Ecological and molecular basis of differential resource use in populations of burying beetles *Nicrophorus vespilloides*

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This thesis is submitted for the degree of Doctor of Philosophy

Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the preface and specified in the text.

It is not substantially the same as any work that has already been submitted before for any degree or other qualification except as declared in the preface and specified in the text.

It does not exceed the word limit prescribed by the Biology Degree Committee.

Swastika Issar

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Abstract

A fundamental prediction of ecological theory is that competition for resources can drive the evolution of specialised resource use. One way in which costly competition can be avoided is via individual specialisation, i.e., the persistence of specialised individuals within a generalist population that utilise a smaller subset of the entire population's resource base. This could occur through the evolution of genetic morphs that specialise on different resources. Although correlational evidence exists that is consistent with this prediction, there is surprisingly little evidence that competition causes resource specialisation.

Burying beetles are an ideal species for testing this prediction. They require the carcass of a small vertebrate such as a mouse or a songbird for reproduction, but carcasses can be unpredictably distributed and competition to secure ownership is correspondingly intense. For my PhD project in Prof. Rebecca Kilner's lab, I tested whether this fierce competition for a carcass breeding resource has driven the evolution of beetles that specialise in breeding on dead mammals or dead birds.

With field experiments at three different woodlands, I tested for evidence of a bias in the type of carcass favoured by *Nicrophorus vespilloides* and if this bias changed across the burying beetle season (from April to October each year). I found spatial and seasonal variation within each of the three populations in the preference for dead mice over dead birds. In two populations, beetles were more likely to be trapped upon dead mice overall, but were occasionally trapped with greater frequencies on dead birds. This trend was completely reversed for the third population, where beetles were more likely to be found in traps baited with dead birds than dead mice.

The patterns of resource use I observed in the field could be due to adaptive partitioning of resource type within populations. To test this hypothesis, I measured the reproductive success of wild beetles induced to breed on different types of carrion. Although I found seasonal variation in beetle reproductive success on different types of carrion, I found no evidence that this resulted from variation in carrion preferences at the individual or population level. Instead, it is more likely to be explained by variation in individual quality.

In collaboration with Dr Michael Sheehan at Cornell University, we sequenced females trapped on each type of carrion within all three woodlands, to test whether carrion specialisation was associated with genetic differences. Consistent with this possibility, we found divergence at ~ 50 loci in each of the three populations. Several of these loci were associated with olfaction and sensory-system development.

In the lab, I set up replicate experimentally evolving populations of *N. vespilloides* which were bred either on mice or chicks for ~ 20 generations. I used these populations to test whether, in principle, beetles within a natural population could become divergently adapted to specialise on different types of carrion. I found no evidence to support this possibility, perhaps because there was insufficient standing genetic variation in the founding populations to select upon. However, there was some indication that the experimental populations might have diverged in cryptic ways that I did not measure directly.

To understand the chemical basis for differential resource use, I carried out several analyses in collaboration with Prof. Patrizia d'Ettorre at Université Paris, using mass spectrometric techniques. We found little evidence that the volatiles emitted from carrion differ substantially between birds and mice. We also found little evidence that a beetle's cuticular hydrocarbons predict the carrion it will be attracted to in nature. However, we did find seasonal variation in the cuticular hydrocarbon profiles of wild-caught beetles that could be related to beetle quality or breeding status.

In short, although we found some evidence for differential carrion use within wild burying beetle populations and some indication that this is associated with genetic differences among individuals, some of this variation is also due to phenotypic variation in individual quality. While it is possible that carrion specialists could evolve within natural populations, we found no strong evidence to suggest that this happens routinely.

Table of Contents

Chapter 1

Introduction

A key challenge for evolutionary biology is to understand how populations of the same species diverge genetically and phenotypically. Individuals of different populations can diverge by adapting to local environments, through the stochastic fixation of beneficial mutations, and via genetic drift. Across a wide geographical range, variable and divergent environments can cause populations to become locally and adaptively differentiated in their behaviour and life history- a process that could ultimately culminate in ecological speciation in some cases (Schluter 1996, Hendry et al. 2007, Nosil 2012).

Ecological niche differentiation could be linked to local adaptation to food resources (Feder et al. 1994, Ackermann & Doebeli 2004, Dieckmann et al. 2004, Huber et al. 2007, Matsubayashi et al. 2010) and their associated microbiota (Shropshire & Bordenstein 2016, Dillard & Benbow 2020), interactions with other species (Taper & Case 1992, Denno et al. 1995, Schluter 2000, Langerhans et al. 2007) and, in the case of species that socially interact with each other, differences in the social environment (Bourke 2011, Drown & Wade 2014, Korb & Heinze 2016). Following this, sexual selection on mating behaviour and associated traits can reinforce and accelerate the process of speciation (Lande & Kirkpatrick 1988, Dieckmann & Doebeli 1999, Panhuis et al. 2001, Boughman 2002, Via 2009). For ecological speciation to occur, there must be a heritable mechanism that links the source of divergent natural selection to a form of reproductive isolation (Schluter 2000, Nosil 2012, Verzijden et al. 2012).

An important catalyst for divergence in resource use is intraspecific competition (Rosenzweig 1978; Dieckmann et al. 2004, Svanbäck & Bolnick 2007). Predictions from foraging theory suggest that, in conditions of high intraspecific competition and scarcity of preferred resources, populations should be composed of generalists that opportunistically utilise the available resource base (Stephens & Krebs 1986, Schoener 1971, Pulliam 1974). However, empirical evidence suggests that many generalist populations are in fact composed of relatively specialised individuals and that high intraspecific competition leads to an increase in individual specialisation within populations (Bolnick et al. 2003, Ackermann & Doebeli 2004, Svanbäck & Bolnick 2007, Araújo et al. 2008). Certain frequency dynamics- such as when novel resources are relatively rare or

ephemeral in the environment- can be conducive to the persistence of generalist and specialist resource-use phenotypes within the same population (Fortin et al. 2008, Bono et al. 2015).

Environmental variation and divergent selection can also result in the evolution of generalised and highly plastic populations (West-Eberhard 2005, Pfennig et al. 2010, Reed et al. 2010, Snell-Rood 2013). Phenotypic plasticity can, therefore, evolve as an alternative to ecologically driven genetic divergence resulting in individuals whose phenotype can vary according to their environmental conditions (Stearns 1989, Pfennig et al. 2010, Nosil 2012). On one hand, phenotypic plasticity can act as a deterrent to genetic divergence and adaptation by eliminating the need for a hereditary mechanism that facilitates novel resource use and, on the other hand, developmental and phenotypic plasticity can aid in the exploitation of novel resources that may eventually lead to adaptive divergence within populations (Price et al. 2003, Scheiner & DeWitt 2004, Svänback et al. 2009, Thibert-Plante & Hendry 2011, Snell-Rood 2013, Forsman 2015). Further work is needed to understand the mechanisms behind divergent resource use in natural populations, and their evolutionary implications.

Carrion resources are widely distributed resources that are uniquely suited to studying the emergence, evolution and maintenance of divergent resource use in nature. While reviewing the life histories of necrophagous insects, Blanckenhorn (2015) identified several key ecological characteristics of animal carcasses: ephemerality; unpredictability in time and space; variable frequency of occurrence at local and global scales; large size range; nutritional quality range; nutritional quantity range; diversity across species; diversity of nutrients within patches; diversity in niches within patches (various organs or parts); and diverse community of consumers.

Carcasses thus comprise a large number of spatiotemporal niches and are, consequently, associated with high levels of intra- and interspecific competition (Finn 2001, Barton et al. 2013, Benbow et al. 2015, Benbow et al. 2019). On land, flies and beetles form the bulk of the arthropod communities found on carrion (Dillon 1997, De Jong & Chadwick 1999, Watson & Carlton 2005, Matuszewski et al. 2010, Merritt & De Jong 2015). Studies have reported as many as four hundred different insect species on animal carcasses, with variations depending on the carrion size, carrion type and time of year (Reed 1958, Payne 1965, Kočárek 2003, Moretti et al. 2008, Merritt & De Jong 2015). There is also biogeographical variation in the insect communities found on carcasses at different stages of decay (Shahid et al. 2000, Verves 2002, Whitworth 2006, Anderson 2010). Most necrophilous species have a limited geographical distribution, or are confined to specific

habitats, though a few exceptions such as the house fly (*Musca domestica*), the hairy maggot blowfly (*Chrysomya rufifacies*) or the hide beetle (*Dermestes maculatus*) are more widespread (Farwig et al. 2014, Merritt & De Jong 2015, Anderson et al. 2019, Babcock et al. 2019, Langer et al. 2019).

Carrion insects have evolved adaptations that enable them to detect and colonise carrion resources at appropriate stages of decay to maximise their utilisation (Norris 1965, Stensmyr et al. 2002, von Hoermann et al. 2011, Picard et al. 2015). For example, blowflies (Diptera: Calliphoridae) and burying beetles (Coleoptera: Silphidae), which are among the first insect visitors on a carcass, can identify and orient towards the sulphur-containing volatile organic compounds that are produced during bacterial decomposition soon after the death of an animal (Stensmyr et al. 2002, Kalinova et al. 2009, von Hoermann et al. 2013). The decomposition process that follows is enhanced by the succession of a huge richness of carrion-associated insects (Peschke et al. 1987, Matuszewski et al. 2008, Sharanowski et al. 2008, Goff 2009, von Hoermann et al. 2012).

Ecological separation and adaptive diversification have occurred in response to this intense competition for a limiting resource (Fuller 1934, Payne & Crossley 1966, Schoenly & Reid 1983, Peschke et al. 1987, Merritt & De Jong 2015). Animal carcasses fulfil multiple roles in the life history of insects that utilise them, they can act as: 1. mating arenas; 2. oviposition sites; 3. a source of food for adults; 4. a source of prey, or hosts, for developing larvae of different species (Putnam 1978, Peschke et al. 1987). Consequently, the insect communities on carrion are composed of multiple taxa with distinct ecological requirements and strategies for utilising carrion resources. Peschke et al. (1987) identified several axes along which necrophilous insect taxa are ecologically separated: macrohabitats (such as forests and clearings), seasonality, stages of decay and microhabitats, which include spatial segregation within a carcass.

Insect succession on decomposing carrion occurs in a predictable sequence that is well documented (Payne 1965, Peschke et al. 1987, Sharanowski et al. 2008, Tomberlin et al. 2011, Cruise et al. 2018). Evans et al. (2020) contextualised this generalisable process of carrion succession in terms of the niche width of colonisers: the necrophilous community is a mix of generalists and specialists. The generalist species have a strategy of using a wide range of resources at a local scale (Evans et al. 2020). The specialists, by contrast, harbour specific adaptations which enable them to locate and consume carcasses scattered across larger spatial scales as their primary resources. Early colonisers of carcasses, such as blowflies and carrion beetles, tend to be specialists that obligately breed on carrion as their restricted dietary niche allows them a narrow timeframe

for colonisation. They can fly long distances and are highly sensitive to carrion odour cues (Norris 1965, Kalinova et al. 2009, Tomberlin et al. 2011, Olea et al. 2019). On the other hand, late colonisers like trogid and dermestid beetles, either have broader dietary requirements or feed on substrate that is only available during the later stages of decomposition (Anderson et al. 2019).

Differentiated use of food resources is a key mechanism of ecological niche differentiation between species (Payne & Crossley 1966, Schoenly & Reid 1983, Peschke et al. 1987, Hocking et al. 2007, Evans et al. 2020). It can also be a source of ecologically mediated population differentiation and genetic divergence within species, which can occur in sympatry but may also accompany geographical differentiation (Ackermann & Doebeli 2004, Funk et al. 2006, Nosil 2012). For instance, the deer bot flies (*Cephenemyia* spp.) in northern Norway have evolved faster development times compared to the same species in the southern region due to the shorter season and cooler climate in the north (Nilssen 1997a). This has been correlated with different dispersal regimes and potential physiological attraction to different host species (reindeer vs. red deer; Nilssen 1997a, Nilssen 1997b, Blanckenhorn 2015).

However, due to the unpredictability and ephemerality of carcasses as a resource, many carrion insects have evolved a high degree of plasticity in their behaviour and life history traits to maximise their fitness (Brundage et al. 2014, Blanckenhorn 2015). Therefore, any evidence for divergence between populations observed in the field could be a result of local environmental variation and reflect phenotypic plasticity, which may or may not be adaptive. Owing to this interplay between genetic and environmental factors, a combination of field studies and controlled laboratory experiments is necessary to uncover whether or not there really is evidence of population divergence in carrion resource use and to understand the mechanisms driving it.

This thesis employs a combination of techniques to address the following general questions:

1. Is there evidence of divergence in resource use between and within natural populations of a carrion-using insect?
2. Is this divergence adaptive?
3. What mechanisms maintain divergence?

We used burying beetles, which are obligate carrion breeders, as a model system to address these questions, focusing primarily though not exclusively on *Nicrophorus vespilloides*. The Silphidae family

(commonly known as carrion beetles) comprises two subfamilies of beetles (Silphinae and Nicrophorinae) that breed obligately on animal carrion. Burying beetles are carrion beetles belonging to the genus *Nicrophorus* (Coleoptera: Silphidae: Nicrophorinae; Fabricius 1775). They are well known for monopolising small vertebrate carrion to brood their larvae in subterranean crypts (Pukowski 1933, Eggert & Müller 1997, Fetherston et al. 1994). Unlike other silphids, which breed on large carcasses, *Nicrophorus* beetles exhibit complex biparental care behaviours (Milne & Milne 1976, Peck & Anderson 1985, Ratcliffe 1996, Scott 1998).

Burying beetles are primarily confined to the temperate regions of the northern hemisphere (Figure 1; Anderson & Peck 1985, Peck & Anderson 1985, Scott 1998, Bedick et al. 1999, Sikes et al. 2002). *Nicrophorus* species have a wide geographical spread within the northern hemisphere: there are at least 49 Old World species, 21 New World species and 2 Holoarctic species (Sikes 2005, Sikes & Venables 2013). Phylogenetic evidence points to an Old World (most likely Asian) origin of the genus (Hatch 1927, Sikes & Venables 2013). Fossil and amber records suggest that biparental care and guarding of small vertebrate carrion for their larvae had evolved in *Nicrophorus* beetles as early as the Cretaceous period (Cai et al. 2014). Burying beetles are completely absent from sub-Saharan Africa, Australia and Antarctica and, with the exception of a few tropical areas where this lineage occurs, they tend to be found in cool habitats at higher elevations (Sikes et al. 2002, Sikes & Venables 2013, Merritt & De Jong 2015). Variation in environmental factors such as climate, seasonality or carrion availability can impose different selection pressures on populations of the same species and result in population diversification. For instance, *N. investigator* populations in higher altitudes tend to be larger due to the changes in temperature and this affects their physiology, behaviour and population dynamics (Smith et al. 2000, Smith 2002). Similarly, *N. nepalensis* populations have locally adapted their breeding season to match the optimal temperature conditions for reproduction across mountain ranges of mainland China, Taiwan, and Japan (Tsai et al. 2020).

Carrion beetles control brood size on the carcass and oviposit asynchronously during periods of high competition, which avoids overcrowding of the carcass and helps the maximum number of larvae to survive (Bartlett 1987, Rauter & Rust 2012). These behaviours can help maintain genetic diversity and haplotypic variation (Picard et al. 2015). The success of their behavioural adaptations ensures that, in environments where they are found, burying beetles monopolise small carcasses. A consequence of this is intense competition for resources between closely related species as well as within individuals of the same populations.

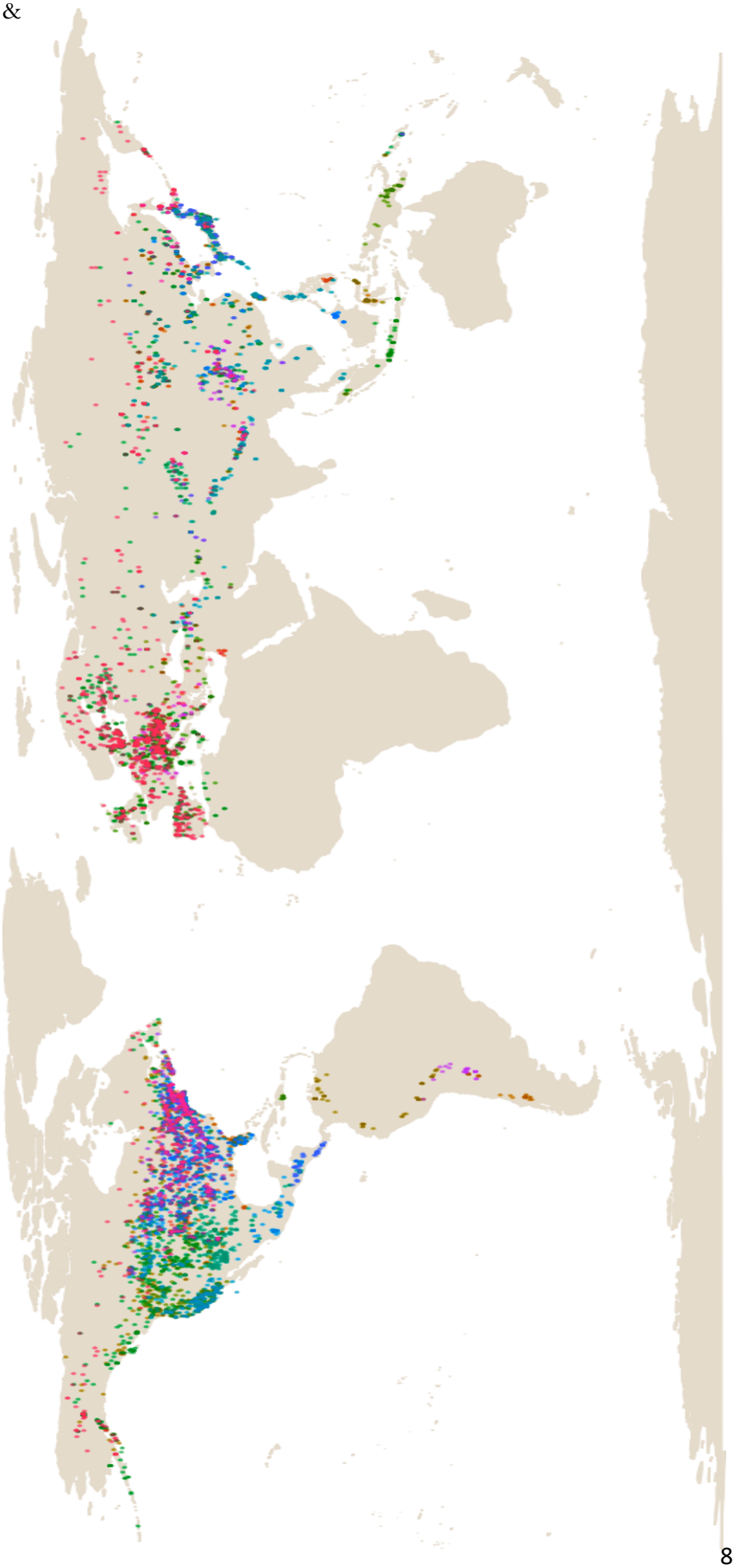
Anderson (1982) identified competition for food resources as the primary factor driving ecological separation in silphids. At the sub-family level, resources appear to be partitioned between carrion beetles by the size of the carcass- with Silphinea beetles preferring large to medium sized carrion (> 300 g) and *Nicrophorus* beetles specialising on small carcasses such as songbirds and rodents (Anderson & Peck 1985, Ratcliffe 1996, Eggert & Müller 1997, Scott 1998, Dekeirsschieter et al. 2011). At the genus level, resources are partitioned through differences in seasonal activity and reproductive period (ranges from early spring to late summer) as well as the macrohabitat associations (coniferous or deciduous forests, meadows, fields or marshy areas). The temporal activity of the beetles also tends to vary, with most silphines being diurnal and most burying beetles being nocturnal (Shubeck 197, Anderson 1982). *N. tomentosus* is an exception to the norm- the diurnal activity of these beetles could have been a result of ecological character displacement that reduces competition from other *Nicrophorus* beetles (Anderson 1982).

Studies of burying beetle community dynamics have indicated that the carrion niche of burying beetles is further differentiated by body sizes of the beetles, because they play a key role in influencing the outcomes of contests for exclusive ownership of the carcass (Scott 1998, Trumbo 1990, Hopwood et al. 2016). Burying beetle communities in Europe and North America differ in their species composition but are remarkably similar in structure: each location tends to host a guild of four to six species that significantly overlap in their spatio-temporal niches but differ in body sizes (Scott 1998) and consequently in the size of the carrion upon which they breed. Intraspecific contests for a carcass are typically won by the largest species and, consequently, larger *Nicrophorus* species appear to be under selection to breed on larger carcasses (Smith & Heese 1995; Hopwood et al. 2016) whereas the smaller species are specialised to breed on smaller carrion. Recent work on a North American beetle, *N. pustulatus* has indicated an astonishing host shift from carrion to snake eggs, making it the only known parasitoid of vertebrate eggs (Blouin-Demers & Weatherhead 2000, Keller & Heske 2001). Laboratory studies have indicated that *N. pustulatus* can utilise both snake eggs and mice carrion. The mechanisms behind this differentiation are as yet opaque (Smith et al. 2007).

Although the natural history of burying beetles is well-characterised in some respects, relatively little is known about how resources are distributed within populations of burying beetles. In particular it is unclear whether high rates of intraspecific competition have given rise to adaptive diversification in resource use within populations of the same burying beetle species. This is the problem I address here.

In Chapter 2, we test for evidence of divergence in resource use between and within natural populations of burying beetles with field experiments in three different woodlands. In Chapter 3, I test whether the patterns of resource use we observe in the field could be due to adaptive partitioning of resource type within populations using "common garden" breeding experiments in the lab. Whether differential resource use in nature is associated with genomic divergence is the focus of the work described in Chapter 4. In Chapter 5, I present the results of experimental evolution, which I used to test whether individuals within natural beetle populations could, in principle, become divergently and locally adapted to breed on different types of carrion. With experiments in Chapter 6, we explore the chemical basis of differential resource use via mass spectrometry to test whether chemical cues and signals could potentially underpin any divergent resource use. Finally, in Chapter 7, I synthesise the results of the previous chapters to address the three questions posed in this chapter and consider topics for further research.

Figure 1: Georeferenced records of the subfamily Nicrophorinae. (Map constructed using Sikes & Venables 2013 dataset)



Chapter 2

Differential resource use in natural populations of burying beetles

Introduction

Specialised resource use has been predicted to evolve as a means of avoiding costly competition for resources (Butlin & Smadja 2018). Local adaptation to, and specialisation upon, different food resources is a putative mechanism for population divergence (Berlocher & Feder 2002, Coyne & Orr 2004), which has been associated with different forms of reproductive isolation. There are several examples of reproductive isolation arising from niche differentiation associated with changes in host specificity in insects (Feder et al. 1994, Groman & Pellmyr 2001, Via et al. 2000):

One such example is that of sympatric apple- and hawthorn-infesting races of the fly *Rhagoletis pomonella*. A sub-population of flies in Grant, Michigan which had previously been reproducing and feeding on hawthorn began to diverge and adapt to the newly introduced apple trees in the span of about a hundred years (Bush 1969). High oviposition preference for the same host species that was used in earlier life-history stages, acted as a premating barrier between these diverging sympatric populations, and subsequently led to reproductive isolation (Feder et al. 1994).

Novel host specialisation can, in turn, lead to significant morphological and phenological differences associated with rapid genetic differentiation (Groman & Pellmyr 2000). *Prodoxus quinquepunctellus* is a highly specialised species found throughout North America whose larvae are known to feed exclusively on the inflorescence of yucca species. Groman & Pellmyr (2000) found that different sub-populations of the moth shifted from feeding on the native *Y. lamentosa* to the newly introduced *Y. aloifolia* plants on the east coast of the United States, and that this led to rapid genetic differentiation between the sub-populations.

Another instance where divergent resource use has created barriers to gene flow comes that of sympatric populations of pea aphids on adjacent fields of alfalfa and red clover (Via 1999, Via et al. 2000). The two populations were found to be locally adapted and highly genetically differentiated, with behaviourally mediated habitat choice restricting most of the gene flow.

Selection against immigrants and hybrids on the alfalfa and red clover host plants acted as additional reinforcements, leading to significant reproductive isolation.

Several theoretical and empirical studies (Bush 1969 & 1975; Rice 1987; Berlocher & Feder 2002) have proposed a direct link between ecological adaptation and the evolution of reproductive isolation. Traits involved in resource adaptation and specialisation can act as pre- and post-mating barriers to gene flow through a variety of mechanisms. Though much of the theory surrounding ecological speciation and resource-based based genetic differentiation stems from work on phytophagous insects (Nosil & Crespi 2006, Matsubayashi et al. 2010), certain core principles can be generalised and applied to other insect groups.

One such principle is that seasonal differences in resource abundance can lead to the synchronisation of the life histories of insects with peak abundance of their respective resources (Tauber et al. 1986). For example, there could be a temporal shift in mating to coincide with resource availability. Alternatively, mating could occur solely on the resource. This peak can vary, depending on the type of resource, and so lead to temporal divergence in reproduction within populations according to the resource that individuals are specialised upon.

A key example of this phenomenon is seen in the shift in life-history timing of the apple-adapted *R. pomonella* to coincide with the earlier fruiting time of apples (Bush 1969). Follow-up field experiments (Mattson 2015) have demonstrated that this shift to match the host-fruiting time can evolve rapidly and repeatably. *R. pomonella* introduced to the Pacific Northwestern USA from larval-infested apples gave rise to apple-, black hawthorn-, and ornamental hawthorn-associated fly populations that exhibited a rapid shift in eclosion and adult flight activity patterns to match the fruiting season of their respective host plants.

In this chapter, I consider whether this sort of principle could apply to burying beetles. Specifically, I investigate whether there is temporal variation in the type of resources available for burying beetles to breed upon.

Burying beetles as a model system for studying resource specialisation and population divergence

Burying beetles are necrophagous insects that breed on vertebrate carcasses. Vertebrate carcasses are unpredictably distributed, ephemeral resources that act as both mating arenas for adult beetles and a food resource for developing larvae (Scott 1998). Carcasses can be scarce, making competition among burying beetles to secure ownership correspondingly intense. Burying beetles have evolved several strategies to reduce inter-specific competition on host carrion (Anderson 1982). Through an elaborate system of bi-parental care (Milne & Milne 1976), burying beetles are able to conceal carrion from rivals and defend it from attack.

Beetles locate the dead body of a small vertebrate such as a mouse or a bird using olfactory cues when in flight. The parents then shave the carcass of its fur or feathers, roll the flesh into a ball and bury it in the soil. During this time, the female also lays her eggs in the soil around this carrion nest. Upon hatching, the larvae crawl into the nest and feed themselves on the flesh. They are also fed by their parents, who guard the larvae, until they disperse from the carcass to pupate in the soil (Milne & Milne 1976, Eggert & Müller 1997).

Across a broad geographical area, burying beetle species and populations appear to have differentially adapted to breed on different species of vertebrates, depending on local vertebrate diversity (Wilson & Fudge 1984, Hocking et al. 2007). There are reports of differential resource use involving sympatric species of burying beetles specialising on aquatic versus terrestrial carrion. For example, one study used stable isotope analysis to reconstruct the dietary niches of *Nicrophorus investigator* and *Nicrophorus defodiens* in a watershed in coastal British Columbia (Hocking et al. 2007). It found that the majority of *N. investigator* individuals were raised on a diet of salmon carrion, while all of *N. defodiens* individuals had a larval diet of carrion from shrews and songbirds. This suggests that resource use influences niche partitioning among species.

In principle, intraspecific competition for resources can likewise drive differential use of vertebrate carrion between sub-populations, perhaps in association with a temporal shift in breeding if different types of carrion are available at different times of the year. However, whether this has ever happened within burying beetle populations remains unknown.

N. vespilloides is found in diverse habitats ranging from open forests in Europe and the Palearctic ecozone, to the bogs and marshes of North America (Anderson 1982, Beninger 1994). This means

populations are spread over an exceptionally broad geographical and ecological range, and potentially breed upon different types of carrion accordingly. Even within a small geographical area, seasonal variation in the availability of different carrion maybe be expected, corresponding to seasonal variation in the mortality of the vertebrate community (Promislow & Harvey 1990, Wettlaufer et al. 2018). The relative abundance of mammalian and avian carcasses available to the beetles could vary accordingly, across the beetle breeding season. For example, there is considerable mortality among fledgling songbirds in late spring/ early summer (Newton 1998, Chase et al. 2005, Clapham 2011, Capstick 2017) whereas mouse populations show high mortality in mid-late summer (Moffat 1910, Harris 1979, Merritt et al. 2001, Haberl & Krystufek 2003, Clapham 2011).

