutus* and *C. russula* is similar in both Ireland (up to ~46%) and Belle Île (up to ~49%; **Table 3.5**) and has been seen at this level in other populations of sympatric species of shrews (Churchfield and Rychlik, 2006). To relieve competitive pressure from this high level of dietary overlap in Belle Île, there are key prey taxa that are largely consumed by one shrew species, but not the other. For example, the Belle Île population of *S. minutus* (SM-Bel) show an increased consumption of the orders Araneae, Hemiptera and Lepidoptera which do not appear in the diet of the Belle Île population of *C. russula* (CM-Bel; **Figure 3.14**). Alternatively, CM-Bel show an increased consumption of millipedes (Glomerida, Julida and Polydesmida) that are likely too big for *S. minutus* to consume. This is either a

result of resource availability or adapted behaviour because there is little evidence of key prey taxa relieving interspecific competition pressure in Ireland.

A significant finding in this study is the changing diet and physiology of *C. russula* during their range expansion in Ireland. At the edge of their invasive range, *C. russula* are 20% larger in body mass (**Figure 3.6**) with a higher proportion of their diet consisting of larger invertebrates such as worms (Haplotaxida), beetles (Coleoptera) and tough shelled millipedes (Glomerida) (**Figure 3.13**). These invertebrates are likely too large for *S. minutus*, reducing dietary overlap to as low as 14%. The fact that a high abundance of *S. minutus* are present in the same sites as *C. russula* at the edge of their expansion means the reduced competition allows their co-existence in Ireland for a period of time (McDevitt *et al*., 2014). However, due to their small territory size, high abundance and wide diet, *C. russula* are known for their ability to exhaust localised resources of food (Genoud, 1985; Brahmi *et al*., 2012). The reduced consumption of larger prey orders by well-established populations of *C. russula* (inside zone; **Figure 3.1**) is countered with an increased consumption of smaller prey taxa (**Figure 3.13**), resulting in an increased overlap in diet with *S. minutus* (up to 46%; **Table 3.5**). This is the point of the invasion where *S. minutus* is no longer present, probably due to increased resource competition. This 'layered' effect of the *C. russula* impacts during its invasion of Ireland highlights that introduced species must be carefully and continuously monitored, regardless of initial risk assessments.

### 5.2.3. Chapter 4

The change of diet along the invasion route of *C. russula* is not reflected by structural changes in the microbial community (microbiome) of their guts. Their microbiome remains relatively diverse and stable between Belle Île and Ireland, suggesting a strong phylogenetic relationship between *C. russula* and their microbiome (Colston and Jackson, 2016). However, the abundance of some groups of bacteria that may be pathogenic (*Mycoplasma*; Waites *et al*., 2013) has been significantly reduced in the invasive population (**Table 4.5**). This could indicate that the 'enemy-release hypothesis' could be reducing the pathogenic load of *C. russula* during their invasion and improving their fitness (Dunn and Hatcher, 2015). A counter argument to this is the higher abundance of *Aeromonas spp.* (potentially zoonotic) in the Irish population of *C. russula* (CR-Ire), particularly at the edge. Although *C. russula* host some pathogenic bacteria, there is no evidence from this study of transmission of these bacteria to the *S. minutus* population.

While it is interesting to see little change in the microbiome structure of *C. russula* populations that have established between one year ago or thousands of years ago, the microbiome of *S. minutus* shows some strong changes between the Belle Île population and the Irish population (**Table 4.5; Figure 4.7**). The main changes seen are due to functionally different bacteria co-evolving with each isolated population over a long time (Eichmiller *et al*., 2016) as intraspecific differences in their diet composition is not shown to dramatically correlate with intraspecific differences in microbiome (**Table 4.4**).

Neither the diet or microbiome dramatically change in *S. minutus* between the outside zone (before *C. russula* arrives) and the edge of the invasion range (recently after arrival of *C. russula*). The microbiome can be affected by various environmental stresses or changes (Hauffe and Barelli, 2019), but the lack of response between these zones suggests that *S. minutus* and *C. russula* may co-inhabit areas in Ireland without much impact at first. Over time, something causes *C. russula* to alter their diet and increase competitive pressure onto *S. minutus* and ultimately leads to their disappearance. Whatever change may be happening to *S. minutus* when competition for resources is increased, it is a rapid impact not fully captured by the microbial composition in these samples.

## 5.3. Recommendations for Future Work

### 5.3.1. Ireland's Invertebrate Community

The shift in diet exhibited by *C. russula* between the invasion zones in Ireland (**Figure 3.13**) could be a response to exhausting the local resources (see Chapter 3 and **Section 5.2.2**), which would alter the invertebrate community structure in Ireland's hedgerows. It is recommended to survey Ireland's hedgerow invertebrate community outside the *C. russula* range, at the edge of the range and inside their range where they have well-established populations. If *C. russula* is indeed altering the invertebrate community structure, this could have implications on other insectivorous species such as hedgehogs, a species already in serious decline in Great Britain (Hof and Bright, 2016). Altered invertebrate communities may also have other less predictable impacts as terrestrial invertebrates are essential for many ecosystem services through decomposition, nutrient cycling and soil structure maintenance (Lavelle *et al*., 2006; Pulleman *et al*., 2012).

### 5.3.2. Pathogen and Parasites

Invasive populations begin as a small subset of individuals, which increases their chance of establishing a population with an impoverished parasitic or pathogenic load, resulting in increased fitness and chance of survival (Enemy-release hypothesis; Dunn and Hatcher, 2015). The invasive population could also co-introduce novel parasites or pathogens into the invaded range, causing negative impacts on the fitness of native populations (Spillover hypothesis; Miller *et al*., 2018). Additionally, the invasive population could provide more hosts to benefit native parasites or pathogens, causing a boom in the pathogen prevalence at the expense of the native populations (Spillback hypothesis; Diagne *et al*., 2016). These can all play a potential role in invasion success, negative impacts on local wildlife and have potential One Health initiative implications (Cunningham, Daszak and Wood, 2017). Although we have screened for intestinal bacteria that could be introduced into the Irish small mammal community, we still lack information on what other kind of parasites and pathogens they could potentially introduce or host. For example, Nally *et al*. (2016) have determined that *C. russula* host a novel strain of *Leptospira* that is transmitted through urine. However, it is unknown if *S. minutus* harbours it also (or even if it originated in the native shrew and was transferred to *C. russula*).

Because of the strict insectivorous diet (invertebrates are important intermediate hosts of parasites), shrews are often infected with at least one species of helminth parasite (Portole *et al*., 2004; Kinsella *et al*., 2008; Kinsella and Tkach, 2009; Hope *et al*., 2016; Greiman *et al*., 2018). The gut contents of shrews from this study were homogenised for DNA extraction, meaning there are no morphological means for identifying parasites in the guts. However, there are tested primers and protocols available that target various gastrointestinal parasites that can be used via DNA metabarcoding techniques (Greiman *et al*., 2018).

Shrews can also be infected with various haemoparasites (blood-borne parasites), many of which as known to be zoonotic, such as *Bartonella spp.* (Bray *et al.*, 2007; Bown *et al*., 2011). Blood samples from the majority of shrews are stored at -80$^{o}$C. Prevalence of haemoparasites can be tested from shrews using inoculation/culturing methods from the blood (Bray *et al*., 2007), or metabarcoding methods can also be applied to the blood samples to screen for a range of haemoparasites (Huggins *et al*.,

2019). Ticks are important vectors for haemoparasites and zoonotic diseases (Bown *et al*., 2011), and have been found on multiple shrews trapped during this study and are currently available stored in absolute ethanol. Future studies can determine the prevalence of potential zoonotic pathogens and their vectors on the invasive *C. russula* and native *S. minutus* to identify the potential threat of these pathogens to both small mammal communities and humans.

As these animals were sacrificed for this study (under license and ethical approvals; see **Section 6.4**), using these samples for further studies is highly encouraged.