Furthermore, *N. vespilloides* is the smallest burying beetle species in the UK and so potentially suffers from intense competition for carrion with sympatric burying beetle species (Scott 1998, Hopwood et al. 2016, Sun et al. 2020). In theory, *N. vespilloides* could avoid competition for carrion by diversifying to breed on different types of carrion. Nevertheless, the extent of competition for carrion differs among *N. vespilloides* populations (Sun et al. 2020), and selection for divergent resource use could vary accordingly among populations. Therefore, we could expect spatial variation in resource use between populations. However, whether or not any of this happens in nature remains to be determined.

We tested for evidence of divergence in resource use between and within three natural East Anglian *N. vespilloides* populations by recording the relative abundance of beetles in traps baited with avian versus mammalian carcasses over the course of the burying beetle's breeding season from May to October. We predicted temporal variation in the abundance of beetles attracted to different carcass types within populations, coinciding with seasonal differences in resource abundance. We also predicted spatial variation in attraction to different carrion types resulting from potential differences in vertebrate diversity, and intensity of competition among *Nicrophorus* beetles between woodlands.

Based on our predictions, we addressed the following key questions:

Question 1: Is there temporal variation in the abundance of *N. vespilloides* beetles trapped on avian versus mammalian carcasses?

Question 2: Is there spatial variation in the number of *N. vespilloides* beetles trapped on avian versus mammalian carcasses?

Question 3a: Do some trapping locations within woodlands attract more *N. vespilloides* beetles when baited with chick carcasses rather than mice?
Question 3b: Is this true when *N. vespilloides* beetles have a paired choice, or when sequentially choosing?

Question 4: Does seasonal variation in population density alter the relative abundance of beetles attracted to avian versus mammalian carcasses?

Question 5: How abundant are other burying beetle species on avian versus mammalian carcasses?

Materials and methods

Study area

We focused on three East-Anglian burying beetle populations from Thetford Forest, Gamlingay Wood and Waresley Wood (Figure 1) during the burying beetle breeding season, which usually falls between April and October every year.

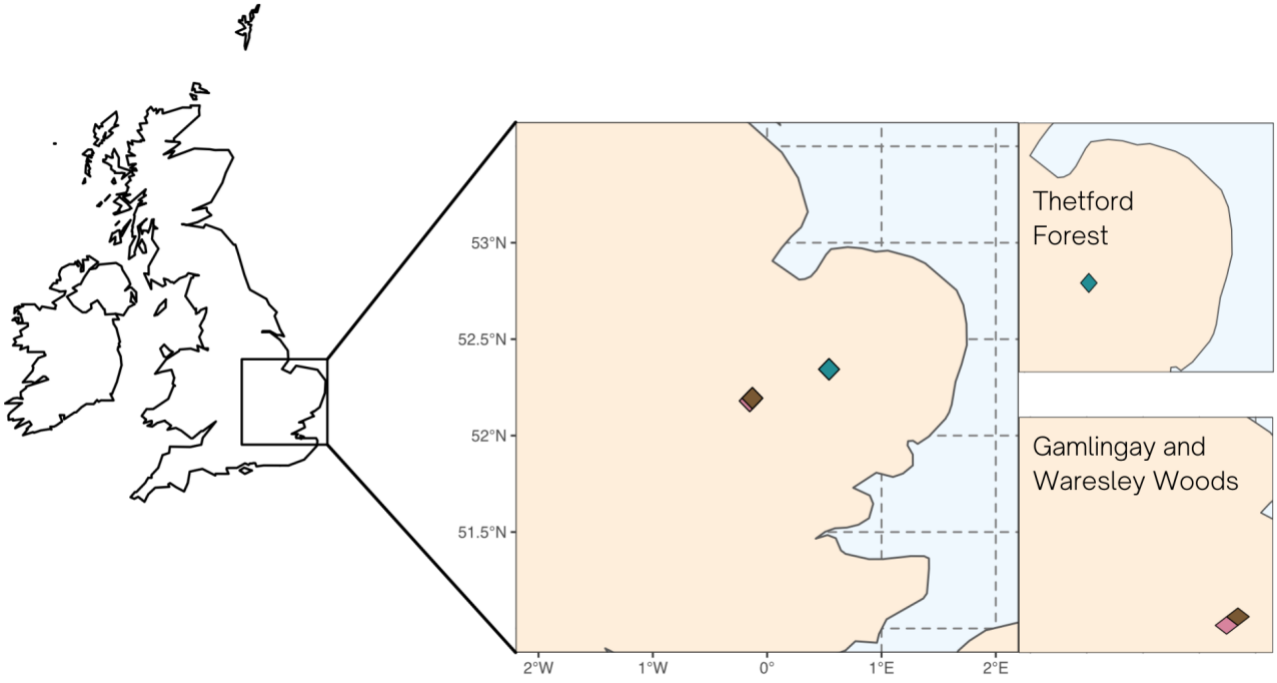


Figure 1: Map depicting the East-Anglian sampling sites.

We sampled the Thetford population from April to October 2017 and then again from May to October 2019 at the trap locations schematically depicted in Figure 2a.

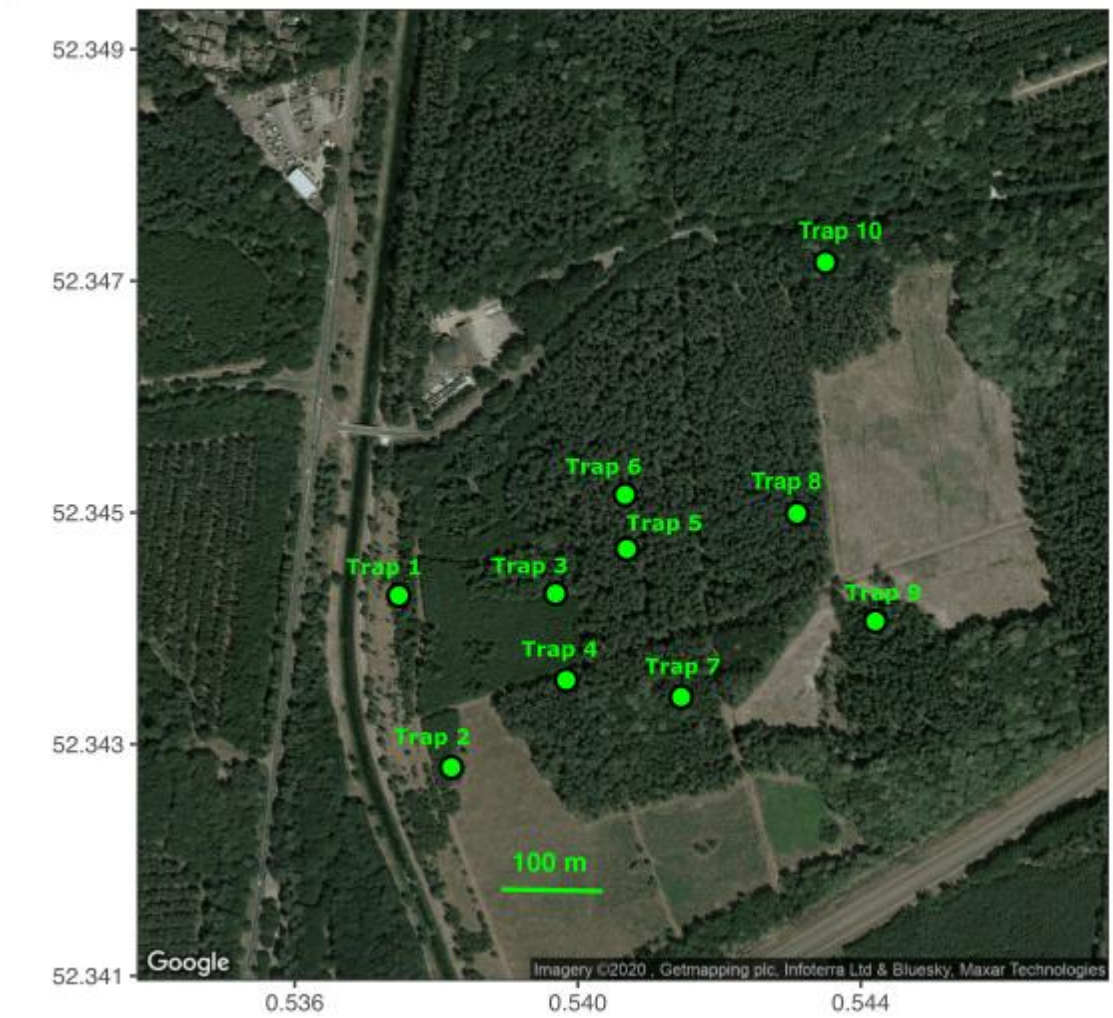


Figure 2a: Map of Thetford Forest beetle trapping locations.

We sampled the Gamlingay population from June to October 2017 at the trap locations schematically depicted in Figure 2b.

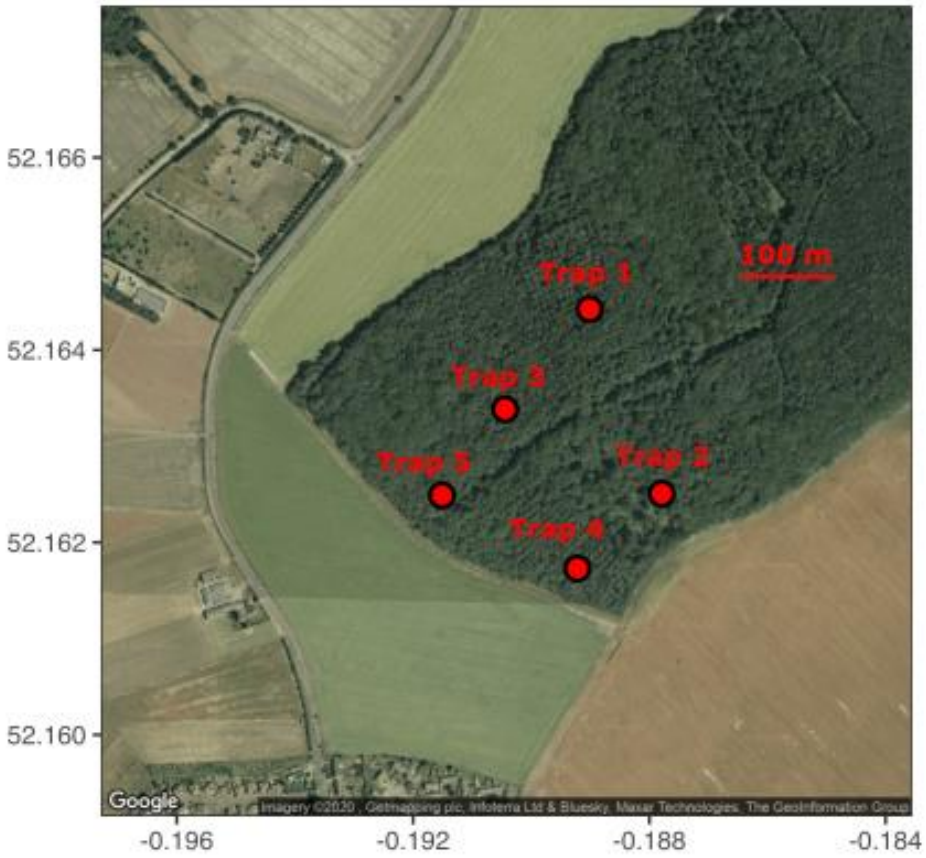


Figure 2b: Map of Gamlingay Wood trapping locations

We sampled the Waresley population from June to October 2017 at the trap locations schematically depicted in Figure 2b.

Figure 2c: Map of Waresley Wood trapping locations

The geographical coordinates of traps at each of the three sites are listed in Table A.2.1 of the appendix.

Beetle collection

Beetle collection at all locations was carried out under permit from Forestry Commission England. We collected beetles at each site using Japanese beetle traps filled with soil and baited with a freshly thawed mouse or chick carcass. We hung the traps from tree branches using cotton rope, so that they were about a metre above the ground.

We returned to the trap locations at regular intervals (listed in Table A.2.2 of the appendix) to collect all *Nicrophorus* beetles present inside each trap. After collecting the beetles, we set up the traps again by adding new soil and a fresh carcass to each of them. On every sampling trip, we emptied the soil and beetles collected in each trap into a large plastic box and carried them back to lab in order to process the contents. Once the beetles were brought to the lab, they were all processed (see 'Processing field beetles') and none were released back into the field.

Trapping in Thetford Forest

In Thetford Forest, beetles were sampled using a paired trap arrangement, in which we placed two beetle traps- one with a dead domestic chick and the other with a mouse carcass- near each other at each trap location and recorded the beetles found in each trap. With this design, beetles were given a simultaneous choice between a dead mouse and a dead chick. Each time we rebaited the trap with carrion, we rebaited it with the alternate carrion type. Therefore, if a mouse carcass had been placed in the trap previously, it was replaced by a chick carcass on the next sampling trip to ensure that the trap location itself did not bias beetle catch. The mice and chick carcasses used were matched in weight (30-40 g). The traps within each experimental pair were placed 1-2 m apart. Pairs of traps were placed 200-400 m apart from each other. Three pairs of traps were used to collect the first three data points (23 May - 4 June 2017) and the 10-pair set up was used on 4 June 2017 (beetles collected from these on 14 June 2017), and thereafter until 11 October 2017, with the exception of 12 July 2017 and 4 August 2017, when 5 pairs of traps were setup. These differing numbers of traps were due to logistical constraints associated with carcass availability.

The 10-pair set up was used during the entirety of the May to October 2019 sampling period. However, the methodology for this season differed from the previous one in that sampling was not continuous, i.e., the traps were only put up for a certain time (4 days on average) during each collection period. We left the traps empty between collection periods so that we were not constantly attracting burying beetles (and preventing them from breeding naturally).

Trapping in Waresley and Gamlingay woods

To determine whether this temporal variation in preference was widespread, and possibly driven by resource availability, we also carried out a slightly different version of the experiment in Gamlingay and Waresley woods. Here, we alternated the carcass types placed in each trap location every two weeks, instead of using a paired trap setup. This modified experimental design eliminated

the possibility of any incidental catches due to trap proximity, and also enabled us to test for any absolute rather than relative carrion preferences.

Trapping in these two woods was part of a long-term study of the *Nicrophorus* guilds within each woodland (Sun et al 2020). Single traps were used at each trap location (five per site) and the carcass type was alternated in each trap at every sampling trip, i.e., if a trap contained a chick carcass it was replaced with a mouse carcass on the next trip. Traps were emptied and rebaited every two weeks. All five traps at both the sites were sampled continuously from 15 June to 19 October 2017.

Processing field-caught beetles

At the lab, we used carbon-dioxide to immobilise each beetle and brush off any mites stuck to it. We recorded the species, pronotum width and sex of every *Nicrophorus* beetle. A pair of beetles from each trap was isolated for 4 hours and then frozen at -80°C for subsequently extracting cuticular hydrocarbons (see Chapter 5). The remaining beetles were used to carry out carcass preference experiments and to establish populations for lab experiments (see Chapter 3).

Statistical analysis

We carried out all statistical analyses to test our predictions using R (RStudio version 1.3.959) with generalised linear models (GLM) and generalised linear mixed models (GLMM) using the lme4, MASS and glmmTMB packages.

Question 1: Is there temporal variation in the abundance of *N. vespilloides* beetles trapped on avian versus mammalian carcasses?

Using field data from all three sites in 2017 (Gamlingay Wood, Thetford Forest and Waresley Wood) and Thetford in 2019, we calculated the median number of beetles per day per trap. We first divided the total number of *N. vespilloides* beetles found on a carcass at any collection date by the number of days the traps had been left out in the sampling site for. This gave us the average number of beetles caught per day at each trapping location of the site. We then calculated the median of these values across all trapping locations to get the median number of beetles per day per trap.

We tested for variation in carrion preference between *N. vespilloides* caught at Thetford Forest in 2017 and 2019, using a GLMM that included carrion type, sampling day and year as fixed effects, and trap ID as random factors with a Poisson error structure. Sampling for this project first began on 23 May 2017 at Thetford Forest and all sampling days in 2017 were calculated from this date. In 2019, sampling days were calculated from 23 May 2019. Here the total beetles found in a trap on the sampling day was used as the response variable.

To compare the beetle catch from all three populations, we combined the data from all three sites and fitted an LMM (linear mixed-effects model). Since data collection dates for Thetford Forest differed from Gamlingay and Waresley Wood, we rescaled the dates such that all the data were now compared from a mid-point which marked the middle of the field season for all sites. The log of the total *N. vespilloides* beetles was taken as the response variable. Carcass type, and sampling site were included as fixed effects, while day of collection, quadratic effect of the day of collection were included as covariates (we included the squared term because of the strong curvilinear nature of the raw data). Trap ID (within each site) was included as a random effect.

Question 2: Is there spatial variation in the number of *N. vespilloides* beetles trapped on avian versus mammalian carcasses?

We tested for spatial variation in carrion preference between *N. vespilloides* caught at Gamlingay Wood, Thetford Forest and Waresley Wood in 2017 using a GLMM that included carrion type and sampling site as fixed effects along with trap ID (within each site) and sampling day as random effects with Poisson error structure.

Question 3a: Do some trapping locations within woodlands attract more *N. vespilloides* beetles when baited with chick carcasses rather than mice?

We used the package ggmap to plot Stamen maps depicting the variation in carrion preference at the trapping locations of Thetford Forest in 2019 and all three sites in 2017.

We tested for spatial variation in carrion preference of *N. vespilloides* at different trapping locations of Thetford Forest in 2019 and 2017 using a GLMM that included carrion type, year and trap ID as fixed effects along with sampling day as a random effect with Poisson error structure.

Question 3b: Is this true when *N. vespilloides* beetles have a paired choice, as with the Thetford data or when sequentially choosing as with Gamlingay and Waresley woods?

We used carrion type and sampling method (paired or unpaired traps) as fixed effects and trap ID, sampling site (Thetford, Gamlingay or Waresley) and sampling day as random effects with Poisson error structure to test for variation in beetle catch due to the trapping methodology used.

Question 4: Does seasonal variation in population density alter the relative abundance of beetles attracted to avian versus mammalian carcasses?

We used a GLMM with a binomial distribution to test the association between carrion preference and population density. We organised the 2019 Thetford data by trap location and used the total number of *N. vespilloides* in both paired traps as the measure of beetle density. We used beetle density as a fixed effect and sampling day and trap ID as random effects. The proportion of beetles in chick-baited traps, weighted by the actual number of beetles caught in the trap (calculated using 'cbind' function) was the response variable.

Question 5: How abundant are other burying beetle species on avian versus mammalian carcasses?

We used a GLMM to test for differences in carrion preference across beetle species per trap in Thetford during the 2019 field season. Beetle species, carrion, sampling day, and their interactions were included as fixed effects, whereas trap ID was included as a random factor. The number of each species per trap was included as a response variable with a Poisson error structure.

Results

Question 1: Is there **temporal variation** in the abundance of *N. vespilloides* beetles trapped on avian versus mammalian carcasses?

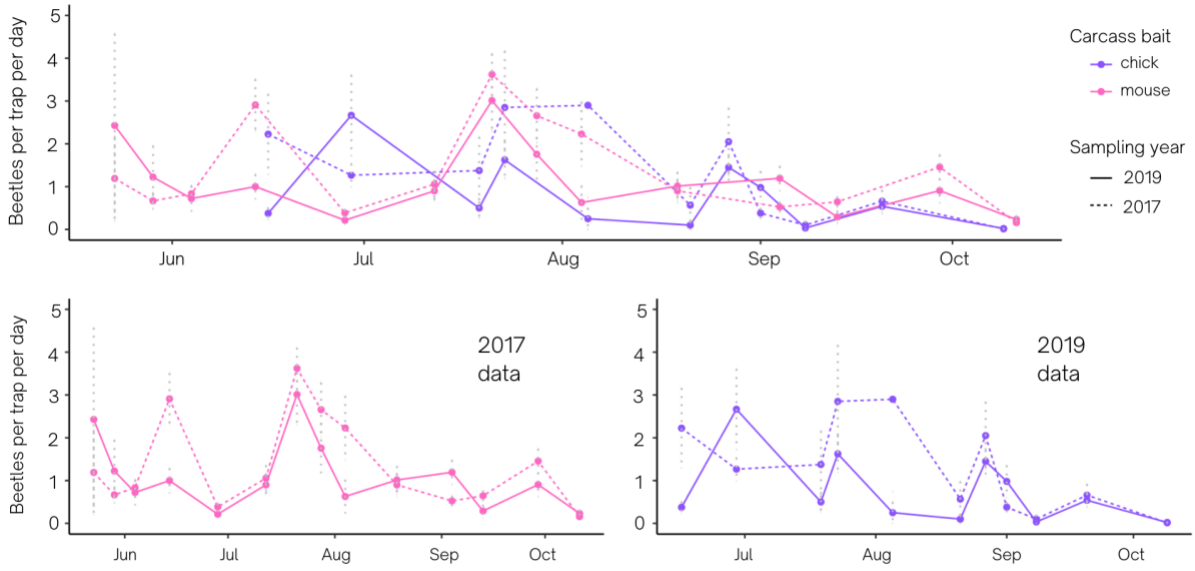


Figure 3a: Summary plot of *N. vespilloide*s collected in chick and mouse trap pairs in 2017 and 2019 at Thetford Forest. Each datapoint represents the median number of beetles per day per trap and the measure of error reported is the median absolute deviation.

Table 1:

a. Results of ANOVA on the effects of carcass type, sampling year, sampling date and their interactions on the number of *N. vespilloides* trapped

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
carcass type	77.0748	1	< 2.2e-16 ***
sampling year	561.8983	1	< 2.2e-16 ***
sampling date	128.3961	1	< 2.2e-16 ***
carcass type x sampling year	4.8645	1	0.02741 *
carcass type x sampling date	16.6824	1	4.419e-05 ***
sampling year x sampling date	5.5816	1	0.01815 *

b. Model summary showing results of the GLMM to test for the effects of carcass type, sampling year, sampling date and their interactions on the number of *N. vespilloides* trapped

Factors	*Estimate*	*Std. Error*
Intercept	1.7976651	0.1744347
Carcass-Mouse	0.6724989	0.0914958
Sampling Year-2017	0.8267861	0.1016716
Sampling Date	-0.0050567	0.0011205
Carcass-Mouse x Sampling Year-2017	-0.1713677	0.0776978
Carcass-Mouse x Sampling Date	-0.0035049	0.0008581
Sampling Year-2017 x Sampling Date	0.0026230	0.0011102

On testing for temporal variation in the number of *N. vespilloides* trapped on each type of carrion at Thetford Forest in 2017 and 2019 (Figure 3a, Table 1), we found that there were significantly more beetles on traps baited with mice than those baited with chicks in 2017 (Tukey post-hoc comparison z-ratio= -6.060, p-value <.0001) as well as 2019 (Tukey post-hoc comparison z-ratio= -5.934, p-value <.0001). On average, there were significantly more beetles trapped during the 2017 field season in both mice-baited (Tukey post-hoc comparison z-ratio= -17.016, p-value <.0001) and chick-baited traps (Tukey post-hoc comparison z-ratio= -17.109, p-value <.0001) compared to 2019. Significant sampling year x sampling date and carcass type x sampling date interactions indicate that both beetle catch, and carrion preference varied significantly across the burying beetle season (Table 1).

From Figure 3a, we can see that there was very high temporal variation in the number of beetles trapped on each type of carrion across sampling days. In 2017, we observed that a greater number of beetles were caught on chick carrion during sampling in early May, late May and early September. In 2019, *N. vespilloides* were trapped in greater numbers on chicks in late June and early September.

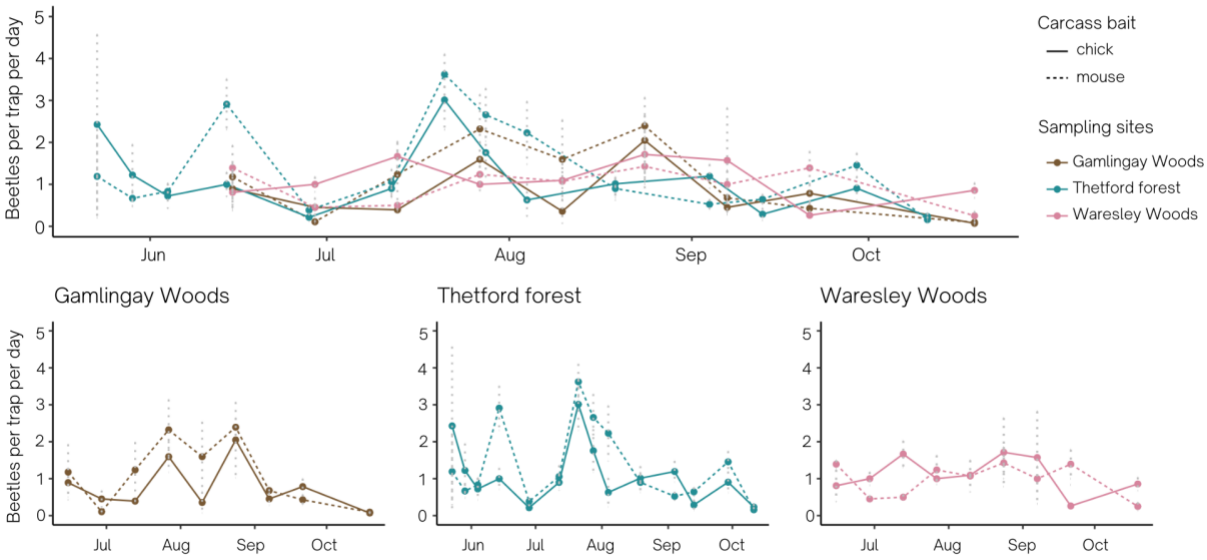


Figure 3b: Summary plot of *N. vespilloides* collected in chick and mouse trap pairs in 2017 at Gamlingay Wood, Thetford Forest and Waresley Wood. Sampling sites and carrion bait are differentiated by colour. Each datapoint represents the median number of beetles per day per trap and the measure of error reported is the median absolute deviation.

Table 2:

a. Results of ANOVA on the effects of carcass type, sampling site, sampling date and their interactions on the number of beetles trapped.

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
carcass type	30.2375	1	3.823e-08 ***
sampling site	0.8772	2	0.644955
sampling date	91.1129	1	< 2.2e-16 ***
carcass type x sampling site	23.0963	2	9.654e-06 ***
carcass type x sampling date	9.6973	1	0.001845 **
sampling site x sampling date	15.3215	2	0.000471 ***

b. Model summary showing results of the GLMM to test for the effects of carcass type, sampling site, sampling date and their interactions on the number of beetles trapped.

Factors	*Estimate*	*Std. Error*
Intercept	2.6659144	0.1998307
Carcass-Mouse	0.3946102	0.1040312
Sampling Site-Thetford	-0.0262161	0.2335334
Sampling Site-Waresley	0.0152747	0.2776648
Sampling Date	-0.0023172	0.0012043
Carcass-Mouse x Sampling Site-Thetford	0.0357840	0.0924101
Carcass-Mouse x Sampling Site-Waresley	-0.3965018	0.1174049
Carcass-Mouse x Sampling Date	-0.0025184	0.0008087
Sampling Site-Thetford x Sampling Date	-0.000974	0.0012077
Sampling Site-Waresley x Sampling Date	0.0037026	0.0015599

On testing for temporal variation in carrion preference in Gamlingay Wood, Thetford Forest and Waresley Wood during the 2017 field season (Figure 3b, Table 2), we found significant carcass type x sampling date interactions, indicating that temporal variation in carrion preference across sampling days was very high. Beetle catch varied significantly over time in all three populations (significant sampling site x sampling date interaction). In Gamlingay Wood, late June and late September marked a marginal increase in the number of beetles caught on chick carrion (Figure 3b). In Waresley Wood, there was a marked increase the number of beetles caught on chicks during sampling days in late June, mid-July, early September and mid-October (Figure 3b). There was a significant carcass type x sampling site interaction, indicating spatial variation in resource use (see Question 2).

Question 1b: Are there any **overall trends** in the number of *N. vespilloides* beetles trapped on avian versus mammalian carcasses across all three sites?

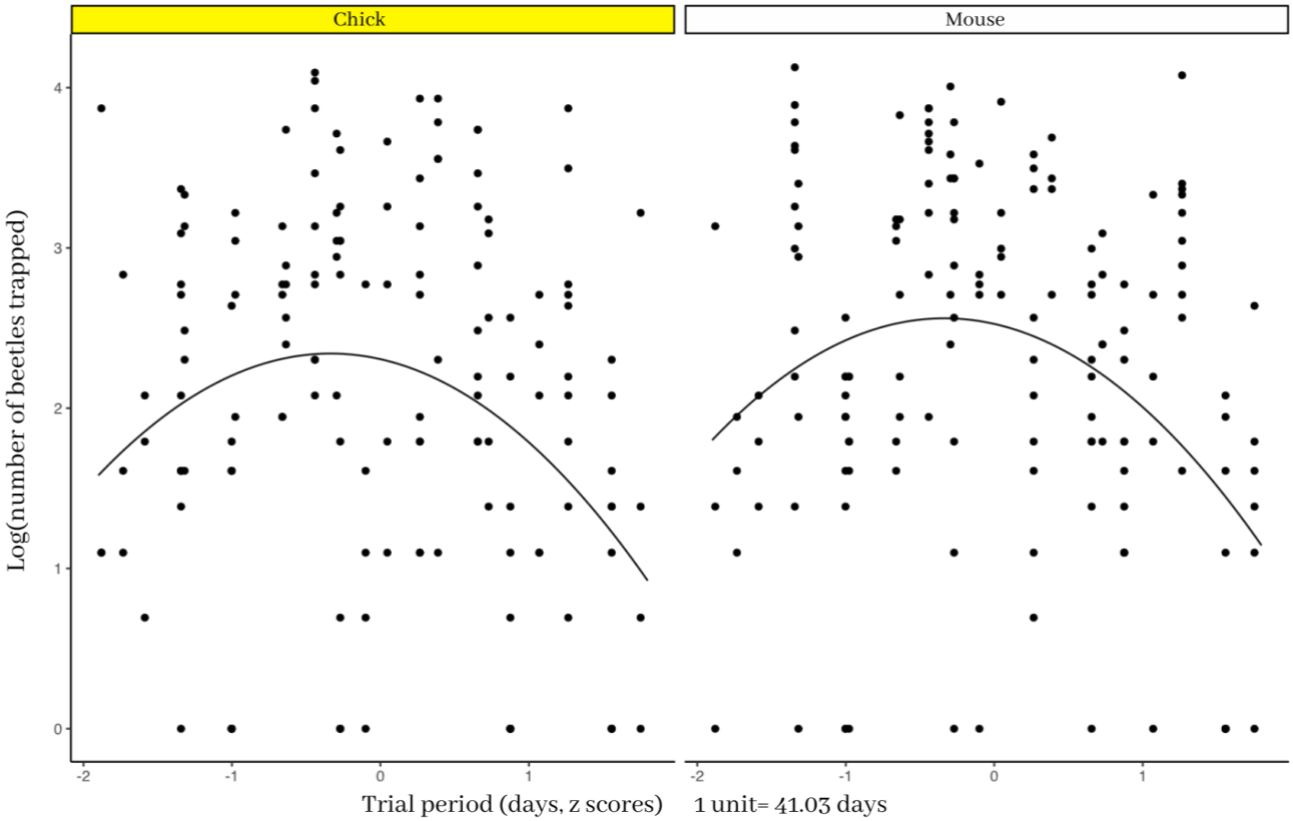


Figure 4: Trend for beetles caught in Thetford Forest, Gamlingay Wood and Waresley Wood across the 2017 field season. The y axis shows the sampling period in days, standardised to be measured with z-scores. Data points under the yellow panel show the log of the number of beetles caught in chick-baited traps. Data points under the white panel depict the log of the number of beetles caught in traps baited with mice.

Table 3:

a. Results of ANOVA analysing overall trends in beetle preference across all sampling sites

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
carcass type	3.6545	1	0.05592
sampling site	3.1187	2	0.2103
(sampling date) ^2	25.882	1	3.629e-07 ***

b. Model summary showing results of the LMM to analyse overall trends in beetle preference across all sampling sites

Factors	*Estimate*	*Std. Error*
Intercept	2.63334	0.22957
Carcass-Mouse	0.21990	0.11546
Sampling Site-Thetford	-0.32717	0.25129
Sampling Site-Waresley	0.02854	0.30459
Sampling Date	-0.20857	0.05843
I(Sampling Date) ^2	-0.31069	0.06053

Comparing the data from all three populations (Figure 4, Table 3), the general trend for total beetles caught was similar across time across all three populations, i.e., site (Thetford, Waresley or Gamlingay) did not have a significant effect on beetle catch. The quadratic effect of date, however, was significant (Table 3). This means that when beetle trapping data were summarised across all sites, it followed the same pattern of lower trappings during the beginning and end of the field season, regardless of the carrion bait in the trap.

Question 2: Is there **spatial variation** in the number of *N. vespilloides* beetles trapped on avian versus mammalian carcasses?

Taking variation in sampling days and number of traps at each site into account, the three sites did not differ significantly in total *N. vespilloides* beetle catch. However, the populations used the carrion in different ways (significant sampling site x carcass interaction, Table 2). In Waresley Wood, more beetles were caught in chick-baited traps than in mice-baited traps across the entire field season (Tukey post-hoc comparison z-ratio= 2.388, p-value= 0.0169). This pattern was reversed in Gamlingay Wood (Tukey post-hoc comparison z-ratio= -2.389, p-value= 0.0169) and Thetford Forest (Tukey post-hoc comparison z-ratio= -6.208, p-value <.0001), where significantly more beetles were caught in mice-baited traps compared to chick-baited traps over the entire field season.

Question 3: Do some **trapping locations** within woodlands attract more *N. vespilloides* beetles when baited with chick carcasses rather than mice?

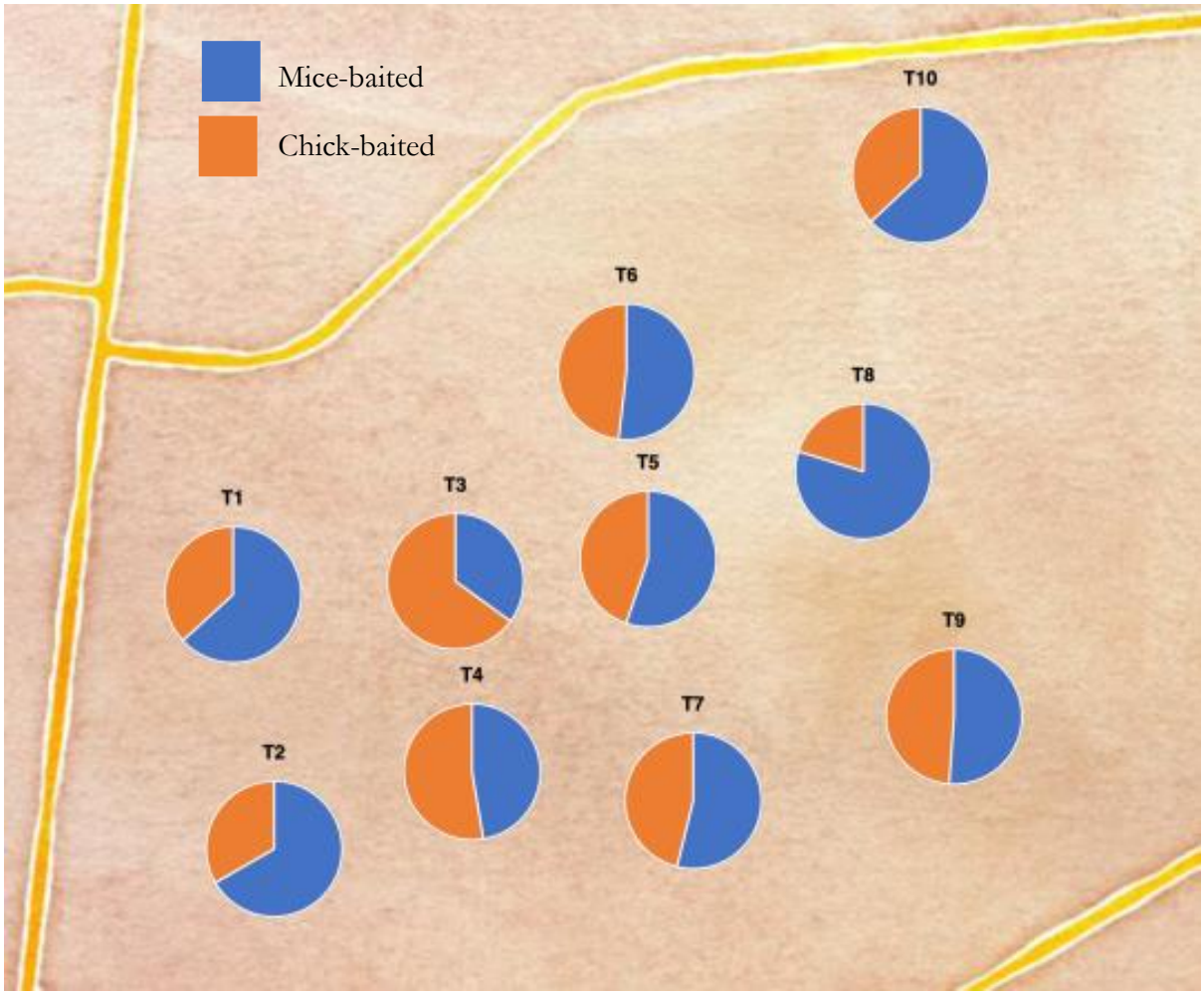


Figure 5a: *N. vespilloide*s collected in chick- and mouse-baited traps pairs in 2019 at Thetford Forest. Pie charts depict the proportion of beetles attracted to each carrion type at the trapping location. Trapping locations at Thetford Forest were baited with chick and mice carrion simultaneously, using a paired trap setup.

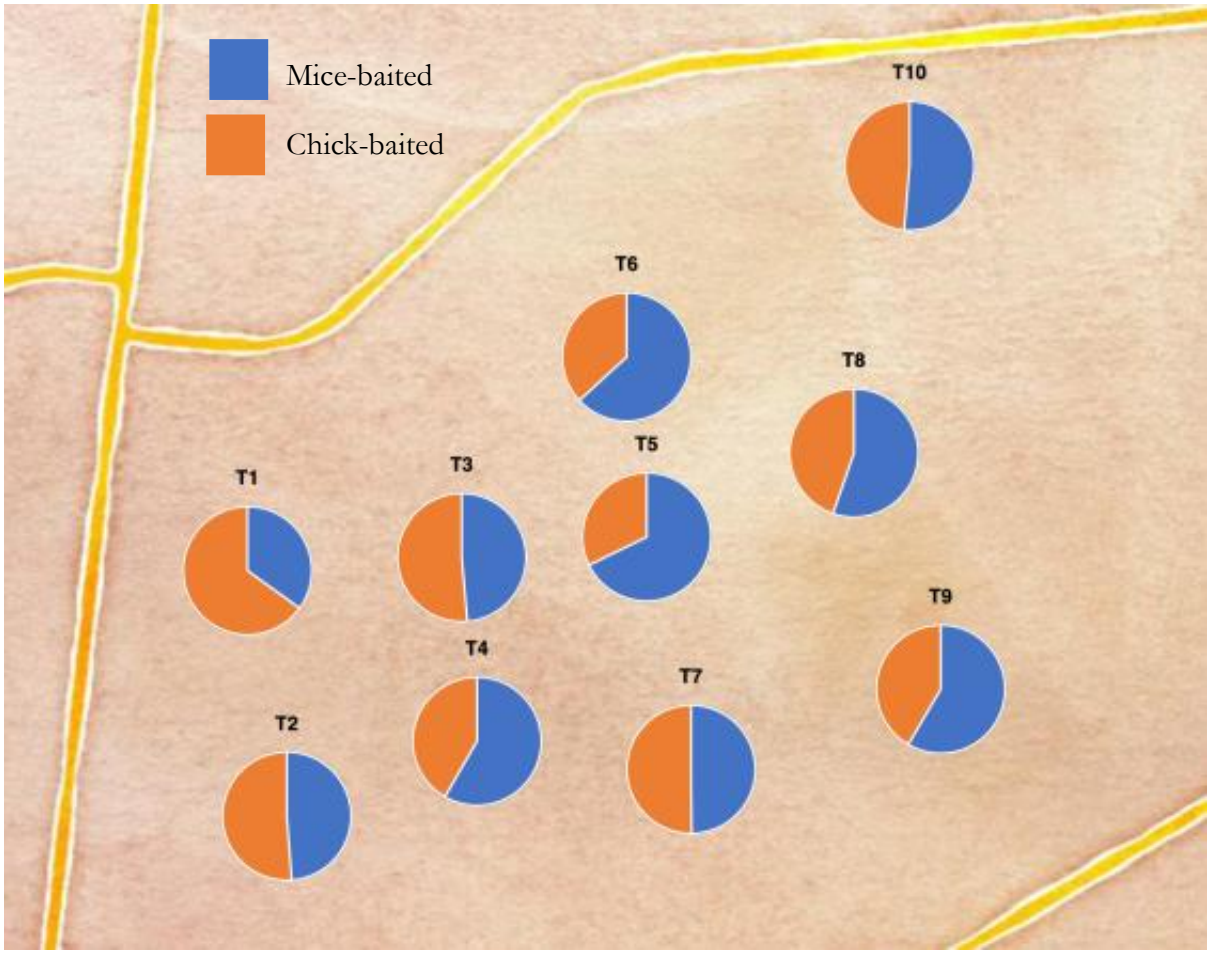


Figure 5b: *N. vespilloide*s collected in chick- and mouse-baited trap pairs in 2017 at Thetford Forest. Pie charts depict the proportion of beetles attracted to each carrion type at the trapping location. Trapping locations at Thetford Forest were baited with chick and mice carrion simultaneously, using a paired trap setup.

Table 4 a: Results of ANOVA on the effects of carcass type, sampling year, trapping location and their interactions on the number of beetles caught.

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
carcass type	66.611	1	3.307e-16 ***
sampling year	12.964	1	0.0003176 ***
trapping location	491.114	9	< 2.2e-16 ***
carcass type x trapping location	59.390	9	1.757e-09 ***
trapping location x sampling year	115.485	9	2.2e-16 ***
carcass type x trapping location x sampling year	58.132	10	8.165e-09 ***

Table 4 b: Model summary showing results of the GLMM to test for the effects of carcass type, sampling year, trapping location and their interactions on the number of beetles caught.

Factors	*Estimate*	*Std. Error*
Intercept	-0.046861	0.437039
Carcass-Mouse	0.538756	0.474479
Trapping Location- T2	-0.120239	0.555770
Trapping Location- T3	-0.120239	0.555770
Trapping Location- T4	0.616143	0.439091
Trapping Location- T5	0.902370	0.420002
Trapping Location- T6	1.471258	0.402499
Trapping Location- T7	1.578158	0.399639
Trapping Location- T8	1.418846	0.402607
Trapping Location- T9	1.705688	0.397747
Trapping Location- T10	1.131322	0.424236
Sampling Year-2017	1.591396	0.490693
Carcass-Mouse x Trapping Location- T2	0.154778	0.688662
Carcass-Mouse x Trapping Location- T3	-1.157525	0.545922
Carcass-Mouse x Trapping Location- T4	-0.644228	0.574684
Carcass-Mouse x Trapping Location- T5	-0.329251	0.534055
Carcass-Mouse x Trapping Location- T6	-0.465887	0.511252
Carcass-Mouse x Trapping Location- T7	-0.384645	0.505895
Carcass-Mouse x Trapping Location- T8	0.833252	0.498648
Carcass-Mouse x Trapping Location- T9	-0.494267	0.504556
Carcass-Mouse x Trapping Location- T10	-0.006055	0.532483
Trapping Location- T2 x Sampling Year-2017	0.017303	0.581939
Trapping Location- T3 x Sampling Year-2017	-0.496368	0.433995
Trapping Location- T4 x Sampling Year-2017	0.020764	0.465371
Trapping Location- T5 x Sampling Year-2017	-0.353355	0.448361
Trapping Location- T6 x Sampling Year-2017	-1.045829	0.435739
Trapping Location- T7 x Sampling Year-2017	-0.493376	0.424389
Trapping Location- T8 x Sampling Year-2017	-0.374565	0.428285

Trapping Location- T9 x Sampling Year-2017	-0.733922	0.424337
Trapping Location- T10 x Sampling Year-2017	0.221549	0.445888
Carcass-Mouse x Trapping Location- T1 x Sampling Year-2017	0.082388	0.496392
Carcass-Mouse x Trapping Location- T2 x Sampling Year-2017	-0.742352	0.530597
Carcass-Mouse x Trapping Location- T3 x Sampling Year-2017	0.563009	0.291977
Carcass-Mouse x Trapping Location- T4 x Sampling Year-2017	0.443288	0.348006
Carcass-Mouse x Trapping Location- T5 x Sampling Year-2017	0.533426	0.274063
Carcass-Mouse x Trapping Location- T6 x Sampling Year-2017	0.467199	0.239998
Carcass-Mouse x Trapping Location- T7 x Sampling Year-2017	-0.160279	0.207555
Carcass-Mouse x Trapping Location- T8 x Sampling Year-2017	-1.158742	0.190119
Carcass-Mouse x Trapping Location- T9 x Sampling Year-2017	0.296253	0.205640
Carcass-Mouse x Trapping Location- T10 x Sampling Year-2017	-0.479114	0.259470

Comparing trapping data in Thetford Forest in 2017 and 2019 (Figures 5a and 5b, Table 4a) we found a significant three-way interaction between carcass type, trapping location and sampling year. Our findings are described in Table 4b. Overall, all trapping locations, other than T8 (Tukey post-hoc comparison z-ratio= -1.403, p-value= 0.1606) recorded significantly more beetles in 2017 compared to 2019.

Table 4 c: Tukey HSD post-hoc comparisons of the interaction between carcass type (chick or mouse), trapping location (T1 to T10) and sampling year (2017 & 2019) on beetles trapped at Thetford Forest

Trapping location	*Results*
T1	* Both mouse-baited (z-ratio= -4.000, p-value= 0.0004) and chick-baited (z-ratio= -3.243, p-value= 0.0065) traps recorded significantly more beetles in 2017 compared to 2019 * In 2017, significantly more beetles preferred mice compared to chicks (z-ratio= -4.259, p-value= 0.0001), but not during 2019
T2	* Chick-baited traps in 2017 attracted significantly more beetles compared to 2019 (z-ratio= -3.112, p-value= 0.0101)

T3	* Both mouse-baited (z-ratio= -4.466, p-value <.0001) and chick-baited (z-ratio= -3.221, p-value= 0.0070) traps recorded significantly more beetles in 2017 compared to 2019
T4	* Both mouse-baited (z-ratio= -5.362, p-value <.0001) and chick-baited (z-ratio= -4.248, p-value= 0.0001) traps recorded significantly more beetles in 2017 compared to 2019 * In 2017, significantly more beetles preferred mice compared to chicks (z-ratio= -2.673, p-value= 0.0378), but not during 2019
T5	* Both mouse-baited (z-ratio= -5.190, p-value <.0001) and chick-baited (z-ratio= -3.459, p-value= 0.0030) traps recorded significantly more beetles in 2017 compared to 2019 * In 2017, significantly more beetles preferred mice compared to chicks (z-ratio= -6.061, p-value<.0001), but not during 2019
T6	* Mouse-baited (z-ratio= -3.051, p-value= 0.0122) traps recorded significantly more beetles in 2017 compared to 2019 * In 2017, significantly more beetles preferred mice compared to chicks (z-ratio= -3.696, p-value= 0.0013), but not during 2019
T7	* Both mouse-baited (z-ratio= -2.896, p-value= 0.0197) and chick-baited (z-ratio= -3.354, p-value= 0.0044) traps recorded significantly more beetles in 2017 compared to 2019
T8	* Chick-baited (z-ratio= -3.665, p-value= 0.0014) traps recorded significantly more beetles in 2017 compared to 2019 * In 2019, significantly more beetles preferred mice compared to chicks (z-ratio= -8.945, p-value <0.0001), but not during 2017
T9	* Both mouse-baited (z-ratio= -3.588, p-value= 0.0019) and chick-baited (z-ratio= -2.631, p-value= 0.0423) traps recorded significantly more beetles in 2017 compared to 2019 * In 2017, significantly more beetles preferred mice compared to chicks (z-ratio= -3.007, p-value= 0.0141), but not during 2019
T10	* Both mouse-baited (z-ratio= -4.021, p-value= 0.0003) and chick-baited (z-ratio= -5.118, p-value <0.0001) traps recorded significantly more beetles in 2017 compared to 2019

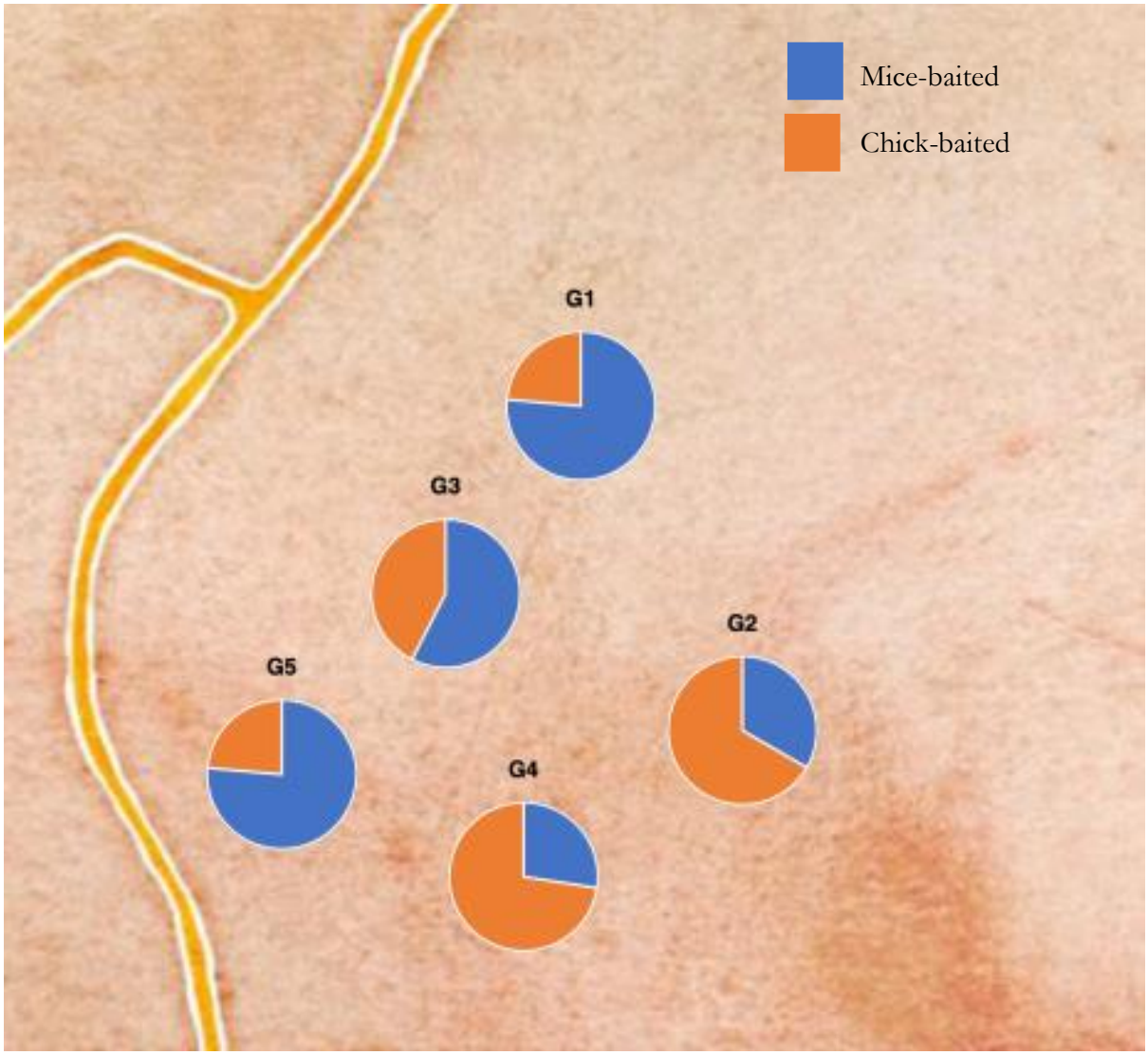


Figure 5c: *N. vespilloide*s collected in chick and mouse trap pairs in 2017 at Gamlingay Wood. Pie charts depict the proportion of beetles caught on each carrion type at the trapping location. Trapping locations at Gamlingay Wood were baited with chick and mice carrion sequentially, alternating carrion type in the traps at every collection trip.

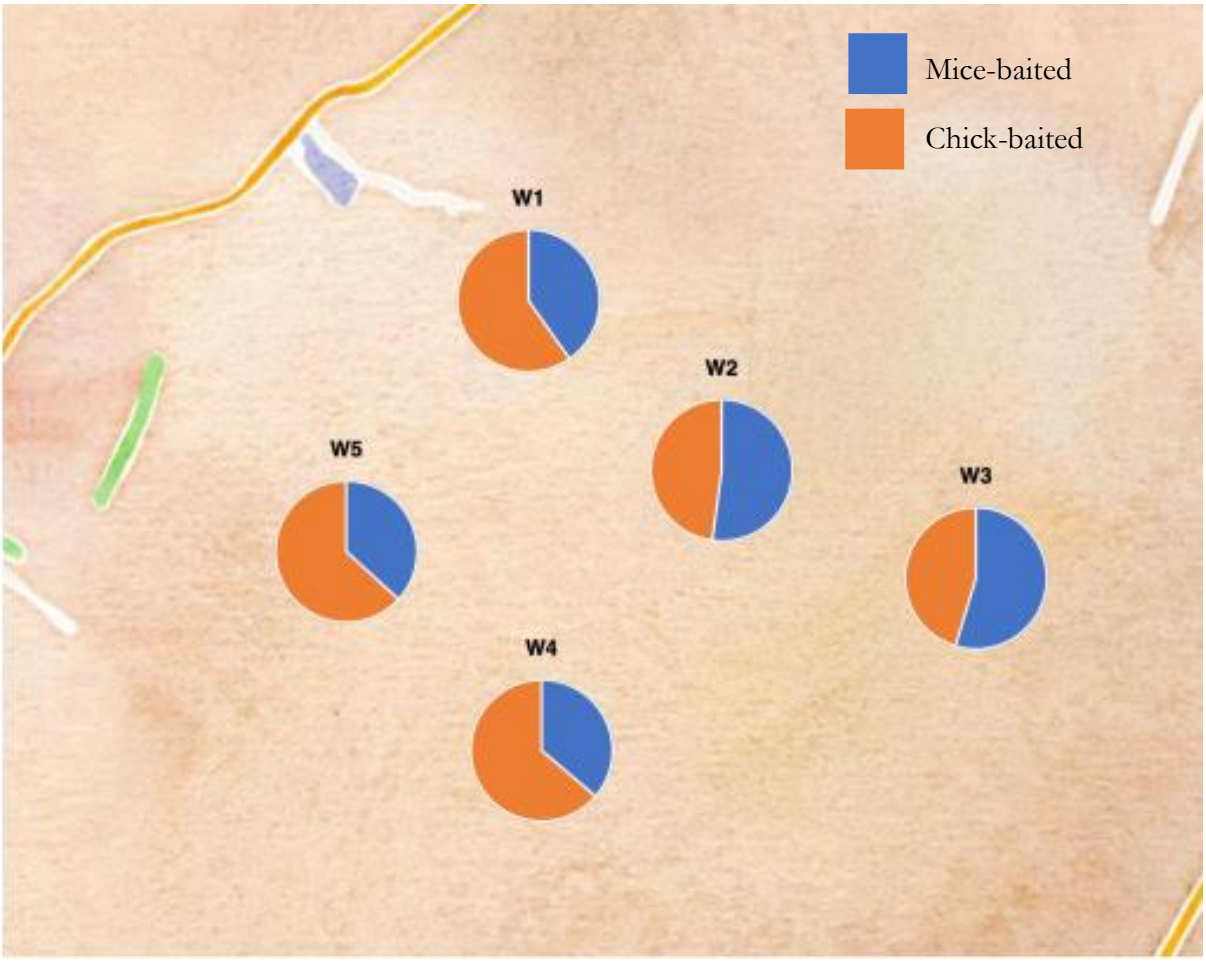


Figure 5d: *N. vespilloides* collected in chick and mouse trap pairs in 2017 at Waresley Wood. Pie charts depict the proportion of beetles attracted to each carrion type at the trapping location. Trapping locations at Waresley Wood were baited with chick and mice carrion sequentially, alternating carrion type in the traps at every collection trip.