### 5.3.3. Adaptation in *Crocidura russula*

The history of *C. russula* expanding its range across Europe, and now Ireland, already suggests they are an adaptable species well suited to dispersal into new habitats/environments. Traits that can factor in on their successful dispersal capabilities are their wide range diet that can shift along their invasive range, coupled with a diverse and stable microbiome (see this study). Other factors outside the scope of this study that may also be playing a role. Selection may be acting at different stages of the invasion for phenotypic traits advantageous to dispersal and establishment. While individuals at the edge of the range expansion exhibit traits associated with dispersal capabilities, longer established populations may exhibit selective pressure towards traits involved in intraspecific competition or reproduction (Burton, Phillips and Travis, 2010; Bonte *et al*., 2012; Travis *et al*., 2012). For example, longer limbs are more efficient for dispersal compared to shorter limbs in the invasive cane toad (*Bufo marinus*) in Australia. This trait has increased in frequency at the expansion front of the cane toad, allowing faster and faster dispersal rates across the Australian landscape (Phillips *et al*., 2006). This study already shows that the largest *C. russula* individuals (by weight and total length) are found at the edge of their expanding range in Ireland compared to the well-established populations (inside zone; **Figure 3.6**).

Genomic regions responsible for traits considered advantageous to dispersal such as reproduction, competitive ability, longer limbs should increase in frequency along the route of the expanding range compared to the inside established populations (Hancock *et al*., 2010). By genotyping sub-populations of *C. russula* at different stages of the expansion route (the path of the range expansion), these shifts in allele frequencies are detectable. White *et al*. (2013) performed a similar study by sampling the invasive

bank vole (*M. glareolus*) in Ireland along transects and using single nucleotide polymorphisms (SNPs) to identify enriched allele frequencies in immune system genes. SNPs are an ideal genetic marker that represent a larger proportion of the genome and can be generated via restriction site-associated DNA sequencing (RAD-seq; Andrews *et al*., 2016).

Performing a genomic analysis for selection, combined with phenotypic measurements will provide further insights to the dispersal capabilities of this invasive species and determine if the rate of expansion may increase. Updated rates of expansion can also be examined by comparing the 2013 survey (McDevitt *et al*., 2014), this study and future trapping to clarify if the range is expanding at an increased rate. This will have implications for the management of any impacts caused by *C. russula* in Ireland.

## 5.4. Conclusions and Future Management

*Crocidura russula* appears to be highly capable species for colonising new areas as they established themselves in Ireland from an initial small population size (Gargan *et al*., 2016), began expanding incredibly rapidly (McDevitt *et al*., 2014) and now show the ability to adapt their diet during their range expansion to optimise their chances of survival. In addition to this, they have a relatively diverse microbiome (which is associated with multiple fitness traits of an individual; Alberdi *et al*., 2016) for their size. Their microbial structure shows little changes despite adapting to novel environments, changing their diet and competing with the native *S. minutus* population. There appears little chance of their range expansion slowing down in Ireland.

The disappearance of *S. minutus* in response to the presence of *C. russula* is most likely due to competition for prey resources, which the invader is winning. The morphological differences between SM-Ire and SM-Bel (this study; Vega *et al*., 2016) and microbiome differences likely reflect long term divergences of these isolated populations. There may be a combined effect of their predatory ability, habitat usage and prey availability that results in *S. minutus* disappearing from areas where the *C. russula* has well-established populations in Ireland.

Based on the current rate of expansion, *C. russula* will likely inhabit the entire island by 2050 (McDevitt *et al*., 2014). With the current state of knowledge and the findings presented here, the future looks bleak for *S. minutus* in Ireland. The invasive population of *C russula* has already reached a size that is unlikely to be managed or

controlled in Ireland. Seymour *et al*. (2005) demonstrated a failure to eradicate an invasive population of shrews from Mauritius islands around 25 hectares in size. Trapping and killing the population in Ireland would not be feasible given the size and density, and toxins would have severe impacts on non-target species such as *S. minutus* (McDevitt, 2019). The best strategy now is to prevent further introductions to neighbouring areas. Given the frequent movement of goods and people, Great Britain may be at risk of invasion from *C. russula,* although it may not have a similar impact as Ireland considering Britain's higher diversity of small mammals (McDevitt, 2019). In addition, there are multiple islands off the coast of Ireland that can act as refuge for *S. minutus* populations (McDevitt *et al*., 2014). Preventing their entry to these islands and the UK will require using stringent border checks. Satellite populations have already been found well outside the main invasive range of *C. russula* so they are clearly moving around with humans within the island of Ireland (McDevitt *et al*., 2014; Gargan *et al*., 2016). Considering that *C. russula* likely arrived with horticultural imports (Gargan *et al*., 2016), border checks should be particularly vigilant for the movement of these goods (McDevitt, 2019). Routine trapping is a cheap and efficient method to monitor shrew abundance and should be considered to monitor each species in different habitat types to determine the long-term viability of *S. minutus* in Ireland. In addition, monitoring bird of prey pellets has proven particularly valuable for tracking their distribution and spread also (Tosh *et al.*, 2008; McDevitt *et al.*, 2014).

Another ray of hope for *S. minutus* is that specific habitat types on the mainland may favour *S. minutus* populations over *C. russula*. For instance, the *C. russula* invasion has not yet reached the primary areas of peatland in Ireland in the west, a habitat in which *S. minutus* tends to be more abundant when co-existing with another shrew species in Britain (Yalden, 1981). Any potential competition between the species in these habitats will not become apparent however until the invasion front reaches these regions. Despite the invasive *C. russula* being discovered in 2007 (Tosh *et al.*, 2008) and its negative impacts on other small mammals being already apparent only several years later (McDevitt *et al*., 2014; Montgomery *et al.,* 2012, 2015), the wider impacts of the invasion remain drastically understudied. Here, we demonstrate for the first time that these impacts affect multiple trophic levels and this clearly requires further investigations into its effects on the wider ecosystem on the island of Ireland.

# 6. Appendices

## 6.1. Power Analysis for Sample Size Estimation

As the study species are required to be killed, it is ethically integral that the correct sample size will be used. The sample size required for this project was therefore calculated using power analyses run on the R package 'powerMediation' (Qiu, 2017). If multiple analyses will be performed on the subject, it is recommended practice to estimate the required sample size based on the analysis that will require the largest sample size.

All power analyses were run to accommodate hypotheses examining prevalence rates of parasites (and pathogens such as *Bartonella*) in *C. russula* and *S. minutus*. Presence/absence data will be used (binary data). A logistic regression approach will be used for the analyses.

The logistic regression model is:

$$log(p / (1 - p)) = \beta\_0 + \beta\_1 X$$

where X is the explanatory variable (a two-level factor).

The formula used to estimate sample size is Formula (2) in (Hsieh, Bloch and Larsen, 1998).

**Power analysis for the greater white-toothed shrew (*Crocidura russula*) in Ireland**

The 'Enemy release hypothesis' has been well documented as a potential contributor to the success of invading species. This states that there is a reduced parasitic load in the animals at the invasion front which allows higher capability to invade with reduced parasitic hindrance (Diagne *et al*., 2016). The following hypotheses on *C. russula* examined are:

Null Hypothesis: There is no difference in prevalence of parasites/pathogens in *C. russula* at the invasion front compared to well-established areas.

Alt Hypothesis: There is a lower prevalence of parasites/pathogens in *C. russula* at the invasion front compared to well-established areas.

This null hypothesis will be tested using logistic regression with the prevalence of parasites/pathogens as the response variable and the site location (a two-level factor with levels: at the invasive wave front and within the well-established area) as a single explanatory variable.

The estimated sample size required to test this hypothesis was **160** *C. russula*. The power analysis used a desired power of 80%, a significance level of 5%, and equal sampling size across the two levels of the factor. The expected prevalence values of parasites/pathogens were estimated as 0.12 and 0.3 (odds ratio of 0.32). These values were estimated from parasite prevalence levels in mice from areas corresponding to different stages of another small mammal invasion (Diagne *et al*., 2016). Mice are used as a proxy for shrews as no literature was identified to examine the enemy release hypothesis in shrews. Shrews have however been shown to host parasites/pathogens of similar prevalence ranges to mice (Kinsella *et al*., 2008).