Table 5:

a. Results of the ANOVA of the effects of carcass type, trapping location and their interactions on beetle preference

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
carcass type	37.21	1	1.061e-09 ***
trapping location	401.07	19	< 2.2e-16 ***
carcass type x trapping location	153.89	19	< 2.2e-16 ***

b. Model summary showing results of the GLMM to test for the effects of carcass type, trapping location and their interactions on beetle preference

Factors	*Estimate*	*Std. Error*
Intercept	2.359936	0.328151
Carcass-Mouse	0.083782	0.301085
Grouped data by site: Gamlingay		
Trapping Location- G2	-0.031544	0.303087
Trapping Location- G3	0.873584	0.244622
Trapping Location- G4	0.342749	0.253426
Trapping Location- G5	-1.440323	0.355096
Grouped data by site: Thetford		
Trapping Location- T1	-0.937168	0.393823
Trapping Location- T2	-1.070446	0.396316
Trapping Location- T3	-0.101648	0.383749
Trapping Location- T4	-0.142585	0.388267
Trapping Location- T5	-0.230494	0.389369
Trapping Location- T6	-0.408785	0.393699
Trapping Location- T7	0.264503	0.384149
Trapping Location- T8	0.249862	0.385347
Trapping Location- T9	0.177383	0.386029
Trapping Location- T10	0.555231	0.382133
Grouped data by site: Waresley		
Trapping Location- W1	0.001299	0.302495
Trapping Location- W2	-0.542328	0.315031
Trapping Location- W3	0.985717	0.233811
Trapping Location- W4	0.880952	0.253073
Trapping Location- W5	-0.076963	0.303935
Grouped data by site: Gamlingay		
Carcass-Mouse x Trapping Location- G2	0.312316	0.502321
Carcass-Mouse x Trapping Location- G3	-0.648666	0.280900
Carcass-Mouse x Trapping Location- G4	-1.468529	0.366521
Carcass-Mouse x Trapping Location- G5	2.180621	0.525709

Grouped data by site: Thetford		
Carcass-Mouse x Trapping Location- T1	0.641525	0.336158
Carcass-Mouse x Trapping Location- T2	-0.028821	0.352330
Carcass-Mouse x Trapping Location- T3	-0.035628	0.322616
Carcass-Mouse x Trapping Location- T4	0.254071	0.326545
Carcass-Mouse x Trapping Location- T5	0.659162	0.325086
Carcass-Mouse x Trapping Location- T6	0.456194	0.334663
Carcass-Mouse x Trapping Location- T7	-0.090009	0.320837
Carcass-Mouse x Trapping Location- T8	0.129472	0.321360
Carcass-Mouse x Trapping Location- T9	0.256981	0.321708
Carcass-Mouse x Trapping Location- T10	-0.030162	0.315547
Grouped data by site: Waresley		
Carcass-Mouse x Trapping Location- W1	0.607988	0.494192
Carcass-Mouse x Trapping Location- W2	1.089727	0.503229
Carcass-Mouse x Trapping Location- W3	-0.985674	0.272770
Carcass-Mouse x Trapping Location- W4	-1.385144	0.300420
Carcass-Mouse x Trapping Location- W5	0.336041	0.510918

Comparing trapping data in Gamlingay, Thetford and Waresley in 2017 (Figures 5b, 5c and 5d, Table 5), we found that beetle catch varied significantly with trapping location in all three sites. In Gamlingay Wood, significantly more beetles were recorded in trap G3 (GLMM summary statistics: z-value= 2.980, p-value= 0.00289), while significantly less beetles were found in trap G5 (GLMM summary statistics: z-value= -2.861, p-value= 0.00422). In Waresley Wood, trap W3 attracted significantly more beetles than any other trap (GLMM summary statistics: z-value= 2.446, p-value= 0.01446). Trapping locations T1 (GLMM summary statistics: z-value= -2.318, p-value= 0.02045) and T2 (GLMM summary statistics: z-value= -4.039, p-value= 5.36e-05) recorded significantly fewer beetles in Thetford Forest compared to all other traps.

We found a significant carcass type x trapping location interaction, with different traps in each of the three sampling sites varying significantly in their beetle catch, depending on the carrion they were baited with. In Gamlingay Wood, traps G3 (Tukey post-hoc comparison: z-ratio= 2.173, p-value= 0.0298) and G4 (Tukey post-hoc comparison: z-ratio= 4.707, p-value <.0001) attracted significantly more beetles whenever they were baited with chicks, while G5 (Tukey post-hoc comparison: z-ratio= -7.030, p-value <.0001) attracted more beetles when baited with mice.

In Waresley Wood, W1 (Tukey post-hoc comparison: z-ratio= -2.585, p-value= 0.0097) and W2 (Tukey post-hoc comparison: z-ratio= -4.133, p-value <.0001) attracted more beetles when baited with a mouse carcass. W3 (Tukey post-hoc comparison: z-ratio= 3.518, p-value= 0.0004) and W4 (Tukey post-hoc comparison: z-ratio= 4.528, p-value <.0001) attracted significantly more beetles when baited with chicks.

In Thetford Forest, half of the trapping locations attracted significantly more beetles in their mouse traps: T1 (Tukey post-hoc comparison: z-ratio= -4.881, p-value <.0001), T4 (Tukey post-hoc comparison: z-ratio= -2.673, p-value= 0.0075), T5 (Tukey post-hoc comparison: z-ratio= -6.060, p-value <.0001), T6 (Tukey post-hoc comparison: z-ratio= -3.696, p-value= 0.0002) and T9 (Tukey post-hoc comparison: z-ratio= -3.007, p-value= 0.0026).

Question 4: Does seasonal variation in **population density** alter the relative abundance of beetles attracted to avian versus mammalian carcasses?

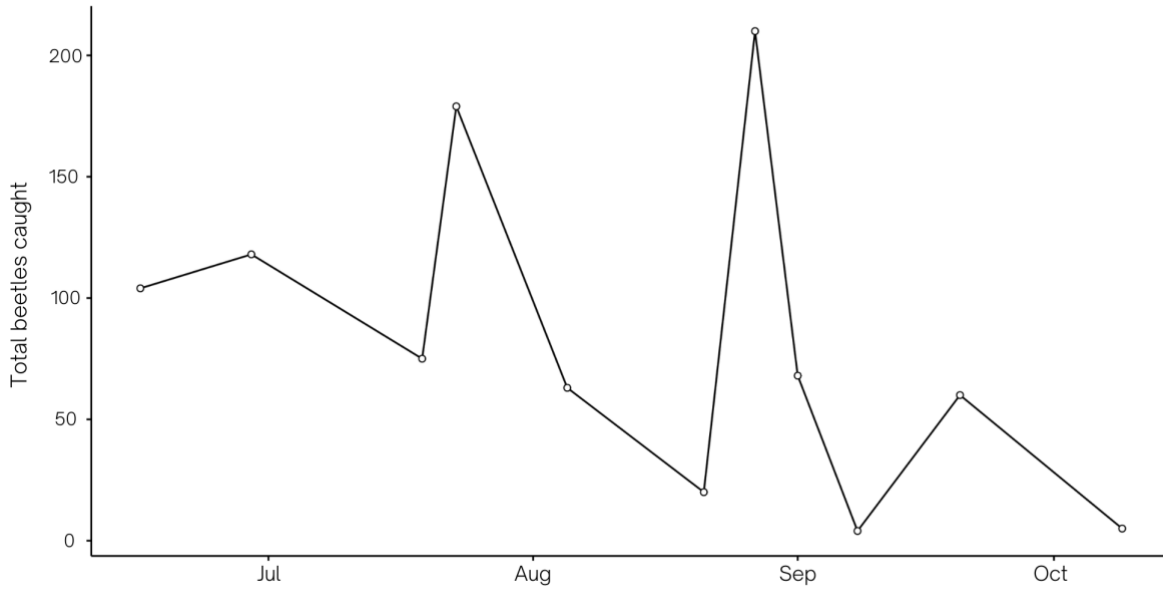


Figure 6: Total *N. vespilloide*s collected in 2019 at Thetford Forest.

Table 6:

a. Results of ANOVA to test the effect of sampling day on total population density

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
sampling day	91.279	1	< 2.2e-16 ***

b. Model summary showing results of the LM to test for the effects of sampling day on total population density

Factors	*Estimate*	*Std. Error*
Intercept	2.8772939	0.0909888
Sampling Day	-0.0038912	0.0004073

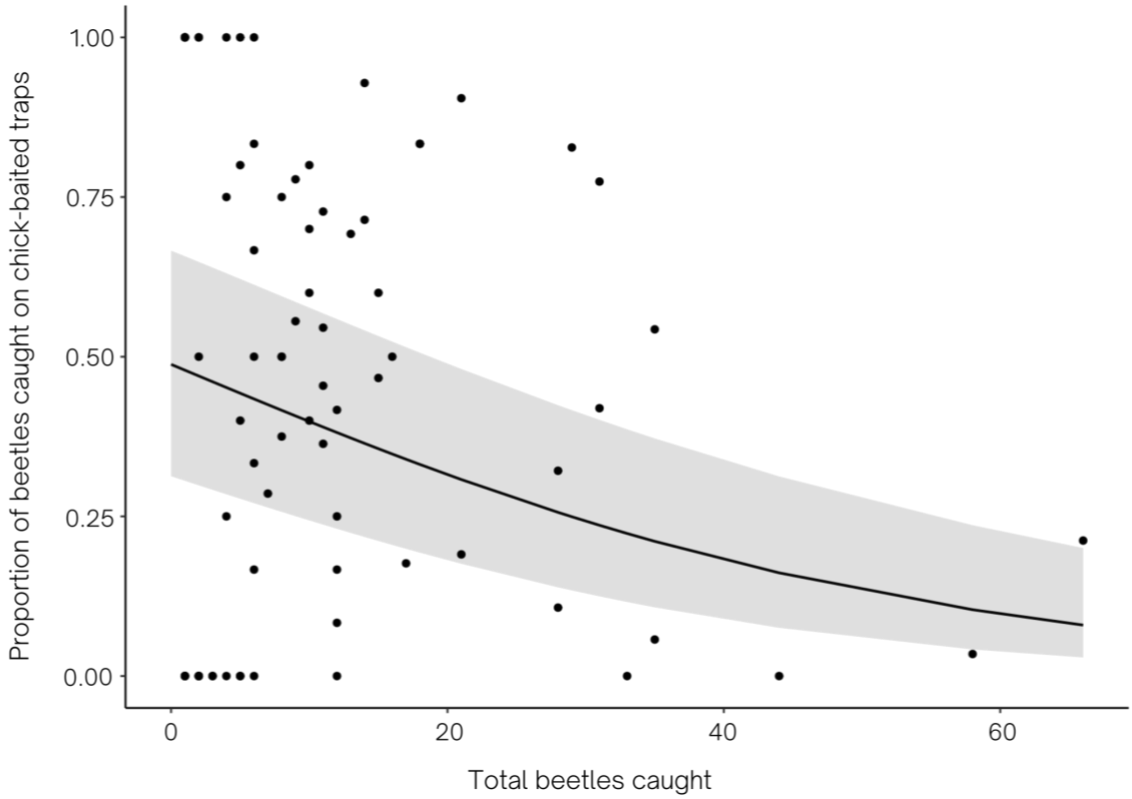


Figure 7: Correlation between population density and the proportion of beetles trapped on chick carrion at Thetford Forest in 2019. Each datapoint represents the proportion of beetles that were caught on chicks, at a given population density.

Table 7:

a. Results of the analysis of deviance to test the effect of total population density on the proportion of beetles trapped on chick carrion

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
total beetles	24.922	1	5.97e-07 ***

b. Model summary showing results of the GLMM to test the effect of total population density on the proportion of beetles trapped on chick carrion

Factors	*Estimate*	*Std. Error*
Intercept	-0.048128	0.376866
Total beetles	-0.036327	0.007277

Population density varied significantly over the entire field season (Figure 6, Table 6). Comparing the distribution of beetles trapped on chick and mice carcasses at different population densities, we found that there was a decrease in the proportion of beetles caught on chicks as population density increased (Figure 7, Table 7).

Question 5: How abundant are other burying beetle species on avian versus mammalian carcasses?

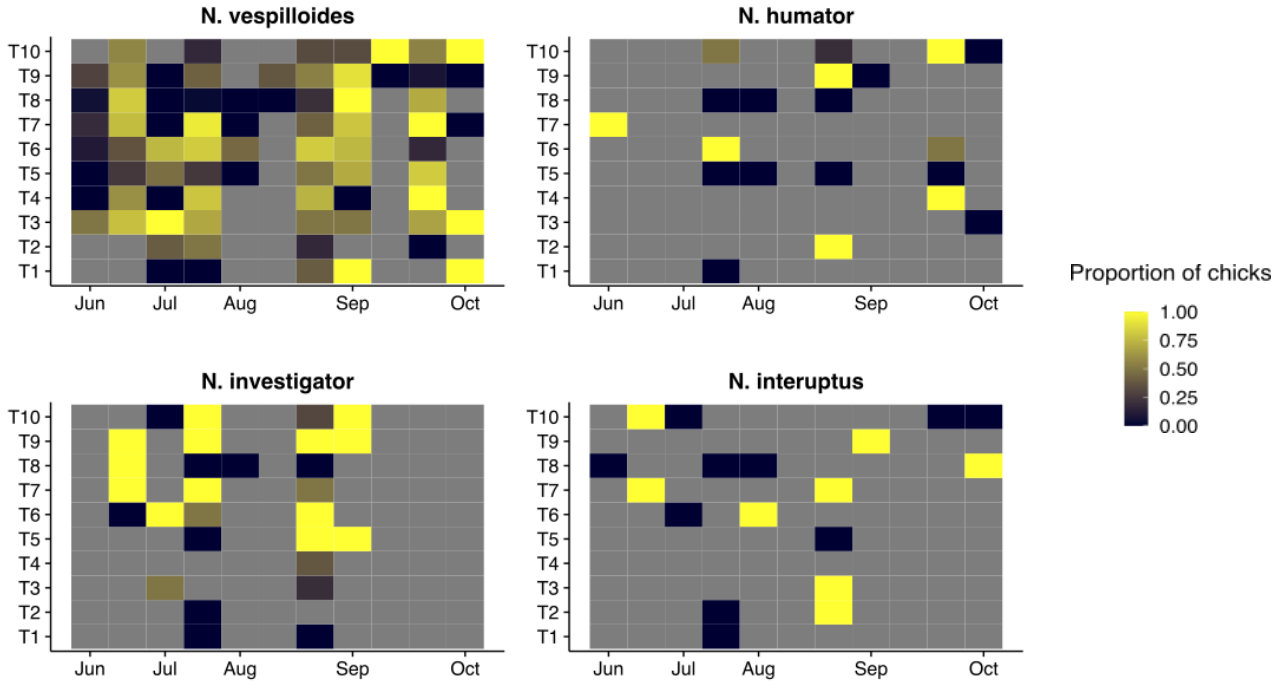


Figure 8: Heatmaps showing temporal and spatial population differences in the number of beetles caught on mice versus chicks Thetford Forest in 2019 for *N. vespilloides*, *N. humator*, *N. investigator* and *N. interruptus*. The trapping locations are on the y-axis while the x-axis represents the time of trapping. The colour intensity in each panel indicates the proportion of individuals in chick traps, compared to the total beetles caught during each sampling period. The grey boxes represent "NaNs", i.e., no beetles of that species were found in the traps on those sampling days.

Table 8:

a. Results of ANOVAs of the effects of carcass type, species, sampling day and their interactions on beetle preference

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
carcass type	42.7050	1	6.365e-11 ***
species	356.9321	4	< 2.2e-16 ***
sampling day	28.4377	1	9.676e-08 ***
carrion type x sampling day	5.4845	1	0.01919 *
species x sampling day	32.7229	4	1.361e-06 ***

b. Model summary showing results of the GLMM to test the effects of carcass type, species, sampling day and their interactions on beetle preference

Factors	*Estimate*	*Std. Error*
Intercept	-0.7341426	0.5499117
Carcass-Mouse	0.7304981	0.1534318
Species-*N. interuptus*	-0.4482186	0.7203114
Species-*N. investigator*	-0.5270676	0.6239997
Species-*N. vespillo*	-0.1301225	1.2847815
Species-*N. vespillooides*	2.4167682	0.5225398
Sampling Day	0.0028093	0.0057050
Carrion-Mouse x Sampling Day	-0.0044796	0.0019128
Species-*N. interruptus* x Sampling Day	-0.0014774	0.0081755
Species-*N. investigator* x Sampling Day	0.0176104	0.0070151
Species-*N. vespillo* x Sampling Day	0.0003466	0.0151668
Species-*N. vespillooides* x Sampling Day	-0.0068404	0.0056815

Combining data from all burying beetle species (*N. vespilloides*, *N. humator*, *N. investigator* and *N. interruptus*) at Thetford Forest in 2019 (Figure 8, Table 8), we found that overall more beetles were caught in mouse-baited traps than in chick baited traps (Tukey post-hoc comparison: z-ratio= -5.975, p-value <0.0001). We did not find any significant species x carcass type interaction: that is, there was no evidence that species consistently diverged in the number caught on mice versus chicks, across the field season. Beetle density, and the carrion type upon which they were trapped,

varied for all species very significantly from one sampling day to another (Table 7). *N. vespilloides* was the most abundant species, comprising of a majority of the beetles found in Thetford across the 2017 beetle activity season (83%, n=912), followed by *N. investigator* (11%, n=112).

Discussion

To investigate whether there is any evidence of differential resource use within wild populations of burying beetles, we posed five questions about carrion use by burying beetles in relation to time of season and location, within and among different woodland populations which we addressed using trapping data.

Questions 1 & 2: Is there spatial and temporal variation in the abundance of *N. vespilloides* beetles trapped on avian and mammalian carcasses?

As predicted, we found very high temporal and spatial variation in the number of *N. vespilloides* caught on different types of carrion (Tables 1 & 2; Figures 3a & 3b). In Thetford Forest, overall, significantly more beetles were caught on mice than on chicks. However, in early May, late May and late September more beetles were trapped on chicks than mice. Likewise, *N. vespilloides* were trapped in greater numbers on mice than on chicks in Gamlingay Wood overall but were found in greater numbers on chicks than on mice in late-June and late-September. However, this pattern was completely reversed in the adjacent Waresley Wood where more *N. vespilloides* were caught on chicks than on mice overall, but greater numbers were trapped on mice only in in mid-June and late-September. These patterns defy a simple explanation. They cannot simply be due to seasonal variation in carrion abundance since we expect greater abundance of avian carrion in late spring or early summer due to greater mortality among fledgling songbirds (Newton 1998, Chase et al. 2005, Clapham 2011, Capstick 2017). Conversely high mortality in mouse populations should lead to abundance in mammalian carrion in mid to late summer (Moffat 1910, Harris 1979, Merritt et al. 2001, Haberl & Krystufek 2003, Clapham 2011). However, measures of seasonal availability of small carrion can often be skewed because of the rate at which these can disappear from the field due to scavenging, predation and the impact of carrion feeders (Crawford 1971, Balcomb 1986). Therefore, it is still possible that the results we observe are correlated with differences in resource use within burying beetle populations, and that each woodland has its own particular pattern of variation in carrion abundance.

Since sampling of the small mammal population in Gamlingay and Waresley woods has indicated no evidence for differential resource availability between them (Sun et al. 2020), it could be possible that the stark differences we observe between these adjacent woods could be related to differential

resource partitioning between the woodlands due to intense resource competition (Hopwood 2016, Sun et al. 2020) as well as localised differences in avian fauna and carrion availability.

Question 3a: Do some trapping locations within woodlands attract more *N. vespilloides* beetles when baited with chick carcasses rather than mice?

On comparing beetle catch in different trapping locations in 2017 and 2019 in Thetford Forest, we found that most traps recorded more beetles in both mice and chick traps during 2017 (Figures 5a & 5b, Table 4a & 4b). This could be simply an artefact of the different methodologies we used to trap beetles during the field season, with continuous trapping in 2017 as opposed to traps only being put up for a certain time (4 days on average) during each collection period in 2019. In addition, at most trapping locations in 2017 recorded more beetles in their mice-baited traps, though this was not the case in 2019, where most trapping locations recorded an equivalent number of beetles on average, in both mice- and chick-baited traps. It is difficult to ascertain whether this result is simply explained by the difference in trapping methodology or due to differences in population dynamics and resource availability between different sampling years.

Question 3b: Is this true when *N. vespilloides* beetles have a paired choice, as with the Thetford data or when sequentially choosing as with Gamlingay and Waresley woods?

Findings from averaged trapping location data in Thetford indicated greater numbers of beetles were trapped on mice at half the trapping locations, while in the other locations very similar numbers of beetles were caught on chicks and mice. The sequential choice set-up used in Gamlingay and Waresley woods prevented us from simultaneously comparing the number of beetles caught on each type of carrion at the same trapping location. However, we could identify locations within the populations that attracted significantly more beetles when baited with mice (traps G5, W1 and W2) or chicks (traps G3, G4, W3 and W4). This could signify that the landscape within populations is composed of patchy habitats that act as hotspots for resource availability (Arthur & Levins 1964, Morris 1987, Fortin et al. 2008).

Question 4: Does seasonal variation in population density alter the relative abundance of beetles attracted to avian versus mammalian carcasses?

The local spatial distribution of resources and populations density can interact and significantly impact population dynamics (Middendorf 1984, Jacobson et al. 2015). We found a density dependent skew in the number of beetles trapped on each type of carrion in Thetford, with fewer beetles attracted to chicks at higher population densities (Figure7, Table 7). Furthermore, certain trapping locations attracted more beetles when they were baited with mice while other traps attracted more beetles when they were baited with chicks. This seemingly contradicts existing theory on generalist and specialist strategies, which predict a decline in specialised resource use with increasing population density (Fretwell 1972, Morris 2003, Fortin et al. 2008). For our analyses, we have assumed that the burying beetle density in the traps are an accurate reflection of population density in the wild. However, it is possible that the population at the time was composed of individuals that specialised on mammalian carrion, and therefore, chose mice carrion in greater numbers whilst also utilising other types of carrion available in the field instead of the chick carcasses we used to bait the traps.

Question 5: How abundant are other burying beetle species on avian versus mammalian carcasses?

We did not find any evidence for specialised resource use between different *Nicrophorus* species, though the population density and numbers trapped on each type of carrion varied significantly for all species over the field season (Figure 8, Table 8). This is consistent with historical data indicating that resource partitioning between burying beetle species is mediated by seasonal patterns and habitat specificity (Anderson 1982).

We measured differential resource use in the wild by actively manipulating resource availability on a local spatial scale in the woodlands. It is, therefore, challenging to compare our findings with previous work on resource use with other insects as most of these studies involve phytophagous insects such as fruit flies, moths and aphids where differential resource use can be quantified in a more natural way by simply measuring population density and occurrence on host plants (Feder et al. 1994, Groman & Pellmyr 2001, Via et al. 2000). Furthermore, our data are cross-sectional snapshots at different moments in time through the breeding season. We were unable to track individuals to see how their behaviour varied seasonally.

Nevertheless, it is possible that the high temporal and local spatial variation in numbers caught on each type of carrion could be due to individual specialisation in carrion use. Individual specialisation is a widely recognised phenomenon in natural populations of many vertebrate and invertebrate taxa (Bolnick et al. 2003, Arau´jo et al. 2011, Bolnick et al. 2011). Generalist populations can be composed of specialised individuals that utilise a smaller subset of the entire population's resource base (van Valen 1965, West 1986, Bolnick et al. 2003) or of a mix of generalist and specialist phenotypes that alternate in frequency within the population (Rainey et al. 2000; Bono et al. 2015). This variation in resource use can be a result of the confluence of several ecological factors such as the level of intra- and inter-specific competition, ecological opportunity and predation (Morris 2003, Arau´jo et al. 2011). We have not proven the existence of individual burying beetle specialists, but it is a plausible hypothesis that could account for the results reported in this chapter.

The following chapters consider this hypothesis further, by exploring whether natural populations of burying beetles are composed of individuals or groups of individuals that specialise on different carrion resources for breeding. We begin these analyses by testing whether the temporal variation in resource use that we report here is adaptive.

Chapter 3

Temporal variation in fitness in natural populations of burying beetles

Introduction

Our first step in testing the idea that are resource specialists within natural *N. vespilloides* populations was to investigate whether seasonal variation in carrion use is related to burying beetle reproductive success.

Seasonality can be a strong and critical source of environmental variability for organisms in temperate environments (Williams et al. 2015). It can impose fluctuating selection pressures on survival and fecundity that can give rise to a great diversity of adaptive responses (Varpe 2017). For instance, insects prepare for periods of dormancy by down-regulating reproduction, up-regulating fat accumulation and hardiness and then down-regulating metabolism (Danks 2007, Koštál 2006, Staples 2016). Two *Drosophila* species (*D. melanogaster* and *D. simulans*) sampled from the same orchard in Pennsylvania, USA in 2011 exhibited a steady seasonal decline in fecundity (Behrman et. al 2015).

Seasonal changes in resource availability and weather conditions can drive population dynamics by directly impacting a species' life history traits (Morgan et. al 2001, Ragland & Kingsolver 2008, Johnson et al. 2016), thereby leading to seasonal variations in reproduction, development and mortality. For example, a 5-year study of the phytophagous ladybird beetle, *Epilachna nipponica*, in two local populations of central Japan revealed that they exhibited distinct patterns of temporal variation in fitness (Ohgushi 1991). Early season cohorts in one population had higher fitness than later cohorts while in the other population, the reverse was true and later cohorts had greater lifetime fitness.

Burying beetles live in seasonal environments. They are active in Europe and North America between April and October, with variation in abundance depending on the species (Dekeirsschieter et al. 2011). At the end of their activity period, burying beetles burrow in the soil

and overwinter as adults or in the pre-pupal stage for some species (Pukowski 1933, Peck & Kaulbars 1987, Ratcliffe 1996).

Older studies comparing reproductive fitness across the burying beetle reproductive season have focused on the effect of environmental conditions such as temperature (Meierhofer et al. 1999), sampling location and carrion size (Wilson & Fudge 1984), or the incidence and duration of parental care (Scott & Traniello 1990).

Reproductive output and its seasonality vary greatly among *Nicrophorus* species. While *N. vespillo* populations studied by Müller et al. (1999) in Bielefeld, Germany, did not differ in the number of offspring produced throughout the season, the period of parental care was significantly higher in spring, compared to early or late summer. This is likely mediated by lower temperatures in spring, which slow down offspring development. Furthermore, natural populations of *N. orbicollis* in southern New Hampshire, United States produced heavier broods in the first few weeks of the breeding season compared to later broods (Scott & Traniello 1990). This has been attributed to less intense competition with flies at the beginning of the season or, potentially, to strategically greater levels of investment in first broods. In other work, Wilson & Fudge (1984) sampled two different sites in Michigan, United States using large and small mice carcasses, and found a large amount of unexplained variation in brood size. At one of the sampling sites, *N. orbicollis* beetles had fewer offspring in early summer (June) while *N. defodiens* had more offspring in late summer (August).

Although these studies have taken variation in carrion size into account, they do not consider how specialisation and local adaptation to particular types of carrion might affect reproductive success. Yet our results from temporal variation in carrion use in the field (Chapter 2) suggest that beetles may be differentially adapted to certain carrion types, which could vary seasonally in their abundance, and that beetles could consequently vary in their reproductive success on different carcasses at different times in the year.

To test this hypothesis, we focussed on burying beetles *N. vespilloides* sampled at Thetford Forest in 2017. *N. vespilloides* was the most abundant species at this site and occurred throughout the field season from May to October (Chapter 2). We previously found that in this wood, *N. vespilloides* beetles from early summer (June) were more likely to be trapped on mice rather than on chicks, whereas beetles from late summer (August) were equally likely to be trapped on mice and chicks.

In this chapter, I test whether the temporal variation in trapping bias I observed in the field is related to reproductive performance on each type of carrion at different points in the year and is, therefore, adaptive. Specifically, I tested the following predictions:

1. Individual beetles have greatest reproductive success on the carrion type they are trapped upon.
2. At a population level, beetles trapped in June have greater reproductive success on mice over chicks.
3. At a population level, beetles trapped in August have equal reproductive success on mice and chicks.

Materials and methods

Study area

I sampled the burying beetle population at Thetford Forest from April to October in 2017 at the trap locations schematically depicted in Figure 1.

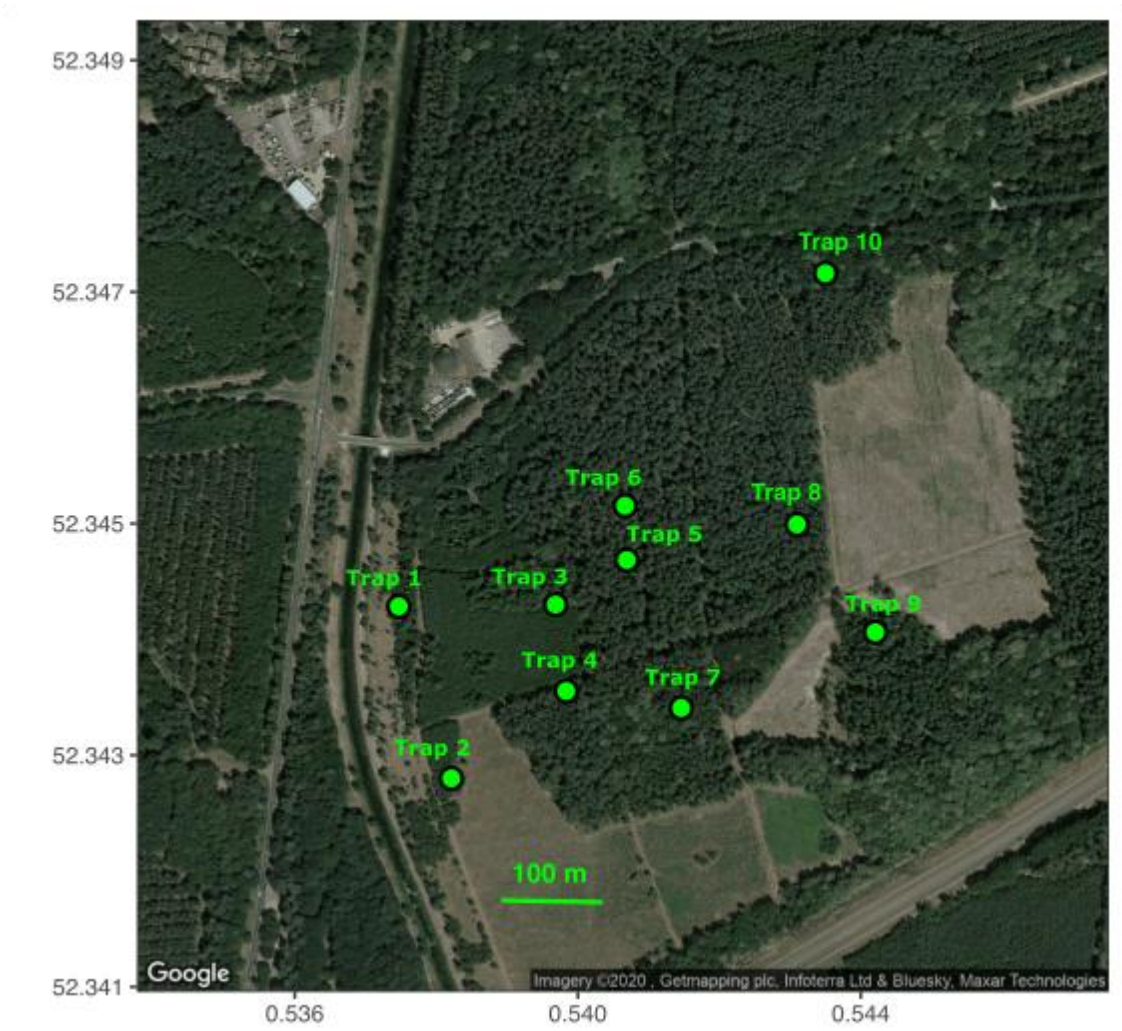


Figure 1: Map of Thetford Forest beetle trapping locations.

Beetle collection

Beetle collection was carried out at Thetford Forest, under permit from Forestry Commission England, as described in Chapter 2.

Beetles were sampled using a paired trap arrangement, in which we placed two beetle traps- one baited with a dead domestic chick and the other baited with a mouse carcass- near each other at each trap location and recorded the number beetles found in each trap. With this design, beetles were given a simultaneous choice between a dead mouse and a dead chick. Each time we rebaited a trap with carrion, we rebaited it with the alternate carrion type. Therefore, if a mouse carcass had been placed in the trap previously, it was replaced by a chick carcass on the next sampling trip to ensure that the trap location itself did not bias beetle catch. The mice and chick carcasses used were matched in weight (30-40 g). The traps within each experimental pair were placed 1-2 m apart. Pairs of traps were placed 200- 400 m apart from each other.

Once the beetles were brought to the lab, they were all processed (see 'Processing field-caught beetles' in Chapter 2) and none were released back into the field.

For the purpose of this study, we compared beetles collected at two different time points during the burying beetle season: the first set were collected in June 2017 after 10 days of trapping between 4 June and 14 June; and the second set were collected in August 2017 after 15 days of trapping between 4 August and 19 August. The 10 trapping locations (as depicted in Figure 1) were the same across both sampling periods.

Measuring reproductive performance

Once the beetles were processed, we put each *N. vespilloides* individual into a small plastic box and fed it 1 g of beef mince. The beetles were stored in the box for 7-10 days before measuring their reproductive performance to ensure that any newly eclosed individuals had had sufficient time to become sexually mature.

A pair of beetles (one male and one female) was placed in a large plastic box half filled with Miracle-Gro compost and provided with either a chick or mouse carcass. Each member of the pair was trapped on the same type of carrion. The mass of the carcass provided for reproduction was recorded and kept consistent within each treatment. The box was then placed inside a cupboard so that it was shielded from light in order to mimic the low light conditions typically experienced by beetles as they breed below ground. Eight days after pairing the beetles (i.e. the point at which the larvae had completed development and were starting to disperse away from the remains of the carcass), we counted and weighed the larvae from each pair. We used brood size and mass at dispersal as a measure of reproductive success.

Prediction 1: Individual beetles have greatest reproductive success on the carrion type they are trapped upon

To test this prediction, I analysed only those beetles caught in June, and established the following four treatments:

Table 1: Experimental design to test the reproductive performance of beetles from June 2017 on different carrion types

Treatment	Caught on	Bred on
CC	chicks	chicks
MM	mice	mice
CM	chicks	mice
MC	mice	chicks

For this experiment, we used data from a total of 37 pairs of beetles caught on chicks (24 for treatment 'CC' and 13 for 'CM') and 95 pairs of beetles caught on mice (53 for treatment 'MM' and for treatment 42 'MC') that successfully produced broods with at least one larva.

Predictions 2 and 3: At a population level, beetles trapped in June have greater reproductive success on mice rather than chicks, whereas beetles trapped in August have equal reproductive success on mice and chicks.

To test these predictions, I used the following treatments:

Table 2: Experimental design to compare the reproductive success of beetles from June 2017 and August 2017 on their preferred carcass

Overall preference	Caught on	Bred on
High preference for mice (June 2017)	chicks	chicks
	mice	mice
Chicks and mice favoured equally (August 2017)	chicks	chicks
	mice	mice

The data collected from June 2017 beetles from experiment described in Table 2 is exactly the same as treatments 'CC' and 'MM' from the experiment in Table 1.

We sampled 53 pairs of beetles on trapped on mice (MM) and 24 pairs of beetles trapped on chicks (CC) that successfully produced broods with at least one larva in June. In August, we used reproductive output data from 16 pairs of beetles trapped on mice (MM) and 25 pairs of beetles trapped on chicks (CC). There were a total of 7 failed broods (2 on mice carcasses and 5 on chick carcasses) in our August 2017 experiment and these were excluded from our data analysis.

Statistical analysis

We carried out all statistical analyses to test our predictions using R (RStudio version 1.3.959) with generalised linear models (GLM) and generalised linear mixed models (GLMM) using the lme4, glmmsr and MASS packages. Analysis-of-variance tables for model objects were calculated using the 'car' package. Post-hoc comparisons using Tukey's HSD test were carried out using the package 'lsmeans'. The asymptotic test for the equality of coefficients of variation (CV) was carried out using the 'cvequality' package (Feltz & Miller 1996).

Quantifying temporal variation in the frequency at which *N. vespilloides* beetles were trapped on avian and mammalian carcasses in June and August 2017

Using field data from Thetford Forest in 2017, we calculated the average number of beetles per day by dividing the total number of *N. vespilloides* beetles found on a carcass during both collection

trips by the number of days the traps had been left out for. Results from our field experiment to study temporal variation in carrion preference across the entire field season have been detailed in Chapter 2. Here we focus on the two different timepoints for which we also measured reproductive outcome, namely June and August 2017, using a GLMM that included carrion type and sampling month as fixed effects, and trap ID as random factors with a Poisson error structure. Sampling for this project first began on 23 May 2017 (day 1) at Thetford Forest and all sampling days thereafter in 2017 were calculated from this date. The total number of *N. vespilloides* beetles found in a trap on the sampling day was used as the response variable.

Testing the relationship between carcass size and reproductive performance in Thetford beetles

To assist our interpretation of the data, I carried out supplementary experiments to investigate the relationship between carcass size and reproductive performance in Thetford beetles. We collected data from a second generation of lab breeding beetles, derived from the wild-caught beetles trapped in Thetford Forest. The beetles were bred in two groups. One group bred on 12 October 2017 and another group bred on 26 October 2017. The first group bred on small chick and mice carcasses (8.23 ±0.70 S.D (g)) and the later on significantly larger chick and mice carcasses (20.64±1.20 S.D (g)). The chicks used for small carcasses were quail chicks and the chicks used for larger carcass treatment were domestic chicks.

Using measures of reproductive performance, we examined the effect of carcass size, and carcass type, and their interactions on:

- the number of dispersing larvae (brood size), using a GLM with a Poisson error term
- average larval mass using a linear model

Testing whether individual beetles have the greatest reproductive success on the carrion type they are trapped on

Using measures of reproductive performance, we examined the effect of carcass preference, carcass environment, and their interactions on:

- brood success, using a multivariate logistic regression model with a binomial error term. We used the following scoring system to record brood success: a 'zero' (0) denoted broods that failed while a 'one' (1) was assigned to those that had at least one larva at 8 days post dispersal.

- the number of dispersing larvae (brood size), using a GLM with a Poisson error term

- average larval mass using a linear model. We also added brood size as an independent variable in the model for average larval mass.
- larval density using a linear model. Larval density refers to the brood size divided by the carrion mass
- carcass use efficiency using a linear model. We calculate carcass use efficiency as the percentage of the carcass that is converted to the brood (i.e., brood mass) using the following formula:

$$carrion\ use\ efficiency\ = \left[\frac{total\ brood\ mass\ (g)}{carrion\ mass\ (g)} \right] \times\ 100\ \%$$

We used a linear model to test the association between average larval mass and larval density.

Testing how the overall preference of the population influence fitness

Using measures of reproductive performance from the experiment described in Table 2, we examined the effect of month trapped, carcass type used for breeding and their interaction on:

- brood success, using a multivariate logistic regression model with a binomial error term. We used the following scoring system to record brood success: a 'zero' (0) denoted broods that failed while a 'one' (1) was assigned to those that had at least one larva at 8 days post dispersal.

- the number of dispersing larvae (brood size), using a GLM with a Poisson error term

- average larval mass using a linear model
- larval density using a linear model. Larval density refers to the brood size divided by the carrion mass
- carcass use efficiency using a linear model

For all the analyses that we used to measure reproductive performance, we removed any broods that failed to produce at least one larva at 8 days post beetle pairing. When arriving at a minimal model using GLMs and GLMMs to explain our results, we removed non-significant terms and interactions using stepwise elimination. When presenting the results from post-hoc analyses, we list all the terms that were tested, and their statistics at the last point when they were retained in the model.

Results

Quantifying temporal variation in the frequency at which *N. vespilloides* beetles were trapped on avian and mammalian carcasses in June and August 2017

As expected from our previous analyses (Chapter 2), there was a significant interaction between month and trap-bait on the number of beetles caught (Table 3, Figure 2). In June, beetles were more likely to be caught on mice rather than chicks (Tukey post-hoc comparison: z ratio= -9.244, p-value <0.0001), whereas by August they were equally likely to be found on both sorts of carrion (Tukey post-hoc comparison: z ratio=1.006, p-value=0.3144).

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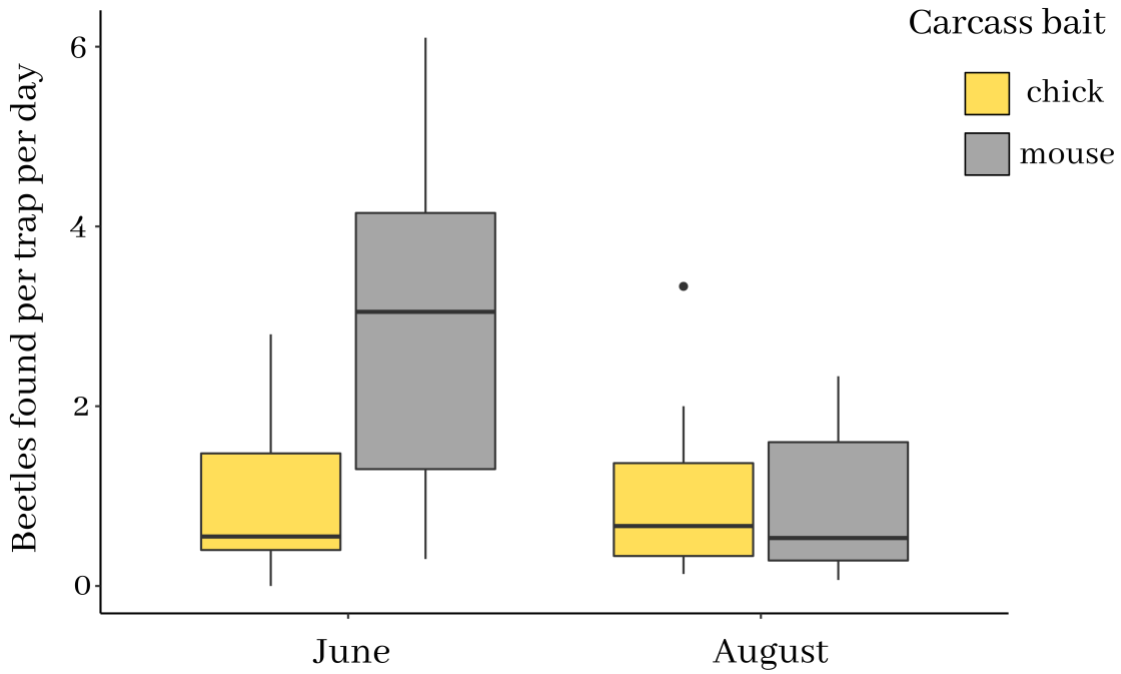


Figure 2: The number of beetles caught per trap per day in traps that were mouse-baited (grey bars) and chick-baited (yellow bars), from June 2017 (N= 391 beetles over 10 days) and August 2017 (N = 287 beetles over 15 days). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

In June 2017, mean catch per trap per day was 1 ±0.29 (SEM – standard error of the mean) beetles on chick carcasses and 2.91 ±0.60 (SEM) beetles on mice. In August 2017, the mean catch per day was 1.01 ±0.32 (SEM) beetles on chick carcasses and 0.9 ±0.27 (SEM) beetles on mice.

Table 3: Model summary showing results of the GLMM to test for the effects of carrion type, sampling month and their interactions on the number of burying beetles trapped using avian versus mammalian carcasses

Fixed effects:	*Estimate*	*Std. Error*	*z value*	*Pr(>|z|)*
Intercept	1.97098	0.16919	11.650	< 2e-16 ***
Carcass-Mouse	-0.04179	0.11838	-0.353	0.7241
Month-June	-0.30768	0.12942	-2.377	0.0174 *
Carcass-Mouse x Month-June	0.96278	0.16598	5.800	6.61e-09 ***

Prediction 1: Individual beetles have greatest reproductive success on the carrion type they are trapped on

We did not find any significant differences in brood success across all our treatments, regardless of the type of bait that beetles were attracted to in the field and the type of carrion they bred upon (Table 4, Figure 3).

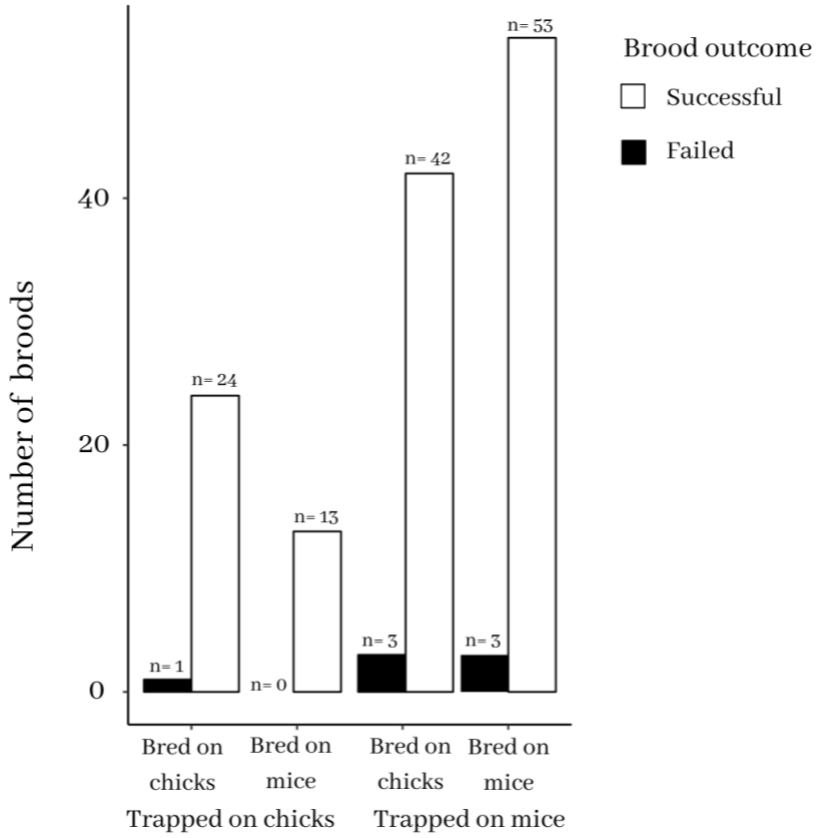


Figure 3: Brood success at larval dispersal of beetles trapped in June 2017 bred on chicks versus mice carcasses. In each treatment, failed broods are represented using a black bar and successful broods with a white bar.

Table 4:

a. Results of the analysis of deviance on the effects of the carcass type that beetles were trapped upon, the carcass type that they bred upon, and their interactions on brood success

Factors	*Deviance resid.*	*Df*	*Pr(>F)*
carcass type for breeding	0.13615	1	0.7121
carcass type trapped upon	0.85714	1	0.3545
carcass type for breeding x carcass type trapped upon	0.65032	1	0.4200

b. Model summary showing results of the GLM to test for the effects of the carcass type that beetles were trapped upon, the carcass type that they bred upon, and their interactions on brood success

Fixed effects:	*Estimate*	*Std. Error*
Intercept	2.8034	0.5149
Carcass bred on-Mouse	0.2877	0.7833
Carcass trapped upon-Mouse	-0.9358	1.1098
Carcass bred on-Mouse x Carcass trapped upon-Mouse	-15.155	1809.055

We found a significant interaction between the type of bait that beetles were attracted to in the field, and the type of carrion they bred upon, on the number of larvae that survived to dispersal (Table 5, Figure 4). However, contrary to our prediction, we found that beetles trapped on mice produced a similar number of larvae on mice and chicks (Tukey post-hoc comparison: z ratio= -0.852, p-value= 0.3941), whereas beetles trapped on chicks produced fewer larvae on chicks than on mice (Tukey post-hoc comparison: z ratio= -3.080, p-value= 0.0021). Overall, beetles that bred on mice carcasses produced larger broods.

Table 5:

a. Results of ANOVA of the effects of the carcass type that beetles were trapped upon, the carcass type that they bred upon, and their interactions on average brood size

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
carcass type for breeding	5.2493	1	0.02196 *
carcass type for breeding x carcass type trapped upon	4.8272	1	0.02801 *
Dropped/ non-significant terms			
carcass type trapped upon	1.2440	1	0.26470

b. Model summary showing results of the GLM to test for the effects of the carcass type that beetles were trapped upon, the carcass type that they bred upon, and their interactions on average brood size

Fixed effects:	*Estimate*	*Std. Error*
Intercept	3.28964	0.03941
Carcass bred on-Mouse	0.19278	0.06258
Carcass trapped upon-Mouse	0.10996	0.04845
Carcass bred on-Mouse x Carcass trapped upon-Mouse	-0.16084	0.07295

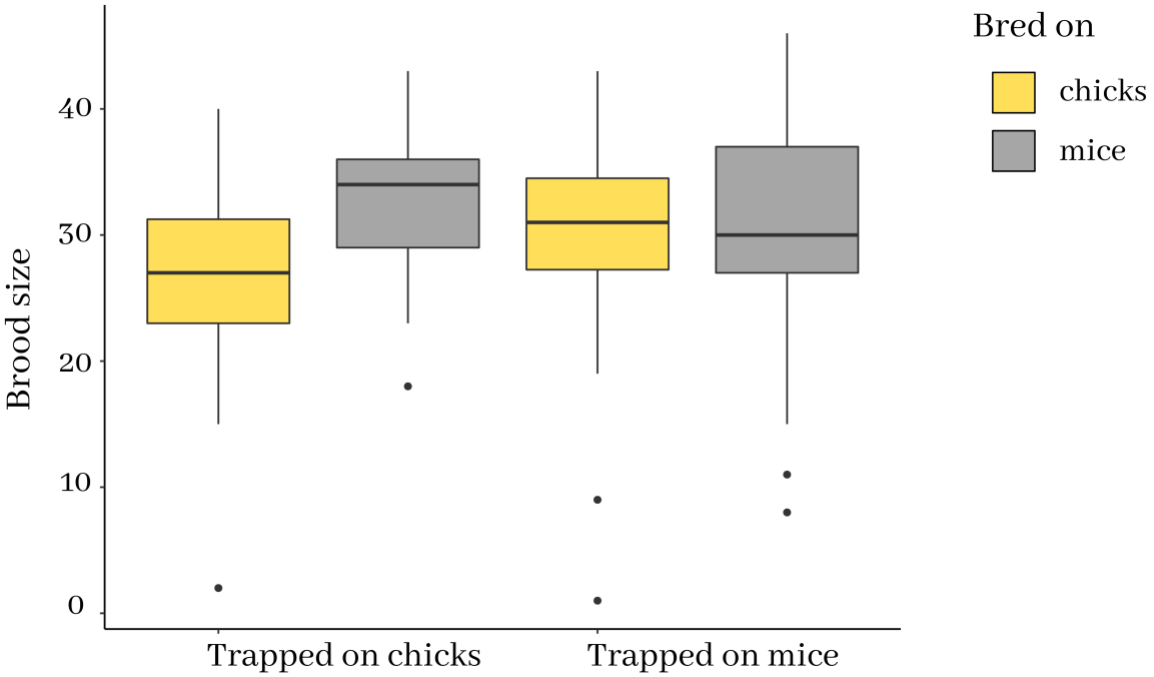


Figure 4: Brood size at larval dispersal of beetles trapped in June 2017 bred on chicks (yellow bars) versus mice (grey bars) carcasses. The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Brood size had a significant effect on larval mass: larvae from larger broods were smaller in size. Furthermore, the type of carrion that beetles were trapped upon had a small but non-significant effect on average larval mass. Beetles trapped on mice carcasses in the field produced slightly smaller larvae, regardless of the carcass type they were bred upon (Figure 5, Table 6) – perhaps because these larvae developed in larger broods.

Table 6:

a. Results of ANOVA of the effects of brood size, the carcass type that beetles were trapped upon, the carcass type that they bred upon, and their interactions on average brood size and their interactions on average larval mass at dispersal

Factor	*Sum Sq*	*Df*	*F value*	*Pr(>|t|)*
brood size	0.021754	1	26.2635	1.07e-06 ***
Dropped/ non-significant terms				
carcass type for breeding	0.000195	1	0.2348	0.62879

carcass type trapped upon	0.003008	1	3.6319	0.05893 .
carcass type for breeding x carcass type trapped upon	0.000844	1	1.0194	0.31457

b. Model summary showing results of the LM to test for the effects of brood size, the carcass type that beetles were trapped upon, the carcass type that they bred upon, and their interactions on average brood size and their interactions on average larval mass at dispersal

Fixed effects:	*Estimate*	*Std. Error*
Intercept	0.2164611	0.0102968
Brood size	0.0062488	0.0100721
Carcass bred on-Mouse	-0.0060145	0.0074292
Carcass trapped upon-Mouse	-0.0016020	0.0003126
Carcass bred on-Mouse x Carcass trapped upon-Mouse	-0.0117653	0.0116526

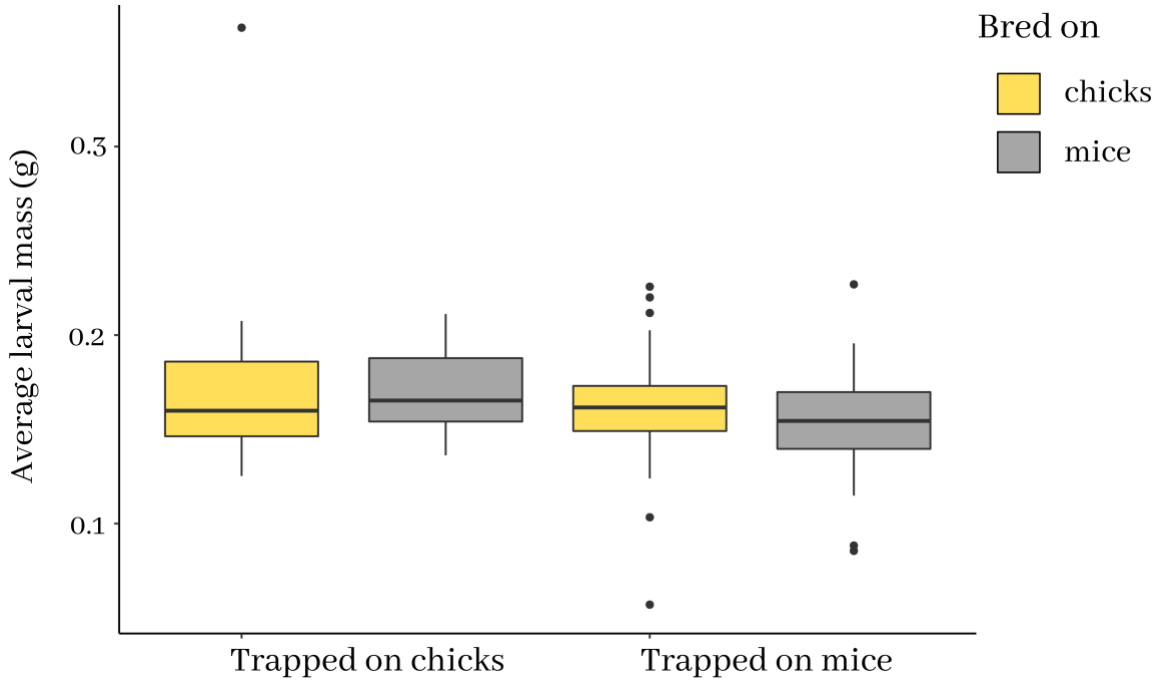


Figure 5: Average larval mass at dispersal of broods bred from adults trapped in June 2017 on chick carcasses (yellow bars) and mice carcasses (grey bars). The box bounds represent the inter-

quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

In general, larvae were at a significantly greater density on mice carcasses than on chicks (Table 7, Figure 6).

Table 7:

a. Results of ANOVA on the effects of the effects of the carcass type that beetles were trapped upon, the carcass type that they bred upon, and the interaction between the two, on larval density in each brood.

Factors	*Sum Sq*	*Df*	*F value*	*Pr(>F)*
carcass type for breeding	0.5613	1	4.026	0.04688 *
Dropped/ non-significant terms				
carcass type trapped upon	0.0848	1	0.6065	0.43753
carcass type for breeding x carcass type trapped upon	0.1145	1	0.8174	0.36764

b. Model summary showing results of the LM to test for the effects of the carcass type that beetles were trapped upon, the carcass type that they bred upon, and the interaction between the two, on larval density in each brood.

Fixed effects:	*Estimate*	*Std. Error*
Intercept	1.35199	0.04596
Carcass bred on-Mouse	0.13042	0.06500
Carcass trapped upon-Mouse	0.05743	0.07375
Carcass bred on-Mouse x Carcass trapped upon-Mouse	-0.13587	0.15028

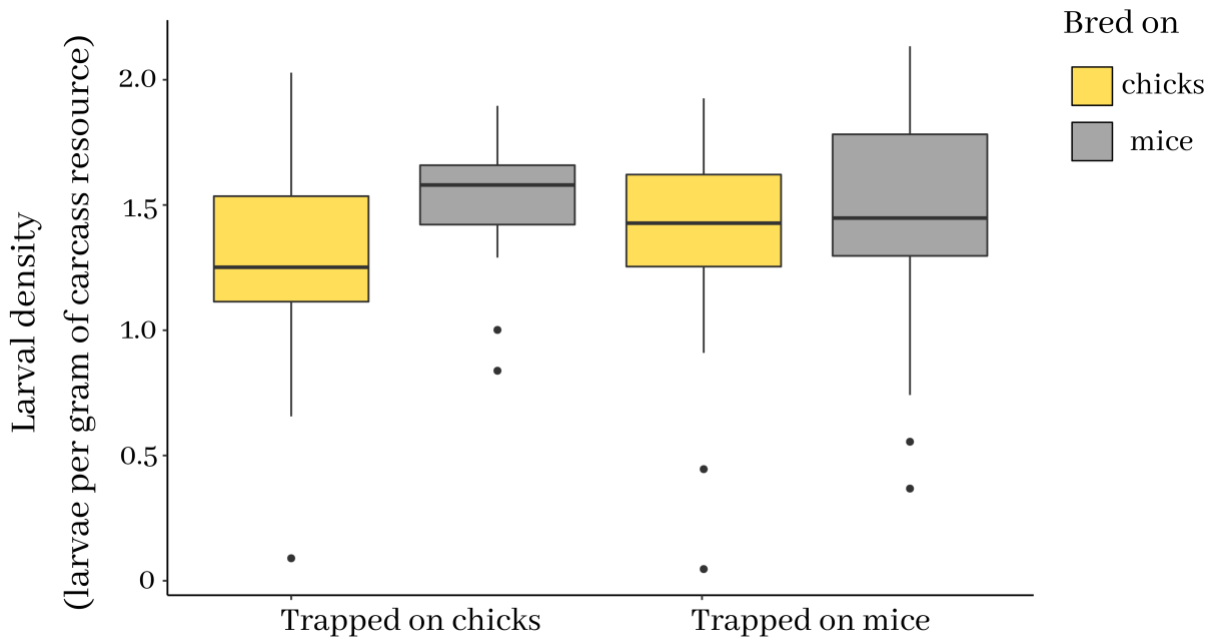


Figure 6: Larval density at dispersal of broods bred from beetles trapped in June 2017 and bred on chick carcasses (yellow bars) and mice carcasses (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

However, we could not detect any effect of the trap-bait on the efficiency with which beetles bred on each type of carrion (Table 8, Figure 7). Instead, we found that the carcass that beetles were bred on and the carcass type they were trapped upon significantly interacted to predict carcass use efficiency. Specifically, where beetles that were trapped on chicks were bred on mice- they used carrion significantly more efficiently than beetles in the other treatments (Tukey post-hoc comparison: t ratio=-2.391, p-value=0.0183).