**Power analysis for the pygmy shrews (*Sorex minutus*) in Ireland.**

The hypothesis to be tested for *S. minutus* is the 'spillback hypothesis' which states that native animals faced by an invading species will gain an increase in parasite load at the invasion front (Edge zone) compared to areas where the invader has not yet reached (Outside zone) (Diagne *et al.*, 2016).

| | |
|---|---|
| Null hypothesis: | There is no difference in prevalence of parasites/pathogens in *S. minutus* at the invasion front compared to non-invaded areas. |
| Alt. Hypothesis: | There is a lower prevalence of parasites/pathogens in *S. minutus* in non-invaded areas compared to the invasion front. |

The expected effect size is the same as for the 'enemy release hypothesis' (Diagne *et al*., 2016). The required sample size of *S. minutus* was therefore estimated at **160**.

**Power analysis for shrews in Belle Île**

We want to know if prevalence rates of parasites/pathogens are shared between both shrew species in Belle Île. This will provide insight into the role of parasites/pathogens in the success of the *C. russula* invasion of Ireland (and hopefully small mammal invasions in general).

Null hypothesis: No difference in parasite/pathogen prevalence between *C. russula* and *S. minutus*

Alt Hypothesis: A difference in parasite/pathogen prevalence between *C. russula* and *S. minutus*

We will test this null hypothesis using logistic regression with the prevalence of parasites as the response variable and the species (a two-level factor with levels: *C. russula* and *S. minutus*) as a single explanatory variable.

The power analysis estimated a required sample size of **80** shrews. The power analysis used a desired power of 80%, a significance level of 5%, and equal sampling size across the two levels of the factor. The expected prevalence values of parasites were estimated as 0.1 and 0.35 (odds ratio of 0.21), based on the natural parasite prevalence range of 7 – 87% in shrews (Kinsella *et al*., 2008), to determine if the shrews of Belle Île have developed different prevalence rates of parasites/pathogens over thousands of years.

## 6.2. Chapter 2 - Appendices

### 6.2.1. Supplementary Tables

**Table S2.1.** The 10 PCR conditions tested with the gillet and zeale primer sets to determine which gave the strongest bands without compensating for quality (i.e. too low annealing temp or too many cycles that could increase PCR related artefacts in the dataset).

| **Conditions 1** | | |
|---|---|---|
| 95°C | | 15:00 |
| x40 | 94°C | 00:30 |
| | 45°C | 00:45 |
| | 72°C | 00:30 |
| 72°C | | 10:00 |
| **Conditions 2** | | |
| 95°C | | 10:00 |
| x16 | 95°C | 00:30 |
| | 61°C | 00:30 |
| | 72°C | 00:30 |
| x24 | 95°C | 00:30 |
| | 53°C | 00:30 |
| | 72°C | 00:30 |
| 72°C | | 10:00 |
| **Conditions 3** | | |
| 95°C | | 15:00 |
| x35 | 94°C | 00:30 |
| | 47°C | 00:45 |
| | 72°C | 00:30 |
| 72°C | | 10:00 |
| **Conditions 4** | | |
| 95°C | | 15:00 |
| x35 | 94°C | 00:30 |
| | 49°C | 00:45 |
| | 72°C | 00:30 |
| 72°C | | 10:00 |
| **Conditions 5** | | |
| 95°C | | 15:00 |
| x40 | 95°C | 00:20 |
| | 55°C | 00:30 |
| | 72°C | 01:00 |
| 72°C | | 07:00 |

| **Conditions 6** | | |
|---|---|---|
| 95°C | | 15:00 |
| x35 | 94°C | 00:30 |
| | 55°C | 00:45 |
| | 72°C | 00:30 |
| 72°C | | 10:00 |
| **Conditions 7** | | |
| 95°C | | 15:00 |
| x35 | 95°C | 00:20 |
| | 55°C | 00:30 |
| | 72°C | 01:00 |
| 72°C | | 07:00 |
| **Conditions 8** | | |
| 95°C | | 15:00 |
| x37 | 95°C | 00:20 |
| | 55°C | 00:30 |
| | 72°C | 01:00 |
| 72°C | | 07:00 |
| **Conditions 9** | | |
| 95°C | | 15:00 |
| x37 | 94°C | 00:30 |
| | 47°C | 00:45 |
| | 72°C | 00:30 |
| 72°C | | 10:00 |
| **Conditions 10** | | |
| 95°C | | 15:00 |
| x10 | 94°C | 00:30 |
| | 49°C | 00:45 |
| | 72°C | 00:30 |
| x30 | 94°C | 00:30 |
| | 47°C | 00:45 |
| | 72°C | 00:30 |
| 72°C | | 10:00 |

**Table S2.2.** Details of samples chosen for this primer trial. Samples are spread across species, sampling zones, transects and season (dates).

| Shrew_ID | Species | Transect | Zone | Date |
|---|---|---|---|---|
| E26 | *C. russula* | East | Edge | 01/10/2017 |
| E27 | *C. russula* | East | Edge | 01/10/2017 |
| E33 | *C. russula* | East | Edge | 06/10/2017 |
| E47 | *C. russula* | West | Inside | 19/02/2018 |
| FR13 | *C. russula* | Belle Île | Belle Île | 11/10/2017 |
| FR20 | *C. russula* | Belle Île | Belle Île | 13/10/2017 |
| FR21 | *C. russula* | Belle Île | Belle Île | 13/10/2017 |
| S33 | *C. russula* | South | Inside | 29/09/2017 |
| S34 | *C. russula* | South | Inside | 29/09/2017 |
| S35 | *C. russula* | South | Inside | 29/09/2017 |
| S47 | *C. russula* | South | Edge | 15/03/2018 |
| S48 | *C. russula* | South | Edge | 15/03/2018 |
| E29 | *S. minutus* | East | Edge | 01/10/2017 |
| E31 | *S. minutus* | East | Edge | 06/10/2017 |
| E32 | *S. minutus* | East | Edge | 06/10/2017 |
| FR14 | *S. minutus* | Belle Île | Belle Île | 12/10/2017 |
| FR15 | *S. minutus* | Belle Île | Belle Île | 12/10/2017 |
| FR16 | *S. minutus* | Belle Île | Belle Île | 12/10/2017 |
| FR17 | *S. minutus* | Belle Île | Belle Île | 12/10/2017 |
| S18 | *S. minutus* | South | Outside | 21/09/2017 |
| S19 | *S. minutus* | South | Outside | 22/09/2017 |
| S21 | *S. minutus* | South | Outside | 22/09/2017 |
| S50 | *S. minutus* | South | Edge | 15/03/2018 |
| S51 | *S. minutus* | South | Edge | 15/03/2018 |
| W12 | *S. minutus* | West | Outside | 22/08/2017 |
| W13 | *S. minutus* | West | Outside | 22/08/2017 |
| W14 | *S. minutus* | West | Outside | 23/08/2017 |

# 6.3. Chapter 4 - Appendices

## 6.3.1. Supplementary Tables

**Table S4.1.** Dunn's post-hoc results for species richness of microbiomes of shrews. Tests are performed for pairwise groups separated to shrew species, season and zone. Benjamini-Hochberg adjusted p-values < 0.05 are in bold.