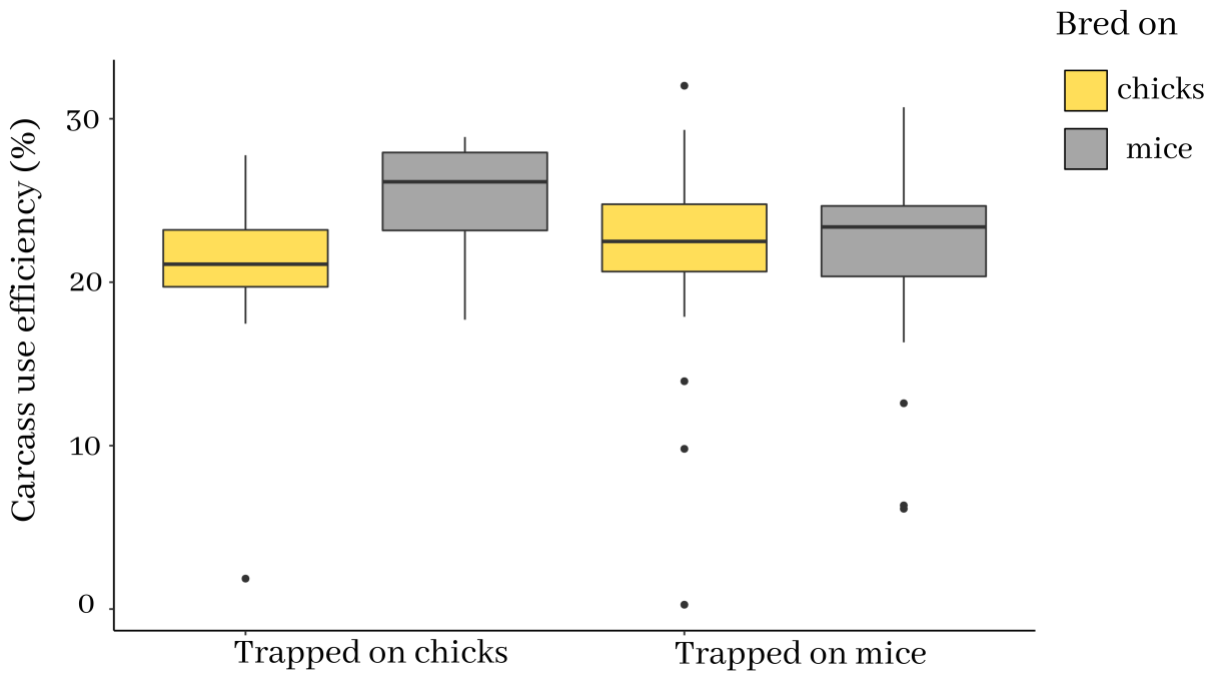


Figure 7: Carcass use efficiency of broods bred from beetles trapped in June 2017 and bred on chick carcasses (yellow bars) and mice carcasses (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Table 8:

a. Results of ANOVA on the effects of the carcass type that beetles were trapped upon, the carcass type that they bred upon, and the interaction between the two, on carcass use efficiency

Factors	*Sum Sq*	*Df*	*F value*	*Pr(>F)*
carcass type for breeding x carcass type trapped upon	104.74	1	4.3572	0.03884 *
Dropped/ non-significant terms				
carcass type for breeding	32.80	1	1.3647	0.24490
carcass type trapped upon	1.01	1	0.0420	0.83788

b. Model summary showing results of the LM to test for the effects of the carcass type that beetles were trapped upon, the carcass type that they bred upon, and the interaction between the two, on carcass use efficiency

Fixed effects:	*Estimate*	*Std. Error*
Intercept	20.860	1.001
Carcass bred on-Mouse	4.037	1.688
Carcass trapped upon-Mouse	1.470	1.255
Carcass bred on-Mouse x Carcass trapped upon-Mouse	-4.110	1.969

Predictions 2 and 3: At a population level, beetles trapped in June have greater reproductive success on mice rather than chicks, whereas beetles trapped in August have equal reproductive success on mice and chicks.

We did not find any significant differences in brood success across all our treatments, regardless of the time of collection in the field and the type of carrion they bred upon (Table 9, Figure 8).

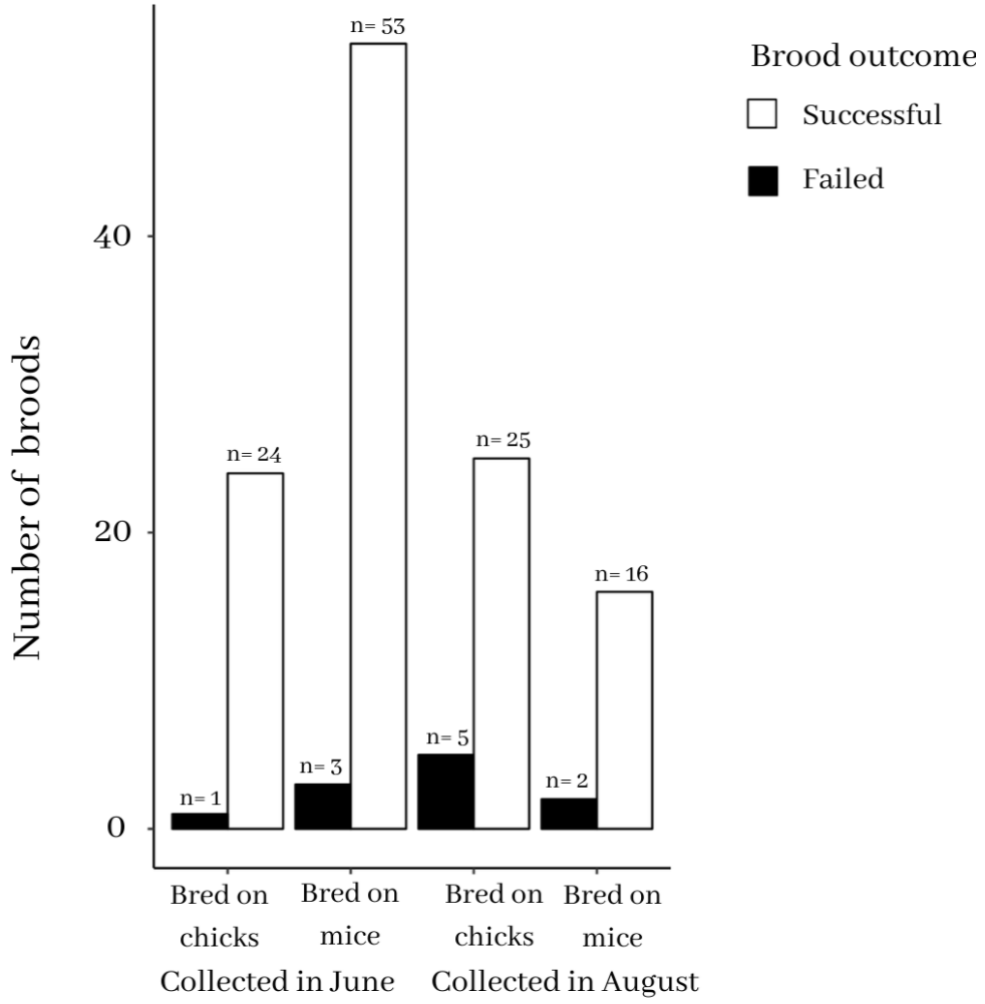


Figure 8: Brood success at larval dispersal of beetles trapped in June and August 2017 and bred on chick carcasses and mice carcasses. In each treatment, failed broods are represented using a black bar and successful broods with a white bar.

Table 9: Results of the analysis of deviance on the effects of month trapped, carcass type used for breeding and their interaction on brood success

Factors	*Deviance resid.*	*Df*	*Pr(>F)*
month trapped	2.83257	1	0.09237 .
carcass type for breeding	0.68863	1	0.4066
carcass type for breeding x month trapped	0.28953	1	0.59052

b. Model summary showing results of the GLM to test for the effects of month trapped, carcass type used for breeding and their interaction on brood success

Fixed effects:	*Estimate*	*Std. Error*
Intercept	2.1001	0.4325
Month trapped on-June	1.1345	0.6886
Carcass bred on-Mouse	0.5246	0.6337
Carcass bred on-Mouse x Month trapped on-June	-0.7764	1.4818

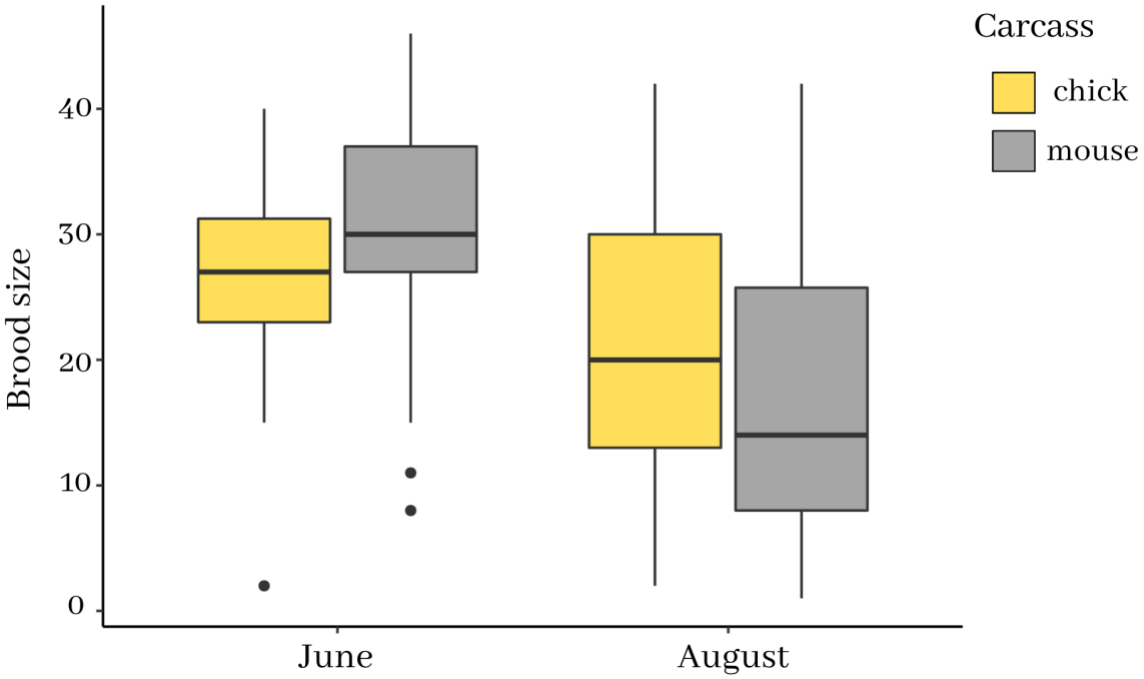


Figure 9: Brood size at dispersal of beetles trapped in June and August 2017 and bred on chick carcasses (yellow bars) and mice carcasses (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

We found that beetles trapped in June produced larger broods than the August-trapped beetles, regardless of the carrion they bred upon (Table 10, Figure 9). In addition, we found that June-caught beetles produced even larger broods on mice than any other treatment (Tukey post-hoc comparison: z ratio= -3.051, p-value= 0.0023). We also found that the June and August beetles had different coefficient of variation in their brood sizes and that this difference was not due to chance (Test for equality of coefficients of variation: test statistic= 26.38341, p-value<0.0001). Beetles bred in August had a greater coefficient of variation in brood size (CV=0.605) compared to those bred in June (CV=0.284).

Table 10:

a. Results of ANOVAs of the effects of month trapped, carcass type used for breeding and their interaction on brood size at dispersal

Factors	*LR Chisq*	*Df*	*Pr(>F)*
month trapped	103.115	1	< 2.2e-16 ***
carcass type for breeding	2.079	1	0.1492942
carcass type for breeding x month trapped	12.375	1	0.0004351 ***

b. Model summary showing results of the GLM to test for the effects of month trapped, carcass type used for breeding and their interaction on brood size at dispersal

Fixed effects:	*Estimate*	*Std. Error*
Intercept	3.00964	0.04441
Month trapped on-June	0.28001	0.05937
Carcass bred on-Mouse	-0.16545	0.07489
Carcass bred on-Mouse x Month trapped on-June	0.30736	0.08816

We found a significant interaction between month of trapping and carrion type used for breeding on average larval mass at dispersal (Table 11, Figure 10). When June-trapped beetles were bred on mice, they produced smaller larvae than any other combination of trapping months and carrion type– probably because the larvae developed in a larger brood (Tukey post-hoc comparison: t ratio= 2.333, p-value= 0.0214).

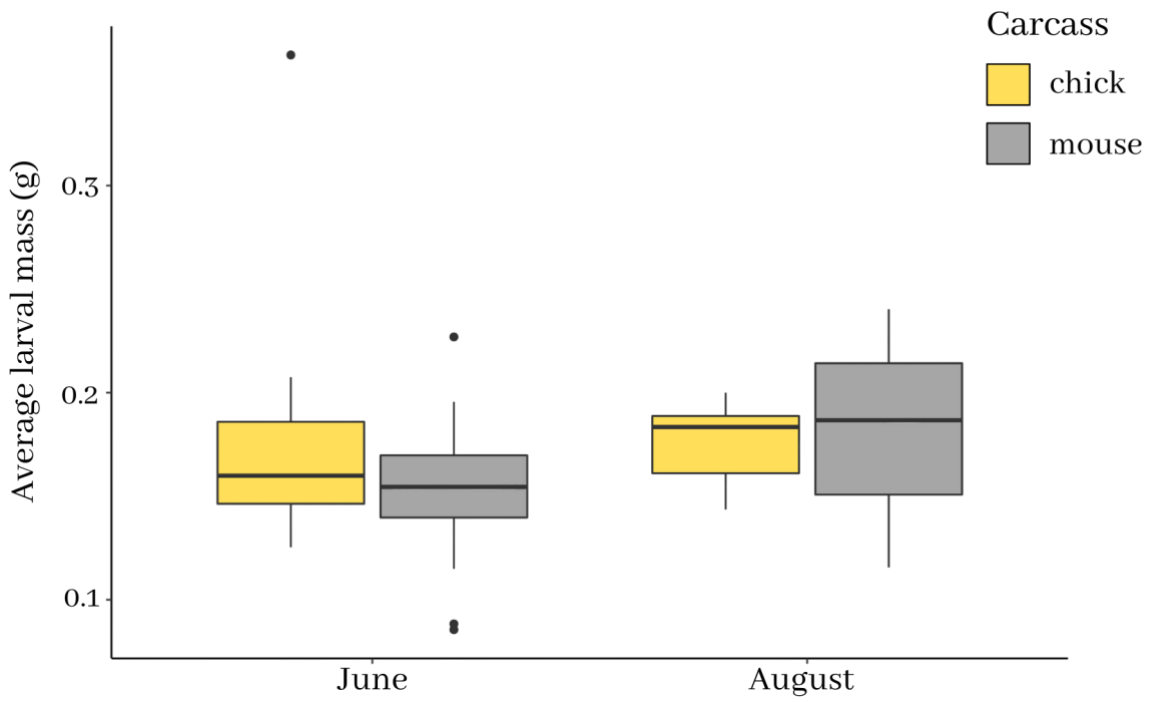


Figure 10: Average larval mass at dispersal of broods bred from beetles trapped in June and August 2017 and bred on chick carcasses (yellow bars) and mice carcasses (grey bars) at eight days post pairing. The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Table 11:

a. Results of ANOVA of the effects of month trapped, carcass type used for breeding and their interaction on average larval mass at dispersal

Factors	*Sum Sq*	*Df*	*F value*	*Pr(>F)*
month trapped	0.006308	1	6.2392	0.01392 *
carcass type used for breeding	0.001676	1	1.6581	0.20046
carcass type used for breeding x month trapped	0.004687	1	4.6364	0.03341 *

b. Model summary showing results of the LM to test for the effects of month trapped, carcass type used for breeding and their interaction on average larval mass at dispersal

Fixed effects:	*Estimate*	*Std. Error*
Intercept	0.174569	0.006359
Month trapped on-June	-0.002187	0.009086
Carcass bred on-Mouse	0.009393	0.010180
Carcass bred on-Mouse x Month trapped on-June	-0.027644	0.012838

We further used larval density and carcass use efficiency to compare reproductive performance between June and August-trapped beetles as these two measures take into account the variation in carcass mass.

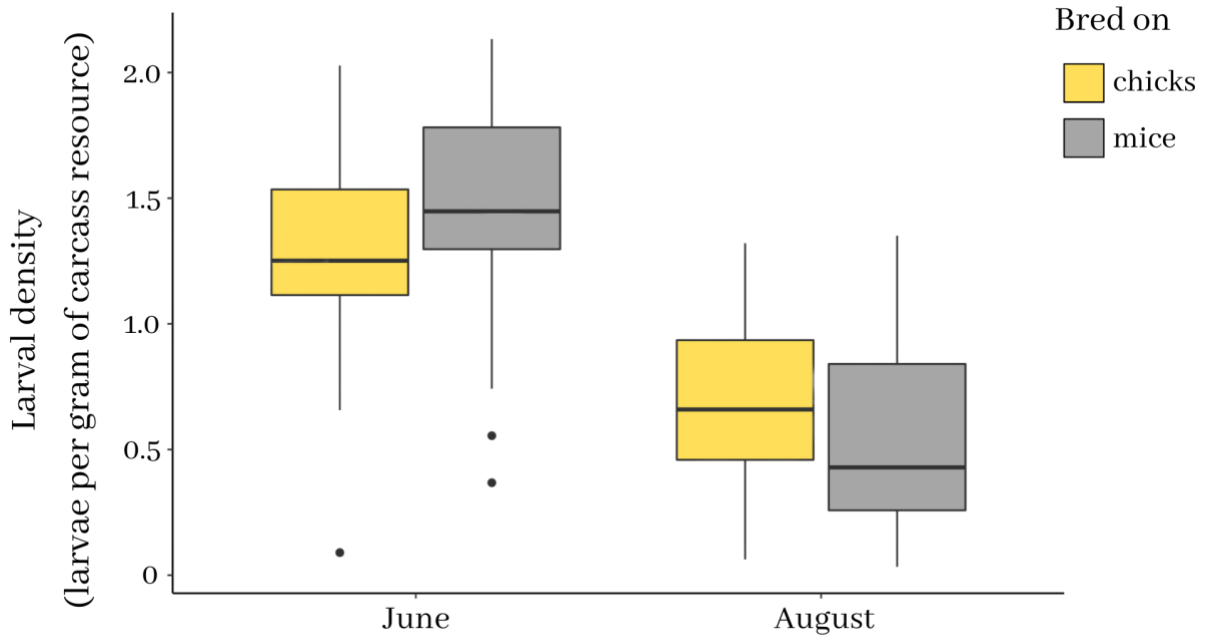


Figure 11: Larval density of broods bred from adults trapped in June and August 2017 and bred on chick carcasses (yellow bars) and mice carcasses (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Broods bred from June-trapped adults had larvae at significantly higher density on the carcass compared to broods bred from August-trapped adults (Table 12, Figure 11). There was a small but non-significant interaction between the type of carcass the beetles bred on and month in which the adults were trapped: beetles trapped on mice carrion in June had broods with a slightly greater larval density than all other treatments.

Table 12:

a. Results of ANOVA of the effects of month trapped, carcass type used for breeding and their interaction on larval density

Factors	*Sum Sq*	*Df*	*F value*	*Pr(>F)*
month trapped	17.277	1	114.87	< 2e-16 ***
Dropped/ non-significant terms				
carcass type used for breeding	0.2003	1	1.3355	0.2502
carcass type used for breeding x month trapped	0.5401	1	3.6857	0.05738 .

b. Model summary showing results of the LM to test for the effects of month trapped, carcass type used for breeding and their interaction on larval density

Fixed effects:	*Estimate*	*Std. Error*
Intercept	0.61267	0.06057
Month trapped on-June	0.80361	0.07498
Carcass bred on-Mouse	0.08731	0.07555
Carcass bred on-Mouse x Month trapped on-June	0.29675	0.15457

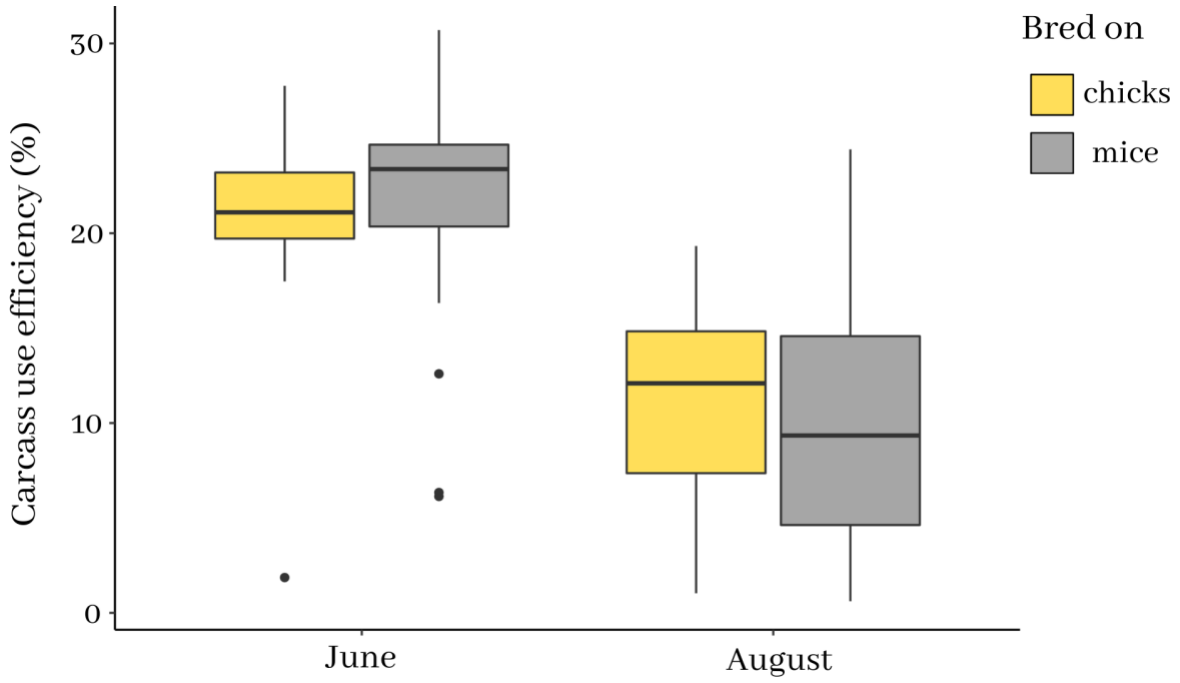


Figure 12: Carcass use efficiency of broods bred from adults trapped in June and August 2017 found and bred on chick carcasses (yellow bars) and mice carcasses (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Beetles trapped in June utilised both chick and mice carcasses significantly more efficiently than beetles trapped in August (Table 13, Figure 12). There was no significant effect of carcass type on how efficiently beetles used the carcasses; nor any significant interaction between carcass type and sampling date. We found that the June and August beetles had a different coefficient of variation in their carcass use efficiency and that this difference was not due to chance (Test for equality of coefficients of variation: test statistic= 43.93225, p-value<0.0001). Beetles bred in August had a greater coefficient of variation in carcass use efficiency (CV=0.320) compared to those bred in June (CV=0.222).

Table 13:

a. Results of ANOVA of the effects of month trapped, carcass type used for breeding and their interaction on carcass use efficiency

Factors	*Sum Sq*	*Df*	*F value*	*Pr(>F)*
month trapped	3298.7	1	116.79	<2e-16 ***
Dropped/ non-significant terms				
carcass type used for breeding	4.4	1	0.1554	0.6942
carcass type used for breeding x month trapped	43.4	1	1.5312	0.2185

b. Model summary showing results of the LM to test for the effects of month trapped, carcass type used for breeding and their interaction on carcass use efficiency

Fixed effects:	*Estimate*	*Std. Error*
Intercept	10.718	0.830
Month trapped on-June	11.104	1.028
Carcass bred on-Mouse	0.4102	1.0406
Carcass bred on-Mouse x Month trapped on-June	2.659	2.149

By chance, beetles trapped in August 2017 were bred on significantly heavier carcasses in the laboratory (30.98 ±2.1 S.D (g)) than beetles trapped in June 2017 (21.20±1.20 S.D (g)), though comparing beetles trapped within each month, carcass mass was consistent between chick and mice treatments (Table 14, Figure 13). The size range used in this experiment still corresponds with the size of carrion that *N. vespilloides* are able to use in nature (Müller et al., 1990; Otronen, 1988), but we carried out further analyses to test whether carcass size alone could account for the results we found, rather than the date of trapping.

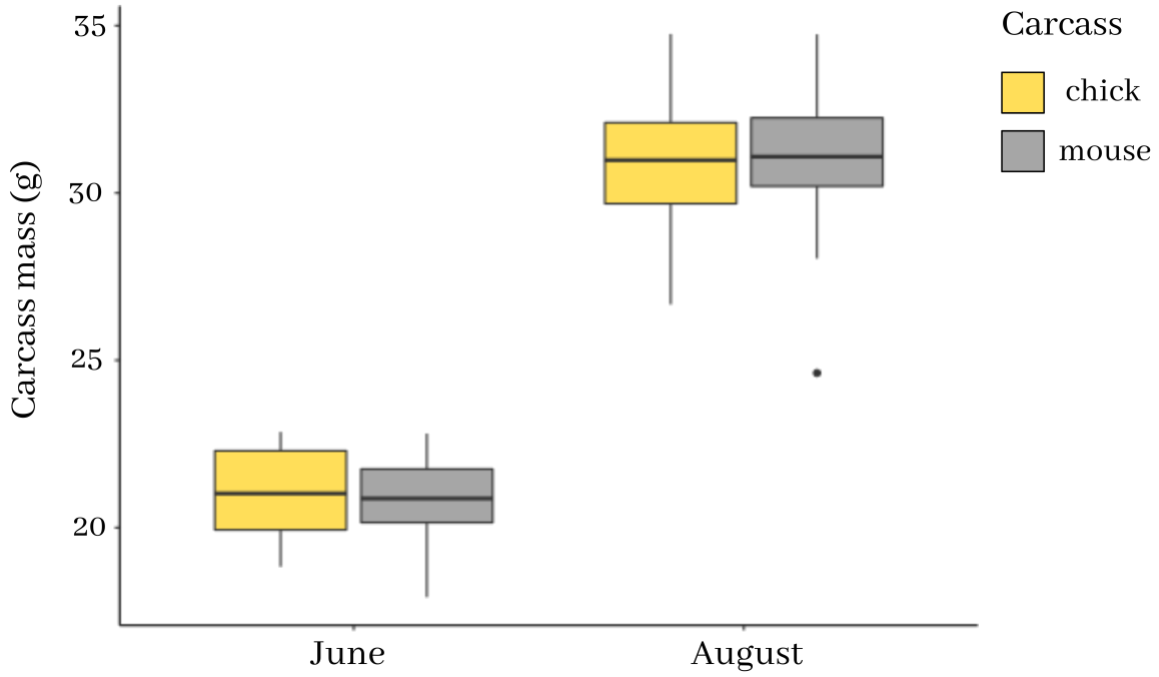


Figure 13: Size ranges of chick and mice carcasses used for measuring reproductive outcomes of beetles from June and August 2017. The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Table 14:

a. Results of ANOVA to test whether the mass of carcasses used for experiments with beetles trapped in June and August 2017 differed significantly

Factors	*Sum Sq*	*Df*	*F value*	*Pr(>F)*
month trapped	2657.96	1	1077.1	<2e-16 ***
Dropped/ non-significant terms				
carcass type	0.15	1	0.0585	0.8094
carcass type x month trapped	1.23	1	0.4915	0.4847

b. Model summary showing results of the LM to test whether the mass of carcasses used for experiments with beetles trapped in June and August 2017 differed significantly

Fixed effects:	*Estimate*	*Std. Error*
Intercept	30.9376	0.2453
Month trapped on-June	-9.9673	0.3037
Carcass bred on-Mouse	-0.07439	0.30771
Carcass bred on-Mouse x Month trapped on-June	-0.4475	0.6383

Testing the relationship between carcass size and reproductive performance in Thetford-derived beetles

Second generation beetles (F1 progeny) from Thetford were bred on small carcasses (8.23 ±0.70 S.D (g)) and large carcasses (20.64±1.20 S.D (g)), and within each group carcass mass was consistent between chick and mice treatments (Table 15, Figure 14).