| Pairwise Comparison | | | Z-ratio | Unadjusted P-value | Adjusted P-value |
|---|---|---|---|---|---|
| GWTS_Summer_Edge | - | GWTS_Summer_France | -0.501 | 0.616 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Summer_Inside | 0.364 | 0.716 | 1.000 |
| GWTS_Summer_France | - | GWTS_Summer_Inside | 0.833 | 0.405 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_Edge | -0.329 | 0.742 | 1.000 |
| GWTS_Summer_France | - | GWTS_Winter_Edge | 0.184 | 0.854 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_Edge | -0.679 | 0.497 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_France | -1.485 | 0.138 | 1.000 |
| GWTS_Summer_France | - | GWTS_Winter_France | -0.946 | 0.344 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_France | -1.795 | 0.073 | 1.000 |
| GWTS_Winter_Edge | - | GWTS_Winter_France | -1.160 | 0.246 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_Inside | -0.046 | 0.964 | 0.964 |
| GWTS_Summer_France | - | GWTS_Winter_Inside | 0.459 | 0.646 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_Inside | -0.409 | 0.683 | 1.000 |
| GWTS_Winter_Edge | - | GWTS_Winter_Inside | 0.285 | 0.776 | 1.000 |
| GWTS_Winter_France | - | GWTS_Winter_Inside | 1.445 | 0.148 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Summer_Edge | 4.456 | 0.000 | **0.000** |
| GWTS_Summer_France | - | Pygmy_Summer_Edge | 4.616 | 0.000 | **0.000** |
| GWTS_Summer_Inside | - | Pygmy_Summer_Edge | 4.059 | 0.000 | **0.003** |
| GWTS_Winter_Edge | - | Pygmy_Summer_Edge | 4.651 | 0.000 | **0.000** |
| GWTS_Winter_France | - | Pygmy_Summer_Edge | 5.397 | 0.000 | **0.000** |
| GWTS_Winter_Inside | - | Pygmy_Summer_Edge | 4.501 | 0.000 | **0.000** |
| GWTS_Summer_Edge | - | Pygmy_Summer_France | 2.595 | 0.009 | 0.369 |
| GWTS_Summer_France | - | Pygmy_Summer_France | 2.889 | 0.004 | 0.182 |
| GWTS_Summer_Inside | - | Pygmy_Summer_France | 2.238 | 0.025 | 0.782 |
| GWTS_Winter_Edge | - | Pygmy_Summer_France | 2.833 | 0.005 | 0.212 |
| GWTS_Winter_France | - | Pygmy_Summer_France | 3.715 | 0.000 | **0.011** |
| GWTS_Winter_Inside | - | Pygmy_Summer_France | 2.637 | 0.008 | 0.343 |
| Pygmy_Summer_Edge | - | Pygmy_Summer_France | -1.571 | 0.116 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Summer_Outside | 5.596 | 0.000 | **0.000** |
| GWTS_Summer_France | - | Pygmy_Summer_Outside | 5.658 | 0.000 | **0.000** |
| GWTS_Summer_Inside | - | Pygmy_Summer_Outside | 5.171 | 0.000 | **0.000** |
| GWTS_Winter_Edge | - | Pygmy_Summer_Outside | 5.758 | 0.000 | **0.000** |
| GWTS_Winter_France | - | Pygmy_Summer_Outside | 6.405 | 0.000 | **0.000** |
| GWTS_Winter_Inside | - | Pygmy_Summer_Outside | 5.642 | 0.000 | **0.000** |
| Pygmy_Summer_Edge | - | Pygmy_Summer_Outside | 0.952 | 0.341 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Summer_Outside | 2.515 | 0.012 | 0.429 |
| GWTS_Summer_Edge | - | Pygmy_Winter_Edge | 2.722 | 0.006 | 0.273 |
| GWTS_Summer_France | - | Pygmy_Winter_Edge | 3.009 | 0.003 | 0.128 |
| GWTS_Summer_Inside | - | Pygmy_Winter_Edge | 2.354 | 0.019 | 0.613 |
| GWTS_Winter_Edge | - | Pygmy_Winter_Edge | 2.961 | 0.003 | 0.147 |
| GWTS_Winter_France | - | Pygmy_Winter_Edge | 3.845 | 0.000 | **0.007** |
| GWTS_Winter_Inside | - | Pygmy_Winter_Edge | 2.765 | 0.006 | 0.250 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_Edge | -1.557 | 0.120 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_Edge | 0.052 | 0.958 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_Edge | -2.528 | 0.011 | 0.424 |
| GWTS_Summer_Edge | - | Pygmy_Winter_France | 2.498 | 0.012 | 0.437 |
| GWTS_Summer_France | - | Pygmy_Winter_France | 2.798 | 0.005 | 0.231 |
| GWTS_Summer_Inside | - | Pygmy_Winter_France | 2.142 | 0.032 | 0.933 |
| GWTS_Winter_Edge | - | Pygmy_Winter_France | 2.739 | 0.006 | 0.265 |
| GWTS_Winter_France | - | Pygmy_Winter_France | 3.628 | 0.000 | **0.015** |
| GWTS_Winter_Inside | - | Pygmy_Winter_France | 2.540 | 0.011 | 0.421 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_France | -1.666 | 0.096 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_France | -0.090 | 0.928 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_France | -2.613 | 0.009 | 0.359 |
| Pygmy_Winter_Edge | - | Pygmy_Winter_France | -0.145 | 0.885 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Winter_Outside | 2.087 | 0.037 | 0.995 |
| GWTS_Summer_France | - | Pygmy_Winter_Outside | 2.419 | 0.016 | 0.529 |
| GWTS_Summer_Inside | - | Pygmy_Winter_Outside | 1.725 | 0.085 | 1.000 |
| GWTS_Winter_Edge | - | Pygmy_Winter_Outside | 2.344 | 0.019 | 0.611 |
| GWTS_Winter_France | - | Pygmy_Winter_Outside | 3.280 | 0.001 | 0.053 |
| GWTS_Winter_Inside | - | Pygmy_Winter_Outside | 2.130 | 0.033 | 0.928 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_Outside | -2.180 | 0.029 | 0.878 |
| Pygmy_Summer_France | - | Pygmy_Winter_Outside | -0.538 | 0.591 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_Outside | -3.173 | 0.002 | 0.075 |
| Pygmy_Winter_Edge | - | Pygmy_Winter_Outside | -0.604 | 0.546 | 1.000 |
| Pygmy_Winter_France | - | Pygmy_Winter_Outside | -0.445 | 0.656 | 1.000 |

**Table S4.2.** Dunn's post-hoc results for Shannon diversity of microbiomes of shrews. Tests are performed for pairwise groups separated to shrew species, season and zone. Benjamini-Hochberg adjusted p-values < 0.05 are in bold.

| Pairwise Comparison | | | Z-ratio | Unadjusted P-value | Adjusted P-value |
|---|---|---|---|---|---|
| GWTS_Summer_Edge | - | GWTS_Summer_France | -0.329 | 0.742 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Summer_Inside | -0.233 | 0.816 | 1.000 |
| GWTS_Summer_France | - | GWTS_Summer_Inside | 0.112 | 0.911 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_Edge | 0.060 | 0.952 | 1.000 |
| GWTS_Summer_France | - | GWTS_Winter_Edge | 0.377 | 0.706 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_Edge | 0.285 | 0.775 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_France | -0.808 | 0.419 | 1.000 |
| GWTS_Summer_France | - | GWTS_Winter_France | -0.464 | 0.643 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_France | -0.597 | 0.550 | 1.000 |
| GWTS_Winter_Edge | - | GWTS_Winter_France | -0.842 | 0.400 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_Inside | 0.437 | 0.662 | 1.000 |
| GWTS_Summer_France | - | GWTS_Winter_Inside | 0.732 | 0.464 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_Inside | 0.665 | 0.506 | 1.000 |
| GWTS_Winter_Edge | - | GWTS_Winter_Inside | 0.364 | 0.716 | 1.000 |
| GWTS_Winter_France | - | GWTS_Winter_Inside | 1.192 | 0.233 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Summer_Edge | 3.801 | 0.000 | **0.008** |
| GWTS_Summer_France | - | Pygmy_Summer_Edge | 3.840 | 0.000 | **0.007** |
| GWTS_Summer_Inside | - | Pygmy_Summer_Edge | 3.994 | 0.000 | **0.004** |
| GWTS_Winter_Edge | - | Pygmy_Summer_Edge | 3.632 | 0.000 | **0.016** |
| GWTS_Winter_France | - | Pygmy_Summer_Edge | 4.151 | 0.000 | **0.002** |
| GWTS_Winter_Inside | - | Pygmy_Summer_Edge | 3.372 | 0.001 | **0.040** |
| GWTS_Summer_Edge | - | Pygmy_Summer_France | 3.023 | 0.003 | 0.118 |
| GWTS_Summer_France | - | Pygmy_Summer_France | 3.128 | 0.002 | 0.088 |
| GWTS_Summer_Inside | - | Pygmy_Summer_France | 3.214 | 0.001 | 0.067 |
| GWTS_Winter_Edge | - | Pygmy_Summer_France | 2.890 | 0.004 | 0.162 |
| GWTS_Winter_France | - | Pygmy_Summer_France | 3.462 | 0.001 | **0.029** |
| GWTS_Winter_Inside | - | Pygmy_Summer_France | 2.620 | 0.009 | 0.325 |
| Pygmy_Summer_Edge | - | Pygmy_Summer_France | -0.543 | 0.587 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Summer_Outside | 4.868 | 0.000 | **0.000** |
| GWTS_Summer_France | - | Pygmy_Summer_Outside | 4.813 | 0.000 | **0.000** |
| GWTS_Summer_Inside | - | Pygmy_Summer_Outside | 5.057 | 0.000 | **0.000** |
| GWTS_Winter_Edge | - | Pygmy_Summer_Outside | 4.657 | 0.000 | **0.000** |
| GWTS_Winter_France | - | Pygmy_Summer_Outside | 5.081 | 0.000 | **0.000** |
| GWTS_Winter_Inside | - | Pygmy_Summer_Outside | 4.424 | 0.000 | **0.001** |
| Pygmy_Summer_Edge | - | Pygmy_Summer_Outside | 0.905 | 0.365 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Summer_Outside | 1.411 | 0.158 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Winter_Edge | 2.892 | 0.004 | 0.165 |
| GWTS_Summer_France | - | Pygmy_Winter_Edge | 3.003 | 0.003 | 0.123 |
| GWTS_Summer_Inside | - | Pygmy_Winter_Edge | 3.088 | 0.002 | 0.099 |
| GWTS_Winter_Edge | - | Pygmy_Winter_Edge | 2.757 | 0.006 | 0.233 |
| GWTS_Winter_France | - | Pygmy_Winter_Edge | 3.347 | 0.001 | **0.043** |
| GWTS_Winter_Inside | - | Pygmy_Winter_Edge | 2.478 | 0.013 | 0.462 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_Edge | -0.767 | 0.443 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_Edge | -0.198 | 0.843 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_Edge | -1.667 | 0.096 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Winter_France | 2.870 | 0.004 | 0.168 |
| GWTS_Summer_France | - | Pygmy_Winter_France | 2.985 | 0.003 | 0.128 |
| GWTS_Summer_Inside | - | Pygmy_Winter_France | 3.062 | 0.002 | 0.106 |
| GWTS_Winter_Edge | - | Pygmy_Winter_France | 2.741 | 0.006 | 0.239 |
| GWTS_Winter_France | - | Pygmy_Winter_France | 3.325 | 0.001 | **0.046** |
| GWTS_Winter_Inside | - | Pygmy_Winter_France | 2.467 | 0.014 | 0.464 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_France | -0.694 | 0.488 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_France | -0.143 | 0.886 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_France | -1.566 | 0.117 | 1.000 |
| Pygmy_Winter_Edge | - | Pygmy_Winter_France | 0.052 | 0.959 | 0.959 |
| GWTS_Summer_Edge | - | Pygmy_Winter_Outside | 2.437 | 0.015 | 0.489 |
| GWTS_Summer_France | - | Pygmy_Winter_Outside | 2.580 | 0.010 | 0.356 |
| GWTS_Summer_Inside | - | Pygmy_Winter_Outside | 2.637 | 0.008 | 0.318 |
| GWTS_Winter_Edge | - | Pygmy_Winter_Outside | 2.314 | 0.021 | 0.661 |
| GWTS_Winter_France | - | Pygmy_Winter_Outside | 2.942 | 0.003 | 0.143 |
| GWTS_Winter_Inside | - | Pygmy_Winter_Outside | 2.023 | 0.043 | 1.000 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_Outside | -1.214 | 0.225 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_Outside | -0.621 | 0.534 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_Outside | -2.129 | 0.033 | 1.000 |
| Pygmy_Winter_Edge | - | Pygmy_Winter_Outside | -0.433 | 0.665 | 1.000 |
| Pygmy_Winter_France | - | Pygmy_Winter_Outside | -0.475 | 0.635 | 1.000 |

**Table S4.3.** Dunn's post-hoc results for Inverse Simpson value of microbiomes of shrews. Tests are performed for pairwise groups separated to shrew species, season and zone. Benjamini-Hochberg adjusted p-values < 0.05 are in bold.

| Pairwise Comparison | | | Z-ratio | Unadjusted P-value | Adjusted P-value |
|---|---|---|---|---|---|
| GWTS_Summer_Edge | - | GWTS_Summer_France | -0.200 | 0.842 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Summer_Inside | -0.660 | 0.509 | 1.000 |
| GWTS_Summer_France | - | GWTS_Summer_Inside | -0.412 | 0.681 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_Edge | -0.017 | 0.986 | 0.986 |
| GWTS_Summer_France | - | GWTS_Winter_Edge | 0.179 | 0.858 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_Edge | 0.624 | 0.533 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_France | -0.686 | 0.493 | 1.000 |
| GWTS_Summer_France | - | GWTS_Winter_France | -0.465 | 0.642 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_France | -0.100 | 0.921 | 1.000 |
| GWTS_Winter_Edge | - | GWTS_Winter_France | -0.655 | 0.512 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_Inside | 0.525 | 0.600 | 1.000 |
| GWTS_Summer_France | - | GWTS_Winter_Inside | 0.684 | 0.494 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_Inside | 1.180 | 0.238 | 1.000 |
| GWTS_Winter_Edge | - | GWTS_Winter_Inside | 0.526 | 0.599 | 1.000 |
| GWTS_Winter_France | - | GWTS_Winter_Inside | 1.147 | 0.251 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Summer_Edge | 3.181 | 0.001 | 0.082 |
| GWTS_Summer_France | - | Pygmy_Summer_Edge | 3.140 | 0.002 | 0.093 |
| GWTS_Summer_Inside | - | Pygmy_Summer_Edge | 3.800 | 0.000 | **0.009** |
| GWTS_Winter_Edge | - | Pygmy_Summer_Edge | 3.107 | 0.002 | 0.100 |
| GWTS_Winter_France | - | Pygmy_Summer_Edge | 3.485 | 0.000 | **0.029** |
| GWTS_Winter_Inside | - | Pygmy_Summer_Edge | 2.667 | 0.008 | 0.329 |
| GWTS_Summer_Edge | - | Pygmy_Summer_France | 2.779 | 0.005 | 0.256 |
| GWTS_Summer_France | - | Pygmy_Summer_France | 2.779 | 0.005 | 0.262 |
| GWTS_Summer_Inside | - | Pygmy_Summer_France | 3.366 | 0.001 | **0.044** |
| GWTS_Winter_Edge | - | Pygmy_Summer_France | 2.724 | 0.006 | 0.297 |
| GWTS_Winter_France | - | Pygmy_Summer_France | 3.129 | 0.002 | 0.095 |
| GWTS_Winter_Inside | - | Pygmy_Summer_France | 2.295 | 0.022 | 0.804 |
| Pygmy_Summer_Edge | - | Pygmy_Summer_France | -0.211 | 0.833 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Summer_Outside | 4.326 | 0.000 | **0.001** |
| GWTS_Summer_France | - | Pygmy_Summer_Outside | 4.183 | 0.000 | **0.002** |
| GWTS_Summer_Inside | - | Pygmy_Summer_Outside | 4.955 | 0.000 | **0.000** |
| GWTS_Winter_Edge | - | Pygmy_Summer_Outside | 4.210 | 0.000 | **0.002** |
| GWTS_Winter_France | - | Pygmy_Summer_Outside | 4.483 | 0.000 | **0.000** |
| GWTS_Winter_Inside | - | Pygmy_Summer_Outside | 3.792 | 0.000 | **0.009** |
| Pygmy_Summer_Edge | - | Pygmy_Summer_Outside | 1.003 | 0.316 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Summer_Outside | 1.160 | 0.246 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Winter_Edge | 2.483 | 0.013 | 0.508 |
| GWTS_Summer_France | - | Pygmy_Winter_Edge | 2.499 | 0.012 | 0.498 |
| GWTS_Summer_Inside | - | Pygmy_Winter_Edge | 3.088 | 0.002 | 0.105 |
| GWTS_Winter_Edge | - | Pygmy_Winter_Edge | 2.432 | 0.015 | 0.570 |
| GWTS_Winter_France | - | Pygmy_Winter_Edge | 2.866 | 0.004 | 0.208 |
| GWTS_Winter_Inside | - | Pygmy_Winter_Edge | 1.986 | 0.047 | 1.000 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_Edge | -0.581 | 0.561 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_Edge | -0.345 | 0.730 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_Edge | -1.570 | 0.117 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Winter_France | 2.718 | 0.007 | 0.289 |
| GWTS_Summer_France | - | Pygmy_Winter_France | 2.722 | 0.006 | 0.292 |
| GWTS_Summer_Inside | - | Pygmy_Winter_France | 3.306 | 0.001 | 0.054 |
| GWTS_Winter_Edge | - | Pygmy_Winter_France | 2.664 | 0.008 | 0.324 |
| GWTS_Winter_France | - | Pygmy_Winter_France | 3.075 | 0.002 | 0.107 |
| GWTS_Winter_Inside | - | Pygmy_Winter_France | 2.234 | 0.025 | 0.867 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_France | -0.271 | 0.787 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_France | -0.057 | 0.955 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_France | -1.222 | 0.222 | 1.000 |
| Pygmy_Winter_Edge | - | Pygmy_Winter_France | 0.287 | 0.774 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Winter_Outside | 2.249 | 0.024 | 0.857 |
| GWTS_Summer_France | - | Pygmy_Winter_Outside | 2.282 | 0.022 | 0.810 |
| GWTS_Summer_Inside | - | Pygmy_Winter_Outside | 2.856 | 0.004 | 0.210 |
| GWTS_Winter_Edge | - | Pygmy_Winter_Outside | 2.205 | 0.027 | 0.906 |
| GWTS_Winter_France | - | Pygmy_Winter_Outside | 2.659 | 0.008 | 0.322 |
| GWTS_Winter_Inside | - | Pygmy_Winter_Outside | 1.752 | 0.080 | 1.000 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_Outside | -0.811 | 0.418 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_Outside | -0.562 | 0.574 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_Outside | -1.807 | 0.071 | 1.000 |
| Pygmy_Winter_Edge | - | Pygmy_Winter_Outside | -0.222 | 0.824 | 1.000 |
| Pygmy_Winter_France | - | Pygmy_Winter_Outside | -0.504 | 0.614 | 1.000 |