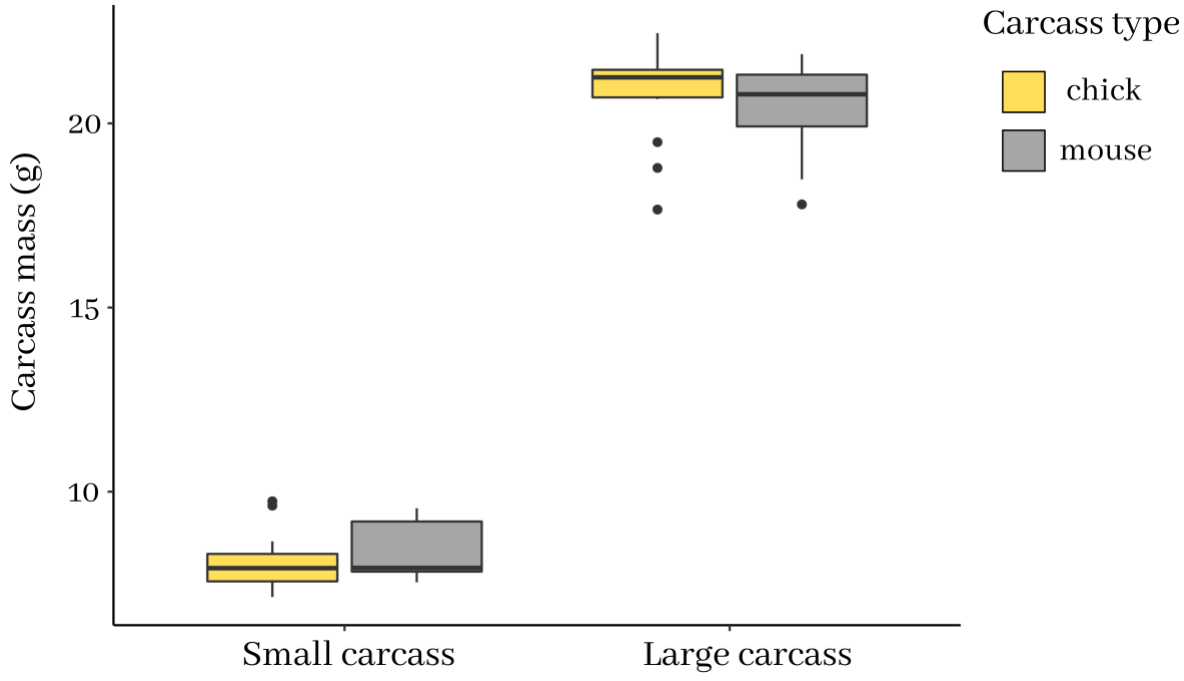


Figure 14: Mass of chick and mice carcasses used for measuring reproductive success of second-generation beetles from Thetford beetles in October 2017. The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Table 15:

a. Results of ANOVA indicating that the 'small' and 'large' carcasses used (Figure 14) were significantly different in size

Factors	*Sum Sq*	*Df*	*F value*	*Pr(>F)*
carcass size	3382.7	1	4042.2	< 2e-16 ***
Dropped/ non-significant terms				
carcass type	0.3	1	0.3335	0.565
carcass size x carcass type	2.4	1	2.9029	0.09187

b. Model summary of the LM to test whether the mass of the 'small' and 'large' carcasses used (Figure 14) were significantly different in size

Fixed effects:	*Estimate*	*Std. Error*
Intercept	8.2291	0.1191
Carcass Size	12.4089	0.1952
Carcass Type-Mouse	0.1103	0.1910
Carcass Size x Carcass Type-Mouse	-0.6667	0.3913

We found that both carrion type and carrion size had an effect on brood size, though there was no interaction between them (Table 16, Figure 15). Beetles bred on mice carcasses tended to have significantly larger broods compared to those bred on chicks. Broods on both larger chick and larger mice carcasses were significantly larger.

Table 16:

a. Results of ANOVA on the effects of carcass size, carcass type, and their interactions on brood size at dispersal of second-generation beetles from Thetford beetles in October 2017

Factors	*LR Chisq*	*Df*	*Pr(>F)*
carcass size	80.581	1	<2e-16 ***
carcass type	170.300	1	<2e-16 ***
Dropped/ non-significant terms			
carcass type x size	0.124	1	0.7248

b. Model summary of the GLM to test the effects of carcass size, carcass type, and their interactions on brood size at dispersal of second-generation beetles from Thetford beetles in October 2017

Fixed effects:	*Estimate*	*Std. Error*
Intercept	1.84597	0.07542
Carcass Size	0.03681	0.00407
Carcass Type-Mouse	0.70937	0.05688
Carcass Size x Carcass Type-Mouse	-0.003139	0.008913

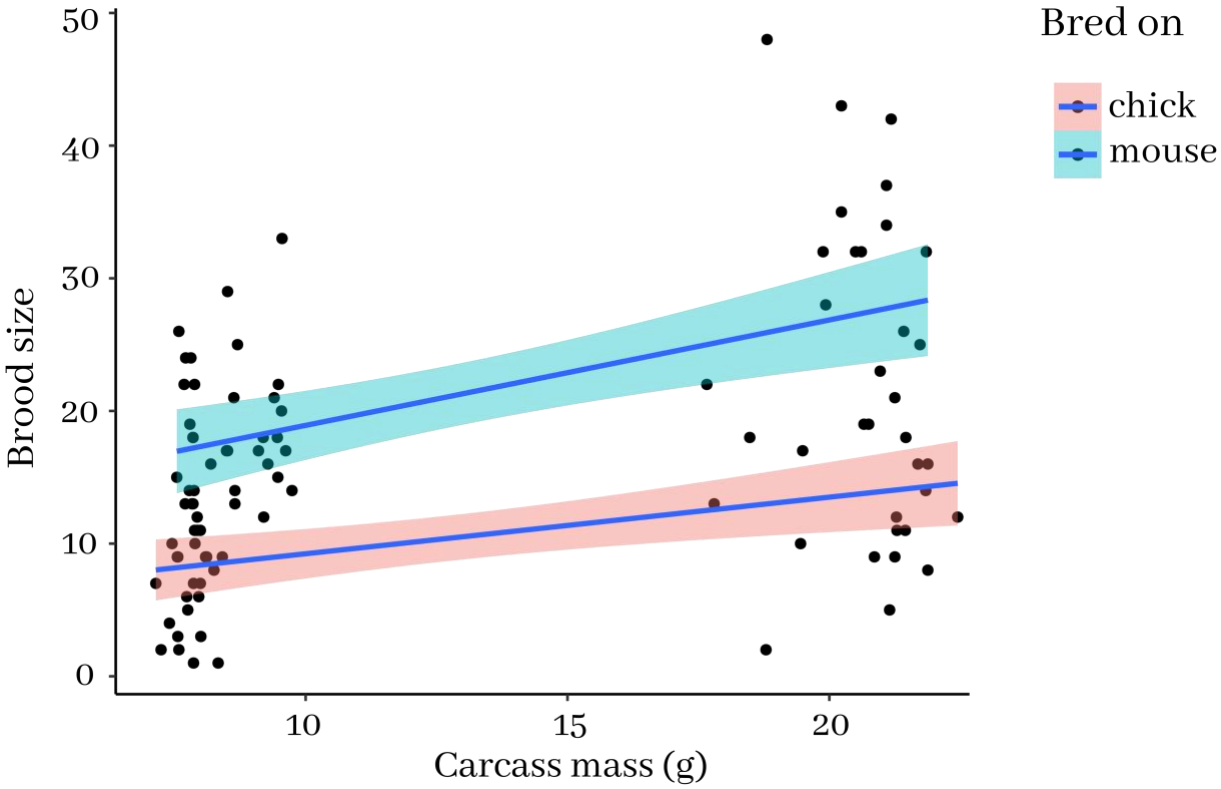


Figure 15: Relationship between brood size and carrion mass on the brood size at dispersal produced by second-generation Thetford beetles, bred on chick and mouse carcasses.

We found that beetles that bred on larger carcasses also produced significantly larger larvae (Table 17). However, mice carcasses within each treatment tended to produce smaller larvae and this is likely explained by our finding that beetles produced larger broods on mice. We did not find any

significant interaction between the carcass type and carcass size on average larval mass at dispersal (Table 17).

Table 17:

a. Results of ANOVA of the effects of carcass size, carcass type, and their interactions on average larval mass at dispersal

Factors	Sum Sq	Df	F value	Pr(>F)
carcass size	0.077438	1	80.4255	3.685e-14***
carcass type	0.004218	1	4.3803	0.03914*
Dropped/ non-significant terms				
carcass type x size	0.003030	1	3.2241	0.07592

b. Model summary of the LM to test the effects of carcass size, carcass type, and their interactions on average larval mass at dispersal

Fixed effects:	*Estimate*	*Std. Error*
Intercept	0.0895873	0.0082728
Carcass Size	0.0047321	0.0005277
Carcass Type-Mouse	-0.0135101	0.0064552
Carcass Size x Carcass Type-Mouse	0.0018785	0.0010462

Discussion

We investigated whether the patterns of seasonal variation in carrion use identified in Chapter 2 might be adaptive, as a first step towards determining whether sub-populations of resource use specialists exist within natural populations of *N. vespilloides*. Specifically, we investigated whether beetles were specialised to breed on the type of carrion they were attracted to in our traps, at different times in the year by measuring their reproductive success on different types of carrion.

Prediction 1

We predicted that beetles would have greater reproductive success on the carcass type that they were trapped on. However, our results do not indicate that breeding on this carcass type confers a fitness advantage to individual beetles. Though beetles from June that were trapped on mice seemed to utilise mice carrion more efficiently compared to chicks (Table 8, Figure 7), they produced a similar number of larvae on both mice and chicks (Table 5, Figure 4). Furthermore, and contrary to our prediction, beetles trapped on chicks produced larger broods on mice than on chicks (Table 5, Figure 4).

Why was our prediction not supported? One possibility is that the extent of reproductive investment we measured was more dependent on the beetle's experience of competition than on the type of carrion *per se*. Experiencing high competition on a resource is likely to modulate preference and reproductive investment on it, either by direct effects of interacting with competitors or through experiencing a poor nutritional environment (Trumbo 1990, Eggert et al. 2008, Hopwood et al. 2014, Lee et al. 2014, Pilakouta et al. 2016). The beetles used in this experiment were collected from traps that were in the field for 10 days, between 4 June and 14 June 2017. Beetles in both traps likely experienced very high competition for the carrion resource during this period and it is possible that this influenced their reproductive investment on the carrion in our experiments.

Alternatively, it could be that this single snapshot of beetle breeding behaviour did not adequately capture how the type of carrion beetles are trapped upon is related to breeding performance. As the wild beetles we trapped were being used for several follow up experiments (Chapters 4, 5 and 6), our sample sizes for different treatments varied a lot: from 53 successful broods of mice-caught beetles to only 13 successful broods of chick-caught beetles that were bred on mice (for treatments 'MM' and 'CM', respectively) in June 2017. Though logistical constraints prevented us from

repeating these experiments in subsequent field seasons, repeatability is an important aspect to consider for future work looking at how patterns of differential resource use could be adaptive. It will be especially important to determine whether individual beetles are consistently trapped on the same type of carrion in the field, or whether individuals vary over the season in the type of carrion they are attracted to.

Predictions 2 and 3

In order to understand trends at the population level, we compared beetles collected at two different timepoints- June 2017 and August 2017 (Table 3, Figure 2). We had previously found that beetles trapped in June had a greater preference for mice while those in August had an equivalent preference for mice and chicks (Chapter 2). Therefore, we predicted that beetles trapped in June would have greater reproductive success on mice over chicks, if this preference is adaptive. We also expected beetles trapped in August to have equal reproductive success on mice and chicks.

Consistent with our predictions, we found that beetles from August had equivalent reproductive success on both chick and mice carrion. All parameters that we measured for quantifying reproductive success (brood size, larval mass, larval density and carcass use efficiency) were similar on both chick and mice carrion in August-trapped beetles (Tables 9-13, Figures 8-12).

Beetles from June that were trapped and bred on mice carrion had significantly greater brood sizes compared to all other treatments, which is consistent with the possibility that they also possess specialised adaptations to breed on mammalian carcasses, at the population level (Table 10, Figure 9). However, we observed that beetles from June also had greater reproductive success on both mice and chick carcasses, compared to August beetles. They had significantly larger broods and significantly higher density on both chick and mice carcasses compared to August beetles (Tables 10 & 12, Figures 9 & 11).

August-trapped beetles were by chance bred on significantly larger carcasses (Table 14, Figure 13), so it is important to consider the role that carcass size played in the results we observe and whether carcass size is a potential confounding effect. Previous work in other labs has shown that larger carcasses are generally associated with larger broods and heavier larvae (Bartlett & Ashworth 1988, Scott & Traniello 1990, Creighton 2005). We found that same pattern in our lab, with second-generation lab beetles that originally derived from Thetford Forest (Table 15, Figure 15). Just as in the previous work, we too found that beetles that bred on larger carcasses produced larger

broods, with larger larvae. Therefore, beetles from Thetford Forest behave in a similar way to other burying beetle populations. If their breeding performance was solely affected by carrion size, then August-trapped beetles should have shown higher reproductive success than June-trapped beetles. Yet we found the opposite pattern. We conclude, therefore, that our results are not caused by the August-trapped beetles being bred on larger carrion.

Though the differences in carcass size make it difficult to directly compare brood size and average larval mass between June- and August-trapped beetles, when we controlled for this in our measures of larval density and carcass use efficiency, we found a similar pattern- with the broods of June-trapped beetles having a greater larval density. Furthermore, June-trapped beetles used both chick and mice carcasses significantly more efficiently than beetles sampled in August.

In short, although we found some evidence that is consistent with June-trapped beetles being specialised on mice, carrion specialisation cannot account for most of the experimental differences in breeding success that we found between June- and August-trapped burying beetles. An alternative interpretation is that the beetles trapped in June were simply of higher quality than those trapped in August. Other ecological and life-history related explanations could thus better explain our results.

For example, there is likely to be seasonal variation in the age structure of natural populations. Several insect species are multivoltine, i.e., they undergo several generations in a year which can result in stage-structured populations (Wagner et al. 1984, Molleman et al. 2006, Carey et al. 2008, Bjørnstad et al. 2016). This variation in age structure can significantly impact population dynamics and has been studied in depth for managing populations of insect pest species (Tauber et al. 1986, Bonsall & Eber 2001, Cook et al. 2008, Rock et al. 2015). Some species persist in cycles of developmental synchrony, which leads to distinct generations while others exist as populations composed of overlapping generations, with individuals of different ages and multiple life stages (Tauber et al. 1986, Gurney et al. 1992, Bjørnstad et al. 2016).

Burying beetles are likely to exhibit intermediate dynamics, with early season separation in generations followed by late season mixing of generations, known as 'generational smearing' (Pukowski 1933, Bjørnstad et al. 2016). This is because *N. vespilloides* beetles in Europe emerge from overwintering in late spring and tend to be the most abundant burying beetles in beetle

communities until late summer, producing multiple generations in a year (Pukowski 1933, Scott 1998).

Since the overwintering individuals begin to emerge in late spring and early summer, it is likely that these beetles would begin producing their first broods during this period meaning that populations in the later part of the burying beetle season are likely to be a combination of older and newly eclosed adults (Pukowski 1933, Urbański & Baraniak 2015). Therefore, populations in August are likely to be a mix of adults that have reproduced in late spring or early summer as well as newly eclosed individuals- which could explain the greater variation in their reproductive outcomes that we observed.

Age structure of the population can also affect reproductive investment through effects of senescence and brood order (Scott & Traniello 1990, Trumbo 2009, Cotter et al. 2011, Billman et al. 2014). Previous lab experiments on *N. vespilloides* have indicated that even when females switch strategies from reproductive restraint to terminal investment, older females have lower reproductive output, due to senescence-related constraints (Cotter et al. 2011). Furthermore, work on natural populations of burying beetles has indicated a decline in brood mass in *N. orbicollis* populations later in the breeding season (Scott & Traniello 1990). Different populations could have different age structures in late summer, depending on local life history strategies, which in turn depend on local ecological conditions. Therefore, this could explain why the quality of individuals in late summer is lower on average than earlier in the year.

In Chapter 2, we suggested that patterns of resource use could be due to adaptive partitioning of resource type within populations. While some evidence from this chapter is consistent with this idea, it also suggests that seasonal variation could be more simply explained by variation in individual quality. Although we find seasonal variation in beetle reproductive success on different types of carrion, we have found no evidence that this variation is directly caused by the type of carrion that beetles are attracted to in nature– either at the individual or population level.
In the next chapter we test the hypothesis that individuals within natural populations are resource specialists in a different way, using genomic data.

Chapter 4

Genomic correlates of differential resource use in burying beetles *N. vespilloides*

Introduction

As discussed in previous chapters, differential resource use within and among populations is common in insect populations (Tauber & Tauber 1989, Johnson et al. 1996, Funk et al. 2002, Drès & Mallet 2002, Forister et al. 2012). It is often linked to the retuned development of sensory systems that affect either patterns of seasonal variation or odour detection (Table 1). Underpinning this divergence, there is often genomic divergence in key functional genes (Johnson et al. 1996, Drès & Mallet 2002, Schluter 2001, Levin 2004, Rundle & Nosil 2005, Matsubayashi et al. 2010, Forister et al. 2012).

Table 1: Differential resource use in insect populations

Species	*Differentially specialised to*	*Phenological and/or physiological basis*	*Molecular basis (if know)*	*References*
Acyrthosiphon pisum (pea aphids)	Alfalfa, pea and red clover	Differences in habitat acceptance, assortative mating and reduced hybrid performance	Whole genome sequencing indicates divergence in genes encoding salivary proteins that could help counteract plant defences (ϕ_{ST}: 0.069–0.17)	Via 1991; Via 1999; Hawthorne & Via 2001; Via & Hawthorne 2001; Via & West 2008; Via 2012; Jaquiéry et al. 2012
Eurosta solidaginis (goldenrod gall fly)	*Solidago altissima* and *S. gigantea*	Oviposition preference, adult emergence time, assortative mating and reduced hybrid performance	Unequivocal evidence of host-associated genetic differentiation from allozyme and mtDNA studies (Allozyme F_{ST}= 0.055; Sequence ϕ_{ST}= 0.116)	Craig et al. 1993; Abrahamson & Weis 1997; Craig et al. 1997; Itami et al. 1997; Stireman et al. 2005; Craig & Itami 2011

Gnorimoschema gallaesolidaginis (solidago gall moth)	*S. altissima* and *S. gigantea*	Oviposition preference, phenology of gall initiation and adult emergence	Divergent allele frequency profiles at several loci. Greater genetic variation in the *altissima* form, suggesting ancestral association with *S. altissima* and subsequent colonization of *S. gigantea* (Allozyme F_{ST} = 0.159; Sequence ϕ_{ST}= 0.544).	Nason et al. 2002; Stireman et al. 2005
Neodiprion abietis (balsam fir sawfly)	Ancestral: pine Derived: spruce, hemlock, Douglas fir and true firs	Population composed of specialists and generalists. Specialists exhibit oviposition preference and phenological differences.	-	Knerer & Atwood 1972; Knerer & Atwood 1973; Johns & Ostaff 2013
Prodoxus quinquepunctellus (bogus yucca moth)	Ancestral: *Yucca lamentosa* Derived: *Y. aloifolia*	Specialisation of moth emergence time and ovipositor morphology	Complex interhost genetic structure suggests independent local host shifts across geographical range (Allozyme F_{ST} = 0.052; mtDNA ϕ_{ST}= 0.07)	Groman & Pellmyr 2000; Althoff et al. 2001
Rhagoletis pomonella (maggot flies)	Ancestral: hawthorn fruit Derived: apples	Seasonal timing (apple trees fruit ~ 3 weeks earlier than hawthorn)	Genomic and transcriptional divergence at loci associated with eclosion, diapause	McPheron et al. 1988; Feder et al. 1993; Feder et al. 2003; Michel et al. 2010; Meyers et al. 2016

			development and termination (Allozyme F_{ST} = 0.012)	
Timema cristinae (walking-stick insect)	*Ceanothus spinosus* (unstriped morph) and *Adenostoma fasciculatum* (striped morph)	Body shape and colour-patterning to aid crypsis on host plant from visual predators. Host-associated mate preference.	Divergence measured using multiple markers: mtDNA, nDNA, AFLPs (Sequence ϕ_{ST} = 0.111, using SNPs)	Sandoval 1994; Nosil et al. 2002; Nosil 2007; Nosil et al. 2008; Nosil et al. 2012
Zeiraphera diniana (larch budmoth)	Larch and pine	Oviposition preference, host-associated larval survival and mate choice (via differentiated female pheromones). Egg hatching of larch form synchronized with flush of larch foliage	Differentiated allozymes: sex-linked isocitrate dehydrogenase (*Idh*), and two unlinked autosomal loci, malate dehydrogenase (*Mdh*) and phosphoglucomutase (*Pgm*). Non-random distribution of divergent AFLP loci. Strongly differentiated *Mdh* maps to strongly differentiated chromosome 6. (Allozyme F_{ST} = 0.065; AFLP loci ϕ_{ST}= 0.216)	Bovey & Maksymov 1959; Day 1984; Guerin et al. 1984; Priesner & Baltensweiler 1987; Emilianov et al. 1995; Emelianov et al. 2001; Emelianov et al. 2003

Here, we examined whether beetles trapped from the same population on different carrion were divergent genomically, and whether the same loci were consistently associated with differential carrion use within three different field populations.

Previous work on population differentiation in burying beetles has been focussed on large-scale genetic differences due to habitat specialisation (Sikes et al. 2016). Using DNA barcoding, Sikes et al. (2016) showed that the Canadian wetland population of *N. vespilloides*, which are bog and marsh specialists, is potentially a different species that is distinct from other *N. vespilloides* populations. However, with the exception of one study (Pascoal & Kilner 2017) there has so far been no detailed population genetic work to analyse the extent of population divergence within *N. vespilloides* in the Palearctic zone.

The assembled and annotated genome of the burying beetle *Nicrophorus vespilloides* became available recently (Cunningham et al. 2015) and has enabled studying the molecular basis of differential resource use within populations of this species. Sun et al. (2020) identified genetic differences between neighbouring populations of Gamlingay Wood and Waresley Wood associated with divergently adaptations in clutch sizes between the two populations.

If the hypothesis outlined in Chapter 2 is correct, and there are indeed sub-populations of resource-use specialists within wild *N. vespilloides* populations, then we can make the following predictions:

1. We should be able to detect associated genetic differences when comparing the genomes of beetles trapped on dead mice versus dead birds.
2. Genetic differences are most likely to be found at loci relevant for finding and utilising carrion, such as olfactory receptors.
3. If specialists are active at specific times in the year (Chapter 2) then this is when the extent of allelic divergence in relation to resource use should be at its greatest.

Materials and methods

We generated and analysed low-coverage whole genome sequences for *N. vespilloides* females from Thetford Forest, Gamlingay Wood and Waresley Wood. The method used is described in detail in Sun et al. (2020).

Beetle trapping and dissection

The beetles used for genomic analysis were collected from the wild as part of the field experiments described in Chapter 2 and stored in absolute ethanol until they were dissected for DNA extraction. Only the head segment of the beetles was used for extracting DNA. The thoracic and abdominal segments were put back in ethanol and stored for future use.

Early season *N. vespilloides* females from Thetford Forest were collected on 23 May 2017 and 14 June 2017. Late season beetles from Thetford were collected between 4 September 2017 and 29 September 2017. In Thetford Forest, beetles were sampled using a paired trap arrangement, in which we placed two beetle traps- one with a domestic chick and the other with a mouse carcass- near each other at each trap location and recorded the beetles found in each trap.

N. vespilloides females used from Gamlingay Wood and Waresley Wood for this work were collected in late summer between 10 August 2017 and 21 September 2017. Here, we alternated the carcass types placed in each trap location every two weeks, instead of using a paired trap setup.

DNA extraction

We extracted DNA individually from beetle heads using DNeasy Blood and Tissue kit (Qiagen), followed by a quality check and quantification using Qubit and NanoDrop (I carried out this work with Sonia Pascoal).

Library prep, sequencing and analyses

We then shipped the DNA to Michael Sheehan's lab at Cornell University where they prepared paired-end 550 bp insert libraries using partial reactions of a Nextera kit by the Cornell Genomics Core. The libraries were sequenced by Novogene (Davis, CA, USA) at an average coverage of 3.4x. After removing adaptors and poor-quality sequences, the trimmed reads were mapped to the *N. vespilloides* reference genome using the Burrows-Wheeler Aligner (version 0.7.13) (Li & Durbin 2009). Sheehan and his colleagues identified SNPs using Picard (version 2.8.2) and GATK (version

3.6) HaplotypeCaller following best practice recommendations (van der Auwera et al. 2013). After alignment and hard filtering, Sheehan et al. calculated the F_{ST} values. They analysed the bam files in ANGSD (version 0.911) (Korneliussen et al. 2014), which is specifically designed for analysis of low-coverage genome sequencing data.

The Sheehan group identified multiple loci that diverged between beetles that preferred chick and mice carrion in the wild by defining a threshold of an F_{ST} value greater than 0.02 (for a 5kb window). Candidate regions that were associated with olfaction, learning, memory and other relevant functionalities were filtered out and the results are displayed in Table 1. A full list of candidate regions can be found in Appendix Table 4.1.

Results

Question 1: Are there differences between the genomes of beetles trapped on dead mice versus dead birds, within populations?

The analyses revealed differences at nearly 50 loci between beetles attracted to chick versus mice carcasses. These are cases where the fixation index is greater than 0.02 for a 5kb window in at least two groups (out of four: early season Thetford Forest, late season Thetford Forest, Waresley Wood and Gamlingay Wood). This has produced a number of interesting candidate regions (Table 2), even though nothing is shared across all three populations. A table indicating all differentiated regions can be found in the appendix (Table A.4.1)

Table 2: Results from whole genomic sequencing of beetles caught on chick- and mouse-baited traps in Thetford Forest (early and late season), Waresley Wood and Gamlingay Wood. The first column gives information about a.) the site at which the populations are differentiated and the genes that are likely involved; and b.) the number of populations (out of c., d., e. and f.) with an F_{ST} value greater than 0.02 between beetles attracted to chicks and mice for a 5kb window. The populations that are significantly differentiated at a particular site are highlighted in grey.

a. Site of differentiation /chromosome Genes within (or near window of high F_{ST})	b. Number of populations with F_{ST} > 0.02	c. Early season Thetford on chicks - Thetford on mice	d. Late season Thetford on chicks - Thetford on mice	e. Waresley on chicks - Waresley on mice	f. Gamlingay on chicks - Gamlingay on mice
NW_017095694.1 Upstream of metabotropic glutamate receptor 7 (LOC108560036)	2	0.0232	0.0214	-0.0062	-0.0057
NW_017096093.1	2	0.0021	0.0450	-0.0041	0.0230

Neogenin (LOC108569519). May be frazzled in *Drosophila*, involved in neural development					
NW_017096128.1 Glutamate-gated chloride channel-like (LOC108569710)	2	0.0059	0.0201	0.0386	-0.0300
NW_017096637.1 cAMP-specific 3',5'-cyclic phosphodiesterase (LOC108557663). Annotated as *dunce* in *Drosophila*, important in learning including olfactory	2	0.0298	-0.0156	0.0281	-0.0056
NW_017096684.1 Glutamate receptor ionotropic, NMDA 2B-like (LOC108557899)	2	0.0327	-0.0143	0.0092	0.0282
NW_017097262.1 Serine proteinase stubble (LOC108559606)	2	-0.0011	0.0785	-0.0143	0.0484
NW_017098369.1 Between putative gustatory receptor 39b (LOC10856226 closest to this and upstream, possible in promoter region) and anosmin (LOC108562266) and mucin-2-like (LOC108562268)	2	0.0327	0.0309	0.0062	-0.0147
NW_017098369.1 Upstream of aryl hydrocarbon receptor protein 1 (LOC108562270). Best match is *spineless* in *Drosophila*	3	0.0262	0.0208	0.0218	-0.0148
NW_017099114.1 Intron of glutamate receptor ionotropic, kainate 2 (LOC108564059)	2	0.0377	0.0321	-0.0133	-0.0123
NW_017099143.1					

Downstream of centrosome-associated protein 350-like (LOC108564093); upstream of protein ecdysoneless (LOC108564094)	2	0.0283	-0.0015	0.0527	0.0036
NW_017099143.1 Downstream of centrosome-associated protein 350-like (LOC108564093); upstream of protein ecdysoneless (LOC108564094)	2	0.0343	-0.0124	0.0665	-0.0130
NW_017099143.1 Downstream of centrosome-associated protein 350-like (LOC108564093); upstream of protein ecdysoneless (LOC108564094)	2	0.0345	0.0091	0.0386	0.0011
NW_017099578.1 Moesin (LOC108564809. Involved in neural development	2	0.0231	0.0172	0.0469	0.0192
NW_017100102.1 mnt (LOC108568128). Involved in cell cycle, regulates body size	2	0.0180	0.0235	0.0161	0.0479

Question 2: Are there differences at loci relevant for finding and utilising carrion, such as olfactory receptors?