**Table S4.4.** Dunn's post-hoc results for Pielou's eveness value of microbiomes of shrews. Tests are performed for pairwise groups separated to shrew species, season and zone. Benjamini-Hochberg adjusted p-values < 0.05 are in bold.

| Pairwise Comparison | | | Z-ratio | Unadjusted P-value | Adjusted P-value |
|---|---|---|---|---|---|
| GWTS_Summer_Edge | - | GWTS_Summer_France | -0.342 | 0.732 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Summer_Inside | -0.633 | 0.526 | 1.000 |
| GWTS_Summer_France | - | GWTS_Summer_Inside | -0.246 | 0.806 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_Edge | 0.016 | 0.987 | 0.987 |
| GWTS_Summer_France | - | GWTS_Winter_Edge | 0.348 | 0.728 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_Edge | 0.631 | 0.528 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_France | -0.667 | 0.505 | 1.000 |
| GWTS_Summer_France | - | GWTS_Winter_France | -0.320 | 0.749 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_France | -0.104 | 0.917 | 1.000 |
| GWTS_Winter_Edge | - | GWTS_Winter_France | -0.665 | 0.506 | 1.000 |
| GWTS_Summer_Edge | - | GWTS_Winter_Inside | 0.449 | 0.654 | 1.000 |
| GWTS_Summer_France | - | GWTS_Winter_Inside | 0.756 | 0.450 | 1.000 |
| GWTS_Summer_Inside | - | GWTS_Winter_Inside | 1.078 | 0.281 | 1.000 |
| GWTS_Winter_Edge | - | GWTS_Winter_Inside | 0.419 | 0.675 | 1.000 |
| GWTS_Winter_France | - | GWTS_Winter_Inside | 1.061 | 0.289 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Summer_Edge | 3.188 | 0.001 | 0.076 |
| GWTS_Summer_France | - | Pygmy_Summer_Edge | 3.286 | 0.001 | 0.057 |
| GWTS_Summer_Inside | - | Pygmy_Summer_Edge | 3.780 | 0.000 | **0.010** |
| GWTS_Winter_Edge | - | Pygmy_Summer_Edge | 3.081 | 0.002 | 0.105 |
| GWTS_Winter_France | - | Pygmy_Summer_Edge | 3.471 | 0.001 | **0.030** |
| GWTS_Winter_Inside | - | Pygmy_Summer_Edge | 2.748 | 0.006 | 0.246 |
| GWTS_Summer_Edge | - | Pygmy_Summer_France | 2.933 | 0.003 | 0.155 |
| GWTS_Summer_France | - | Pygmy_Summer_France | 3.055 | 0.002 | 0.110 |
| GWTS_Summer_Inside | - | Pygmy_Summer_France | 3.494 | 0.000 | **0.028** |
| GWTS_Winter_Edge | - | Pygmy_Summer_France | 2.843 | 0.004 | 0.197 |
| GWTS_Winter_France | - | Pygmy_Summer_France | 3.249 | 0.001 | 0.063 |
| GWTS_Winter_Inside | - | Pygmy_Summer_France | 2.519 | 0.012 | 0.424 |
| Pygmy_Summer_Edge | - | Pygmy_Summer_France | -0.065 | 0.948 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Summer_Outside | 4.183 | 0.000 | **0.002** |
| GWTS_Summer_France | - | Pygmy_Summer_Outside | 4.195 | 0.000 | **0.002** |
| GWTS_Summer_Inside | - | Pygmy_Summer_Outside | 4.786 | 0.000 | **0.000** |
| GWTS_Winter_Edge | - | Pygmy_Summer_Outside | 4.037 | 0.000 | **0.003** |
| GWTS_Winter_France | - | Pygmy_Summer_Outside | 4.338 | 0.000 | **0.001** |
| GWTS_Winter_Inside | - | Pygmy_Summer_Outside | 3.726 | 0.000 | **0.012** |
| Pygmy_Summer_Edge | - | Pygmy_Summer_Outside | 0.856 | 0.392 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Summer_Outside | 0.871 | 0.383 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Winter_Edge | 2.680 | 0.007 | 0.287 |
| GWTS_Summer_France | - | Pygmy_Winter_Edge | 2.818 | 0.005 | 0.208 |
| GWTS_Summer_Inside | - | Pygmy_Winter_Edge | 3.258 | 0.001 | 0.062 |
| GWTS_Winter_Edge | - | Pygmy_Winter_Edge | 2.593 | 0.010 | 0.352 |
| GWTS_Winter_France | - | Pygmy_Winter_Edge | 3.023 | 0.003 | 0.120 |
| GWTS_Winter_Inside | - | Pygmy_Winter_Edge | 2.255 | 0.024 | 0.772 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_Edge | -0.393 | 0.694 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_Edge | -0.309 | 0.757 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_Edge | -1.233 | 0.217 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Winter_France | 2.805 | 0.005 | 0.212 |
| GWTS_Summer_France | - | Pygmy_Winter_France | 2.936 | 0.003 | 0.156 |
| GWTS_Summer_Inside | - | Pygmy_Winter_France | 3.367 | 0.001 | **0.043** |
| GWTS_Winter_Edge | - | Pygmy_Winter_France | 2.718 | 0.007 | 0.263 |
| GWTS_Winter_France | - | Pygmy_Winter_France | 3.134 | 0.002 | 0.090 |
| GWTS_Winter_Inside | - | Pygmy_Winter_France | 2.391 | 0.017 | 0.555 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_France | -0.191 | 0.848 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_France | -0.120 | 0.905 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_France | -1.002 | 0.317 | 1.000 |
| Pygmy_Winter_Edge | - | Pygmy_Winter_France | 0.187 | 0.852 | 1.000 |
| GWTS_Summer_Edge | - | Pygmy_Winter_Outside | 2.500 | 0.012 | 0.435 |
| GWTS_Summer_France | - | Pygmy_Winter_Outside | 2.650 | 0.008 | 0.306 |
| GWTS_Summer_Inside | - | Pygmy_Winter_Outside | 3.079 | 0.002 | 0.104 |
| GWTS_Winter_Edge | - | Pygmy_Winter_Outside | 2.417 | 0.016 | 0.532 |
| GWTS_Winter_France | - | Pygmy_Winter_Outside | 2.863 | 0.004 | 0.189 |
| GWTS_Winter_Inside | - | Pygmy_Winter_Outside | 2.075 | 0.038 | 1.000 |
| Pygmy_Summer_Edge | - | Pygmy_Winter_Outside | -0.571 | 0.568 | 1.000 |
| Pygmy_Summer_France | - | Pygmy_Winter_Outside | -0.477 | 0.634 | 1.000 |
| Pygmy_Summer_Outside | - | Pygmy_Winter_Outside | -1.417 | 0.157 | 1.000 |
| Pygmy_Winter_Edge | - | Pygmy_Winter_Outside | -0.172 | 0.864 | 1.000 |
| Pygmy_Winter_France | - | Pygmy_Winter_Outside | -0.354 | 0.723 | 1.000 |

## 6.3.2. Supplementary Figures

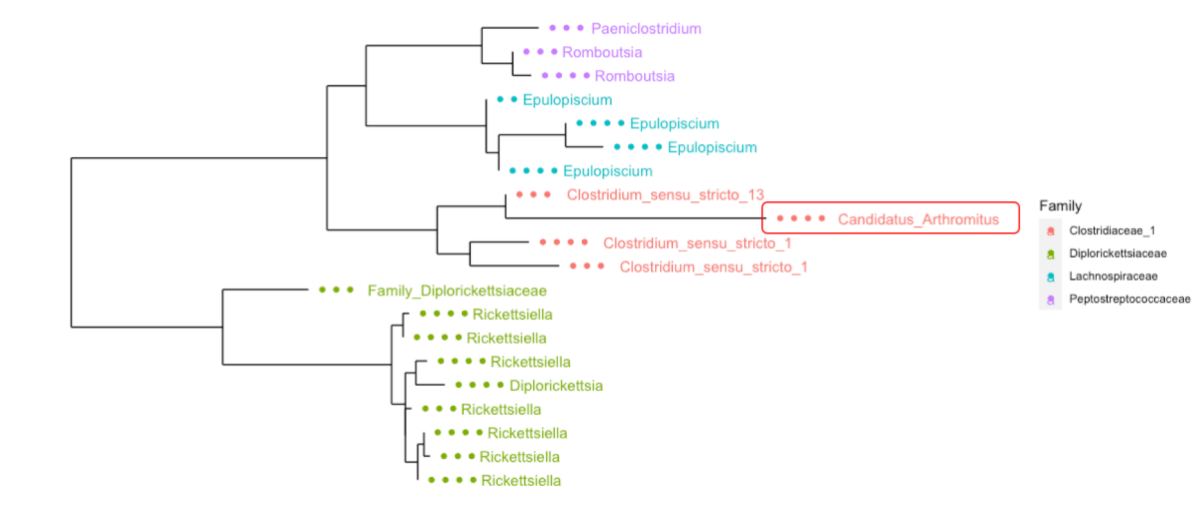



**Figure S4.1.** Maximum likelihood tree of ASVs detected from the four families Clostridiaceae, Diplorickettsiaceae, Lachnospiraceae and Peptostreptococcaceae. *Candidatus Arthromitus* is clustered within Clostridiaceae rather than Lachnospiraceae. This means it has been misidentified and is actually *Candidatus Savagella.*

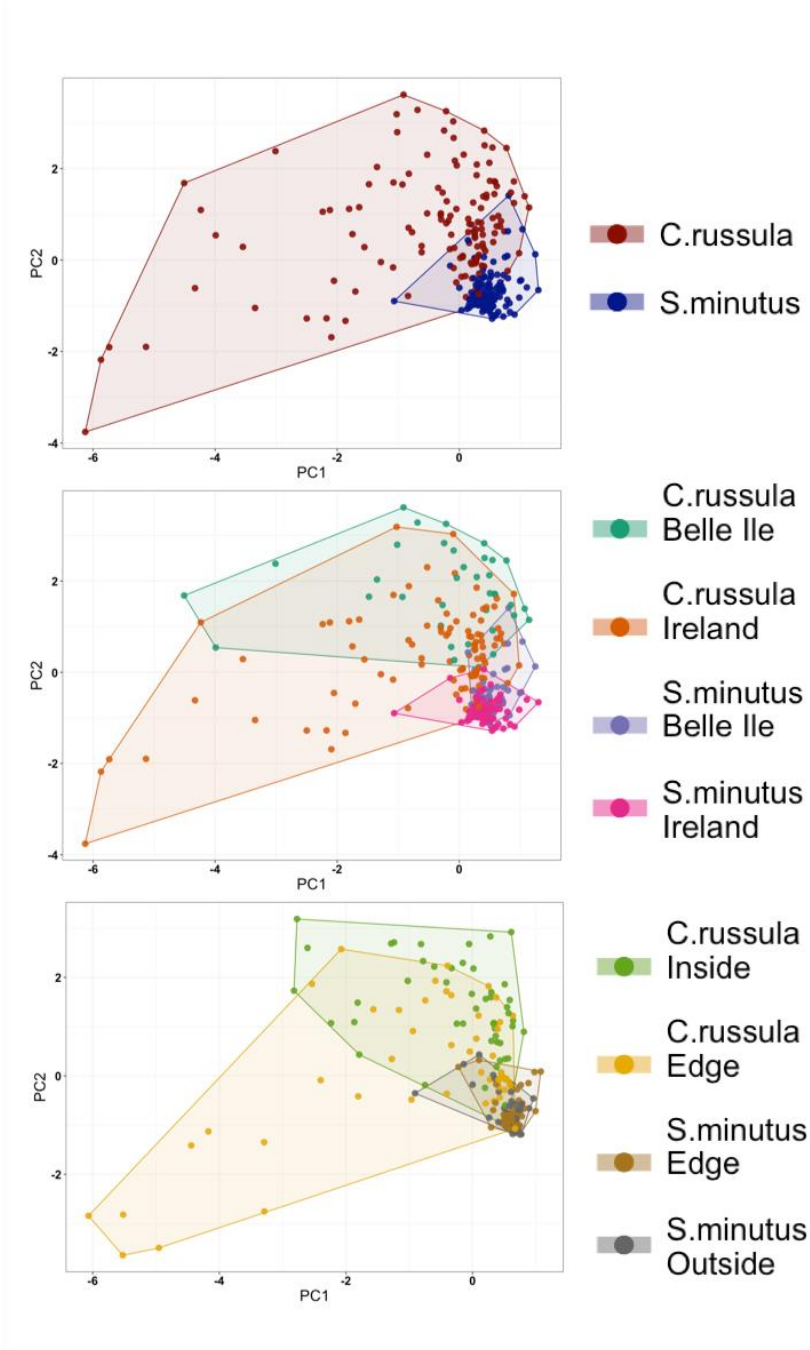



**Figure S4.2.** PCA of clr-transformed data. Top plot shows samples coloured according to species of shrew. Middle plot is the same plot coloured according to both species of shrew and country of origin. Bottom plot shows Irish samples coloured according to species of shrew and invasion zone

## 6.4. Certificates and Licenses

### 6.4.1. Trapping License from National Parks and Wildlife Services (NPWS) Ireland

An Roinn Ealaíon, Oidhreachta,
Gnóthaí Réigiúnacha, Tuaithe agus Gaeltachta

Department of Arts, Heritage,
Regional, Rural and Gaeltacht Affairs

**Licence No. C21/2017-Amended**

**NATIONAL PARKS & WILDLIFE SERVICE**

**Wildlife Acts 1976 to 2012 – Sections 23 and 34**

**LICENCE TO CAPTURE PROTECTED WILD ANIMALS FOR EDUCATIONAL, SCIENTIFIC OR OTHER PURPOSES**

The Minister for Arts, Heritage, Regional, Rural and Gaeltacht Affairs in exercise of the powers conferred on her by Sections 9, 23 and 34 of the Wildlife Acts 1976 to 2012 authorises:

**Samuel Browett**
**School of Environment and Life Sciences, University of Salford,**
**Salford, M5 4WT United Kingdom**

To disturb specimens of the species specified in Column 1 of the Schedule hereunder in the area specified in Column 2 by the means specified in column 3 for scientific educational or other purposes during the period beginning **27 April 2017** and ending on **31 December 2018, (as amended by this licence)** subject to the conditions listed overleaf.

**SCHEDULE**

| 1 | 2 | 3 |
|---|---|---|
| **Species** | **Area** | **Means of capture** |
| **≤160 Pygmy Shrew *(Sorex minutus)*** | **Counties Carlow, Laois, Limerick, Offaly, Wexford, Cork, Kilkenny, Tipperary, Waterford & Westmeath, Wexford** | **Captured via live-trapping and euthanized dislocation** |

**Dated 8 August 2017**

**For the Minister for Arts, Heritage, Regional, Rural and Gaeltacht Affairs**

National Parks & Wildlife Services
LICENCE

## 6.4.2. HPRA Individual Authorisation

**TERMS OF THE AUTHORISATION**

**1 INDIVIDUAL AUTHORISATION NUMBER**

AE18982/I323

**2 PURPOSE FOR WHICH THE INDIVIDUAL IS AUTHORISED PURSUANT TO PART 8 OF S.I. NO. 543 OF 2012**

Performing euthanasia

**3 ESTABLISHMENT(S) AT WHICH THE INDIVIDUAL IS AUTHORISED FOR THE PURPOSE SPECIFIED ABOVE PURSUANT TO S.I. NO. 543 OF 2012 AS AMENDED**

University College Dublin

**4 SPECIES / CATEGORY OF ANIMALS WITH RESPECT TO WHICH THE INDIVIDUAL IS AUTHORISED FOR THE PURPOSE SPECIFIED ABOVE PURSUANT TO S.I. NO. 543 OF 2012 AS AMENDED**

Wild mammals

Rodents

**5 CATEGORY OF PROCEDURE / METHOD OF EUTHANASIA (I.E. ACTIVITIES) WHICH THE INDIVIDUAL IS AUTHORISED FOR THE PURPOSE SPECIFIED ABOVE PURSUANT TO S.I. NO. 543 OF 2012 AS AMENDED**

Euthanasia using anaesthetic overdose

Euthanasia using carbon dioxide

Euthanasia using cervical dislocation

## 6.4.3. Certificate for Completing Laboratory Animals in Science Training (LAST) Course

LAST IRELAND

LAST – Ireland
9th November 2016

Category; A,B,C  Species : Wildlife

This is to certify that

Samuel Browett

Attended a course and passed an assessment of the course, based on the **EU EWG guidelines contained in the framework document, which fulfill the requirements under article 23 and 24 of directive 2010/63 EU for the Categories of persons stated below.*
**A skills attainment record should be presented with this certificate.**

| Module | Title of Core Module |
|---|---|
| 1 | National Legislation (Irish SI 543/12) |
| 2 | Ethics, Animal welfare and the 3 Rs ( level 1) |
| 3.1 | Basic and Appropriate Biology (species noted on certificate ) |
| 4 | Animal care, health and management ( species noted on certificate ) |
| 5 | Recognition of pain, suffering and distress ( species noted on certificate ) |
| 6.1 | Humane methods of euthanasia ( theory) |
| 7 | Minimally invasive procedures without anaesthesia. |
| 9 | Ethics, Animal welfare and the 3 Rs ( level 2) |
| 10 | Design of procedures and projects ( level 1) |
| 11 | Design of procedures and projects ( level 2) |
| | **Title of Practical Module** |
| 3.2 | Basic and appropriate biology ( species noted on certificate ) |
| 8 | Minimally invasive procedures without anaesthesia |
| 6.2 | *Humane methods of euthanasia (performed as part of establishment training) |
| | **Title of Additional Module** |
| 20 | Anaesthesia for minor procedures |
| 22 | Principles of surgery |
| 30 | Introduction to local environment |

** extract from National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes A working document on the development of a common education and training framework to fulfill the requirements under Articles 23 and 24 - Replacing consensus document I of 22-23 March 2012 – 18/19 Sept 2013

**Signed**

Date: 2016.11.27 16:50:27 Z

**Peter F. Nowlan** (Course organiser)
**Contact information:**
PO Box 12172 Glenageary Co. Dublin

*Category A: Persons carrying out procedures on animals, Category B; Persons designing procedures and projects
Category C; Persons taking care of animals, Category D; persons performing euthanasia

## 6.4.4. University of Salford Ethical Approval

**Research, Innovation and Academic Engagement Ethical Approval Panel**

Research Centres Support Team
G0.3 Joule House
University of Salford
M5 4WT

T +44(0)161 295 5278

www.salford.ac.uk/

20 June 2017

**Samuel Browett**

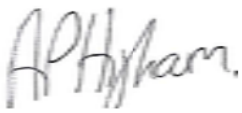
Dear Samuel,

**RE: ETHICS APPLICATION ST1617-55 - Know your enemy: A multi-disciplinary approach to determine how the pygmy shrew (Sorex minutus) in Ireland succumbs to the invasion of the greater white-toothed shrew (Crocidura russula).**

Based on the information you provided, I am pleased to inform you that your application ST1617-55 has been approved.

If there are any changes to the project and/ or its methodology, please inform the Panel as soon as possible by contacting S&T-ResearchEthics@salford.ac.uk

Yours sincerely,



Dr Anthony Higham
Chair of the Science & Technology Research Ethics Panel

## 6.4.4. University College Dublin Ethical Approval

**UCD Office of Research Ethics**

Roebuck Castle
University College Dublin
Belfield, Dublin 4, Ireland

T +353 1 716 8767

**An Oifig Eitic Thaighde UCD**

Caisleán an Ruabhoic
An Coláiste Ollscoile, Baile Átha Cliath
Belfield, Baile Átha Cliath 4, Éire

submissions.arec@ucd.ie
www.ucd.ie/researchethics

14th July 2017

Dr Jonathan Yearsley
UCD School of Biology and Environmental Science
O'Brien Centre for Science - East
Belfield
Dublin 4

**Re:** **AREC-17-14-Yearsley:** *Know your enemy: A multi-disciplinary approach to determine how the pygmy shrew (Sorex minutus) in Ireland succumbs to the invasion of the greater white-toothed shrew (Crocidura russula)*

Dear Dr Yearsley

Thank you for your correspondence to the Animal Research Ethics Committee [12/07/17]. The Decision of the Committee is to **grant approval** for your request to amend and extend this protocol which is still subject to the following conditions.

- This approval is for the work specified in the above protocol and is granted until **31/12/18.**
- Please also note that I request that you immediately notify the Animal Care Staff / Designated Veterinarian should unforeseen adverse effects occur in the course of your research and, in the event of agreed severity bandings being exceeded, provide written notification to the Animal Welfare Board on the Unforeseen Events Report Form detailing the circumstances and the actions taken to alleviate suffering.
- Should it prove necessary to amend the protocol, or extend the time period, please complete an AREC Amendment Form providing details of the revised protocol/time period, with appropriate justification, and submit to the Animal Research Ethics Committee (email: submissions.arec@ucd.ie ), quoting the above protocol reference;
- Please note that you may require copies of submitted documentation relating to this approved protocol and therefore we advise that you retain copies for your own records.
- Approved protocols are subject to a Research Ethics Compliance Review.

If you have queries, please contact the Office of Research Ethics.

Yours sincerely,

Finola Leonard

Dr Finola Leonard
Chair, Animal Research Ethics Committee

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