The gene *spineless*, which is involved in olfactory system development (Burgess and Duncan 1990, Duncan et al. 1998), is differentiated between chick- and mice-trapped beetles in all populations except those caught in Gamlingay Wood.

A cAMP-specific 3',5'-cyclic phosphodiesterase, which is annotated as *dunce* in *Drosophila* is differentiated in both early season Thetford beetles and those from Waresley Wood. It is considered to play a role in learning and olfaction (Dudai et al. 1976, Byers et al. 1981, Qiu & Davis 1993).

Genes responsible for neural development and putative gustatory receptors (for example: Moesin and Neogenin receptors) are also differentiated within populations, depending on the bait on which chicks are caught.

We also found that all four types of glutamate receptors diverged between chick- and mouse-baited beetles in different combinations within at least two different populations (Table 1). Glutamate is a major neurotransmitter involved in learning and memory (Riedel et al. 2003). Ionotropic glutamate receptors, which are differentiated in Thetford, Waresley and Gamlingay populations (Table 1) have been identified as chemosensory receptors in *Drosophila* (Benton 2009).

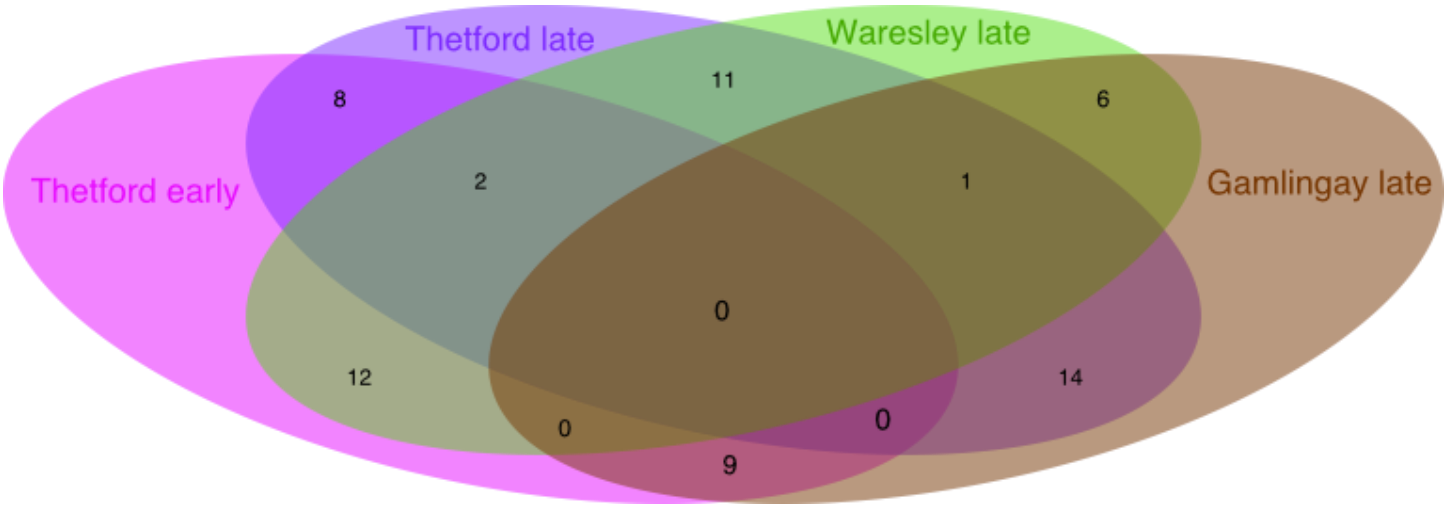


Figure 1: Venn diagram indicating overlaps in divergent loci across all four treatment groups: Thetford Forest (early and late season), Waresley Wood and Gamlingay Wood.

Question 3: Does the extent of allelic divergence in relation to resource use vary across the season?

Beetles in the early season from Thetford diverged at a total of 31 sites (F_{ST} range: 0.0214-0.0942) between those attracted to chicks versus mice (Appendix Table 4.1, Figure 1). Of these, divergence was shared at 12 sites with Waresley, 9 with Gamlingay and 8 with late season Thetford beetles. Divergence from mice-baited beetles was shared at a further two sites between beetles attracted to chicks in Thetford early season, Thetford late season and Waresley Wood.

In the late season at Thetford Forest, chick-baited beetles diverged from mice-baited beetles at a total of 36 different sites (F_{ST} range: 0.0201-0.0785) across the genome (Appendix Table 4.1, Figure 1). Along with the divergence shared above, there were another 11 sites of shared divergence between chick- and mice- baited beetles from Waresley Wood, 14 sites with Gamlingay Wood and an additional 1 shared site between all three populations sampled late in the season.

The sequencing data indicated a total of 32 sites (F_{ST} range: 0.0204-0.0611) that diverged between beetles baited on chicks and mice in Waresley Wood and 30 sites (F_{ST} range: 0.0210-0.0761) in Gamlingay Wood (Appendix Table 4.1, Figure 1). The woodlands share 6 of these sites, along with others shared with Thetford Forest.

Discussion

We found some support for all three predictions, after testing beetles trapped on chicks versus mice within Thetford Forest, Gamlingay Wood and Waresley Wood:

1) We were able to detect associated genetic differences when comparing the genomes of beetles trapped on dead mice versus dead birds.
We also found spatial variation in loci that diverged between chick- and mouse- baited beetles, with the Gamlingay Wood population showing the least amount of divergence between them.

2) Genetic differences were most likely to be found at loci relevant for finding and utilising carrion, such as olfactory receptors.
One noteworthy finding was that the gene *spineless*, which is involved in olfactory system development, was differentiated between chick- and mouse- preferring beetles in both early and late season Thetford beetles as well as Waresley Wood. There were also multiple loci associated with learning and memory that appeared to be differentiated between the beetles in all three populations. Variation in traits related to learning ability have been linked to differences in forging preferences (Latshaw & Smith 2005) and prey recognition (Gibbons et al. 2005) in other systems.

3) There were some differences in which loci were divergent in beetles trapped early versus late season within Thetford Forest.
This could be due to temporal variation in the extent of competition for carrion, within and between *Nicrophorus* species (Chapter 2; Anderson 1982, Scott 1998, Trumbo 1994) and consequently the strength of disruptive selection experienced by a population (Svanbäck & Bolnick 2007).

But do our results indicate the restricted gene flow that is characteristic of "host races" observed in many phytophagous species? In other words, does it mean there are sub-populations of resource specialists that are currently capable of interbreeding but between which gene flow is restricted by resource preference? Compared to the F_{ST} values observed in other insects, our findings are similar to those at the lower end of the spectrum observed in the very recently evolved host races such as *Acyrthosiphon pisum, Prodoxus quinquepunctellus* and *Rhagoletis pomonella* (Table 1). However, F_{ST} values indicating host-associated differentiation are much higher in other insects than the values we

observe in the *N. vespilloides*, suggesting weaker preferences and less resource specialisation within our beetle populations.

Abrahamson et al. (2001) propose five criteria that must be met for insect populations to be considered host races: 1. They should exist in sympatry 2. They should show genetic differences 3. There should be some form of allochronic isolation, for example due to through differences in emergence time of adults belonging to different races. 4. There should be oviposition preference driven by resource preference 5. There should be assortative mating driven by resource preference.

For *N. vespilloides*, we have observed differential resource use in sympatry (Chapter 2) and some evidence for genetic differentiation between beetles that prefer different resources (this chapter). However, it does not appear that beetles which prefer different resources occur in allochronic isolation, because could find no evidence that differential resource use at different times in the year was adaptive (Chapter 3). Instead, seasonal differences in resource use appear more likely to be due to phenotypic differences in individual quality.

Studying criteria #5 through behavioural observations has been beyond the scope of this project due to time and logistical constraints. However, we come back to this suggestion in Chapter 6, we test it indirectly through cuticular hydrocarbon analyses. In the next chapter, we investigate whether beetles consistently favour their natal carrion type for reproduction, when given a choice, and therefore whether there is any evidence for an oviposition preference.

It is important to reiterate that the findings we present in this chapter are preliminary due to time constraints, and thorough statistical analyses of the data are currently pending. Nevertheless, the picture that is building so far is that wild *N. vespilloides* populations are not composed of genetically distinct races, each specialising on different types of carrion. Instead, it seems more likely that wild populations are composed of a mix of relative generalists and relative specialists that vary in frequency, depending on ecological conditions. High competition for resources, a well-known feature of burying beetle ecology (Scott 1998, Trumbo 1994), could be an important factor in maintaining this mixture of genotypes by preventing the competitive exclusion of specialist individuals by the generalists (Smith & Skulason 1996, Rozen & Lenski 2000, Bono et al. 2015). In the next chapter, we test in principle how easily the balance of specialists versus generalists might be perturbed in natural populations by attempting to evolve populations of resource specialists experimentally, in the lab.

Chapter 5

Local adaptation and population differentiation in the lab

Introduction

Our finding that the patterns of resource use we observed in the field (Chapter 2) are associated with genetic divergence (Chapter 4) led us to investigate whether sub-populations might diverge due to the adaptive partitioning of different carrion resources by individuals.

Theory predicts that ecological divergence is more likely to occur in sympatry if divergent natural selection acts on traits that govern both survival and reproduction (Gavrilets 2003, Smadja & Butlin 2011, Nosil 2012). For burying beetles, vertebrate carrion are defensible resources that act as arenas for mating, oviposition sites and a food resource for both adults and larvae (Pukowski 1933, Eggert & Müller 1997, Milne & Milne 1976, Peck & Anderson 1985). As discussed previously, the ephemerality and unpredictability of carrion resources makes them limiting and can promote ecological separation and adaptive diversification associated with their differential use in nature (Chapter 1, Chapter 2; Benbow et al. 2015, Benbow et al. 2019). Divergent resource use may be associated in behavioural as well and life-history traits (Blanckenhorn 2015). However, direct evidence of the mechanisms that drive this process is difficult to find, especially in natural populations (Chapter 1).

Experiments in the lab could provide ideal conditions for a controlled test of the idea that selection via resource use can result in intra-population divergence. Nosil & Harmon (2009) make a convincing case for how experimental studies in the lab are unique opportunities for testing these principles. One strength of experiments involving artificial selection, for example, is that the total strength of selection imposed can be precisely controlled while replicated lines can be selected divergently on one or multiple traits (Rice & Hostert 1993, Nosil & Harmon 2009, Nosil 2012).

Experimental evolution is a different approach because traits are not selected directly by the experimenter. Instead, replicate populations are exposed to different environments and the divergent selection pressures they produce, and any resulting evolutionary change in diverse traits can be tracked across the generations. Pioneering work in experimental evolution on *Drosphila* species revealed that divergent environmental conditions such temperature, humidity, light and food resources resulted in rapid sexual and behavioural isolation (Kilias et al. 1980, Dodd 1989). Since then, this approach has been largely restricted to studies involving *Drosophila* (Rice & Hostert 1993, Fry 2009). Previous work on parental care in burying beetles has demonstrated that this system lends itself particularly well to experimental evolution studies in the lab (Schrader et al. 2015, Jarrett et al 2017, Schrader et al. 2017).

To test in principle whether differential resource use could cause traits to diverge within populations, we experimentally evolved populations of the burying beetles *N. vespilloides* collected from Thetford Forest by breeding them either on chick or on mouse carcasses for over 20 generations. In addition to populations derived from Thetford, we set up replicate lines on chick and mice carrion using a stock population derived from four different woodlands in Cambridgeshire (Byron's Pool Local Nature Reserve, Gamlingay Wood, Thetford Forest and Waresley Wood) to investigate whether putative differences in genetic variation between our founding populations would influence subsequent evolutionary trajectories.

We predicted that the selection we imposed by exposing beetles to contrasting breeding resources would cause populations to diverge adaptively to become specialists on bird or mice carrion, according to the type of resource we had bred them on. After multiple generations of breeding populations in the lab on either birds or mice, we tested for evidence of local adaptation to that breeding resource. Specifically, we addressed two questions:
Question 1: Does beetle performance on a carcass type improve after several generations of evolving on it?

Question 2: Do beetles have the greatest reproductive success on the carrion type they evolved on?

We also investigated the mechanisms underpinning any possible local adaptation by focusing on measures of fecundity and survival, by addressing these two questions:

Question 3: Is differential reproductive performance on carrion a result of differences in clutch size?

Question 4: Is differential reproductive performance on carrion associated with life history trade-offs such as increased lifespan?

With our lab populations, we exposed beetles ourselves to the same type of carrion for generation after generation. However, in nature, local adaptation to different types of carrion could only happen if lineages bred faithfully on the same resource in successive generations. This means there would have to be a mechanism to return beetles to breed on the same type of carrion that they were raised upon. We tested whether beetles preferentially prepare their natal carrion type for reproduction, when given a choice. In addition, we asked:

Question 5: Does beetle preference for their natal carcass increase after several generations of evolving on it?

Materials and methods

Beetle collection

We established experimental populations in the lab using beetles collected during the 2017 field season from Byron's Pool Local Nature Reserve, Gamlingay Wood, Thetford Forest and Waresley Wood, under permits from Natural England and Forestry Commission England.

In Thetford Forest, we sampled beetles using a paired trap arrangement, in which we placed two beetle traps- one baited with a domestic chick carcass and the other baited with a mouse carcass- near each other at each trap location and recorded the number beetles found in each trap. The mice and chick carcasses used were matched in weight (30-40 g). The traps within each experimental pair were placed 1-2 m apart. Pairs of traps were placed 200- 400 m apart from each other.

Trapping in the other three woods was part of a long-term study of *Nicrophorus* beetles within each woodland. For the purpose of this particular study, we only used beetles that were trapped on mice carcasses from Byron's Pool, Waresley Wood and Gamlingay Wood. The trapping methodology used at Thetford Forest, Gamlingay Wood and Waresley Wood is described in greater detail in Chapter 2. In Byron's Pool, we used single traps baited with dead mice (set up by Sue Aspinall) at six different trapping locations. Traps were emptied and rebaited every two weeks during May to October 2017.

Processing field-caught beetles

At the lab, we used carbon-dioxide to immobilise each beetle and brush off any mites stuck to it. We recorded the species, pronotum width and sex of every *Nicrophorus* beetle. Two *N. vespilloides* beetles from each trap were isolated for 4 hours and then frozen at -80°C for subsequently extracting cuticular hydrocarbons (see Chapter 6). The remaining *N. vespilloides* beetles were used to extract DNA (see Chapter 4), measure reproductive performance (see Chapter 3) and to establish populations for the lab experiments described below. We did not return any of the species caught during our trapping experiment back into the field.

Establishment and maintenance of experimental populations

Once the beetles were processed, we put each *N. vespilloides* individual into a small plastic box (12 cm × 8 cm × 2 cm) and fed it 1 g of beef mince. We stored the beetles in the box for 7-10 days before breeding them to establish experimental populations in the lab. This was done to ensure that any newly eclosed individuals had had sufficient time to become sexually mature.

Beetles from Thetford Forest

We used beetles caught in late August and early September 2017 at Thetford Forest to establish two replicate populations in the lab (T1 and T2). Beetles that were trapped on a chick carcass in the field were bred on chick carcasses in the lab (T1C and T2C) and those trapped on dead mice were bred on mouse carcasses in the lab (T1M and T2M). Details of the number of individuals used to establish the populations are listed in Table 1.

T1 and T2 populations were bred approximately 10 days apart from each other throughout the course of their evolution in the lab. T1C and T2C evolved on chick carcasses for 21 and 20 generations respectively, until January 2020. T1M and T2M evolved on mouse carcasses for 21 and 20 generations respectively, until January 2020. We measured the reproductive performance of the beetles at every generation (see 'Measuring reproductive performance'). We measured their preference for mouse and chick carcasses every 4-6 generations (see 'Measuring carrion preference'). At the end of the selection experiment, we measured the reproductive success of T1C and T1M on both chick and mice carrion to test if individual beetles had the greatest reproductive success on the carrion type they had evolved upon (Table 2). We were unable to repeat this experiment in time for the T2 population as the T2C population underwent a significant crash in its numbers at generation 21.

Beetles from multiple locations

In addition to the replicate populations from Thetford, we established a further population (M) that comprised a mix of beetles from multiple locations. The beetles used for this population were trapped on mice carrion in July 2017 at Byron's Pool Local Nature Reserve, Gamlingay Wood, Thetford Forest and Waresley Wood, which were then interbred. Details of the number of individuals used to establish the populations are listed in Table 1.

To ensure that the gene pool of this population was well mixed, we bred them on mice as one stock population for 5 generations. Then in February 2018, at generation 6, the beetles were split

into two groups- one set which continued to be bred on mice (MM) and the other set which was bred on chick carcasses for another 14 generations. Generation 15 beetles were tested for carrion preference, but the lines were not continued further. We measured the reproductive performance of the beetles at every generation (see 'Measuring reproductive performance'). We measured their preference for mouse and chick carcasses every 5 generations (see 'Measuring carrion preference').

Table 1: Founding size and origin of experimental populations

Population	Carcass type evolved on	Origin	Founding size
T1	Mice (T1M)	Thetford Forest	18 pairs
	Chicks (T1C)		30 pairs
T2	Mice (T2M)		28 pairs
	Chicks (T2C)		38 pairs; 19 pairs on domestic chicks and 19 pairs on great tit carcasses
M	Mice (MM)	Byron's Pool Local Nature Reserve (B), Gamlingay Wood (G), Thetford Forest (T) and Waresley Wood (W)	40 pairs (16 B females, 4 B males; 7 G females, 11 G males; 12 T males, 10 T females; 7 W females, 13 W males)
	Chicks (MC)		

Since the beetles from the field are of unknown mating status and relatedness, we paired them up randomly to establish the lab populations. To prevent inbreeding depression thereafter, we bred the lab populations in a more systematic manner to ensure that siblings and cousins were not paired with each other.

After the T1, T2 and M beetles were bred on their respective carcass type, we placed the larvae from these broods in 25-celled eclosion boxes (10 cm × 10 cm × 1.8 cm), with one larva in each cell (2 cm × 2 cm × 1.8 cm), covered them with damp peat, and left them to metamorphose into adults for 18 days. Most individuals had eclosed by 18 days but we left those that had not in the eclosion box to give them more time (usually an additional 1-2 days).

After eclosion, we sexed the individuals and then placed individual beetles in small plastic boxes (12 cm × 8 cm × 2 cm) with damp compost and 1 g of beef mince. Adult beetles remained in

these boxes until they were sexually mature (at least 2 weeks) and were fed beef mince once a week. We bred beetles from each population 14-21 days post eclosion, as described below.

Measuring reproductive performance

We placed a pair of beetles (one male and one female) in a large plastic box (17 cm x 12 cm x 6 cm) half filled with Miracle-Gro compost and provided them with either a chick (T1C, T2C, MC) or mouse carcass (T1M, T2M, MM). The mass of the carcass provided for reproduction was recorded and kept consistent within each treatment. The box was then placed inside a cupboard so that it was shielded from light in order to mimic the low light conditions typically experienced by beetles as they breed below ground. Eight days after pairing the beetles (i.e., the point at which the larvae had completed development and were starting to disperse away from the remains of the carcass), we counted and weighed the larvae from each pair. We used brood size and mass at dispersal as a measure of reproductive success.

Testing if beetles have greatest reproductive success on the carrion type they have evolved upon

To test this prediction, we used Generation 19 T1C and T1M beetles and established the following four treatments:

Table 2: Experimental design to test for evidence of local adaptation, following experimental evolution on different types of carrion

Treatment	Evolved on	Bred on
T1CC	chicks	chicks
T1CM	chicks	mice
T1MC	mice	chicks
T1MM	mice	mice

For this experiment, we tested 144 pairs in all: 72 pairs each of T1C and T1M beetles and 36 per treatment. This experiment was carried out in collaboration with Hyun Woo Park, an M.Phil. student in the Kilner lab.

Measuring clutch size

To test whether any differences in the brood sizes of beetles bred on chicks and mice could be explained by differences in clutch size, we bred 20 pairs each of Generation 20 T1C and T1M beetles on chick carrion and another 20 pairs of each line on mice carrion (as in Table 2). We used an equal volume of soil in each box and left the box inside a cupboard for 53 hrs, to ensure that the beetles had prepared the carcass and laid eggs. We then took out the soil in each box in small batches and recorded all the eggs inside each box as a measure of the clutch size. We also inspected the prepared carcasses to ensure we didn't miss any eggs. This experiment carried out in collaboration with Hyun Woo Park, an M.Phil. student in the Kilner lab.

Measuring lifespan

We compared the lifespan post-breeding of 36 pairs each of Generation 18 T1C, T1M, T2C and T2M beetles as well as Generation 14 MC and MM populations. After the beetles produced a brood, we removed the parents at dispersal and retained them individually in a small plastic box. We measured their pronotum width and fed them 1 g beef mince once every 4 days and checked them for mortality. We recorded any beetles that had died before discarding them and continued the experiment until we had a 4-day window of mortality for all 216 beetles. A single T2C beetle escaped during the experiment and was discarded from the dataset.

Measuring carrion preference

In order to test whether beetles had evolved a preference for the carrion type they developed upon as larvae, we gave sexually mature beetles a simultaneous choice between a chick and a mouse carcass. We placed individual beetles in the middle of a choice chamber (31cm x 17 cm x 10 cm), containing a chick and a mouse carcass (Figure 1). The carcasses used were allowed to decompose for 72 h before presenting to the beetles to allow for optimal volatile release.

I watched the beetles bury themselves into the soil of the central panel (which had holes on either side) and then left the boxes in the dark for 30- 32 hrs. At the end of this period, I noted down the carcass the beetle had processed as well as the one I found the beetle on in each case. I

considered a carcass 'processed' if the beetle had stripped it of fur or feathers and attempted to ball up the carcass and bury it in the soil.

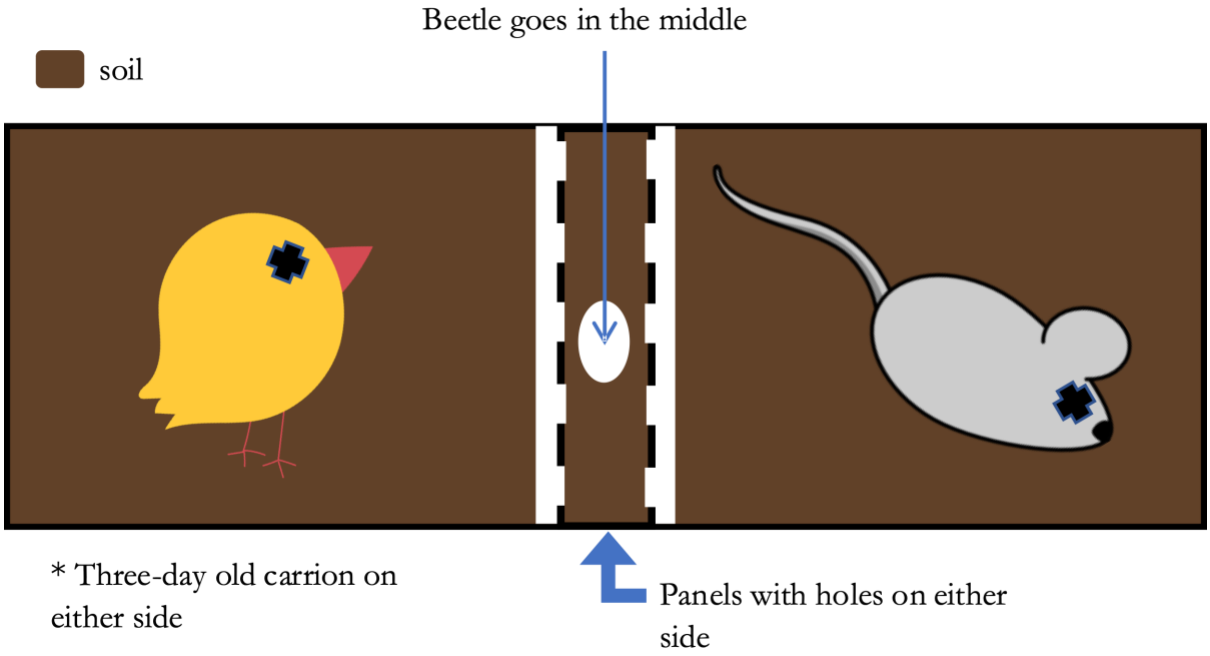


Figure 1: Schematic representation of carrion preference experiment

I tested the carrion preference of generations 5, 10 and 15 of population M; generations 9 and 19 of population T1; and generations 13 and 19 of population T2 using the setup described above.

Statistical analysis

We carried out all statistical analyses to test our predictions using R (RStudio version 1.3.959) with generalised linear models (GLM) and generalised linear mixed models (GLMM) using the 'lme4', 'glmmsr' and 'MASS' packages. Post-hoc comparisons using Tukey's HSD test were carried out using the package 'lsmeans'.

Question 1: Does beetle performance on a carcass type improve after several generations of evolving on it?

We tested for increased reproductive performance by examining the effect of the carrion type that beetle's evolved upon, generation, population (T1, T2 or M), and their interactions on:

- the number of dispersing larvae (brood size), using a GLM with a Poisson error term

- average larval mass using a linear model. We also added brood size as an independent variable in the model for average larval mass.
- larval density using a linear model. Larval density refers to the brood size divided by the carrion mass
- carcass use efficiency using a linear model. We calculate carcass use efficiency as the percentage of the carcass that is converted to the brood (i.e., brood mass) using the following formula:

$$carrion\ use\ efficiency\ = \left[\frac{total\ brood\ mass\ (g)}{carrion\ mass\ (g)} \right] \times\ 100\ \%$$

Question 2: Do beetles have the greatest reproductive success on the carrion type they evolved on?

Using measures of reproductive performance, we examined the effect of the carcass the beetle's lineage had evolved upon ('natal carcass'), the carcass that beetles were given to breed upon ('carcass environment'), and their interactions on:
- the number of dispersing larvae (brood size), using a GLM with a Poisson error term
- average larval mass using a linear model. We also added brood size as an independent variable in the model for average larval mass.
- larval density using a linear model
- carcass use efficiency using a linear model

Question 3: Is differential reproductive performance on carrion a result of differences in clutch size?

To test whether evolution on a particular carcass type is associated differences in clutch size, we looked at the effect of the carcass the beetle's lineage had evolved upon ('natal carcass'), the carcass that beetles were given to breed upon ('carcass environment'), and their interactions on the total number of eggs laid by females (clutch size), using a GLM with a Poisson error term.

Question 4: Is differential reproductive performance on carrion associated with life history trade-offs such as increased lifespan?

We calculated the post-reproductive lifespan of the beetles by subtracting the date they were found dead from the date that they were paired for breeding. We tested the effect of the carcass the beetle's lineage had evolved upon, the specific population they came from and sex on the lifespan of the beetle using a GLM with a Poisson error term

Question 5: Does beetle preference for their natal carcass increase after several generations of evolving on it?

We used the following scoring system to record carrion preference: a 'zero' (0) denoted beetles that did not choose their natal carrion while a 'one' (1) was assigned to those that did. We used a multivariate logistic regression model with a binomial error term to test the effect of carrion type, population, generation and sex on carrion preference. Beetles that processed both or none of the carcasses were excluded from the analysis.

For all the analyses that we used to measure reproductive performance, we removed any broods that failed to produce at least one larva at 8 days post beetle pairing. When arriving at a minimal model using GLMs and GLMMs to explain our results, we removed non-significant terms and interactions using stepwise elimination. When presenting these analyses, we list all the terms that were tested, and their statistics at the last point when they were retained in the model.

Results

Question 1: Does beetle performance on a carcass type improve after several generations of evolving on it?

We found significant variation in brood sizes over the generations of selection imposed (Figure 2, Table 3). There was a significant 3-way interaction between the generation of selection experiment, the carcass type the beetles were evolving on and population (Table 3). On average, broods on chick carcasses were significantly smaller than those on mice in all populations (Tukey post-hoc comparisons| Population M: z ratio= -36.908, p-value <.0001; Population T1: z ratio= -32.835, p-value <.0001, Population T2: z ratio= -40.654, p-value <.0001).

Over time, there was a significant decrease in brood sizes of beetles evolving on chick carcasses compared to those evolving on mice (Tukey post-hoc comparison: z ratio= -63.053, p-value <.0001). Across all generations, population M produced broods with more larvae compared to T1 (Tukey post-hoc comparison: z ratio= 8.556, p-value <.0001) and T2 (Tukey post-hoc comparison: z ratio= 5.917, p-value <.0001). T1 beetles produced significantly smaller broods than T2 beetles (Tukey post-hoc comparison: z ratio= -3.076, p-value= 0.0060).

Table 3:

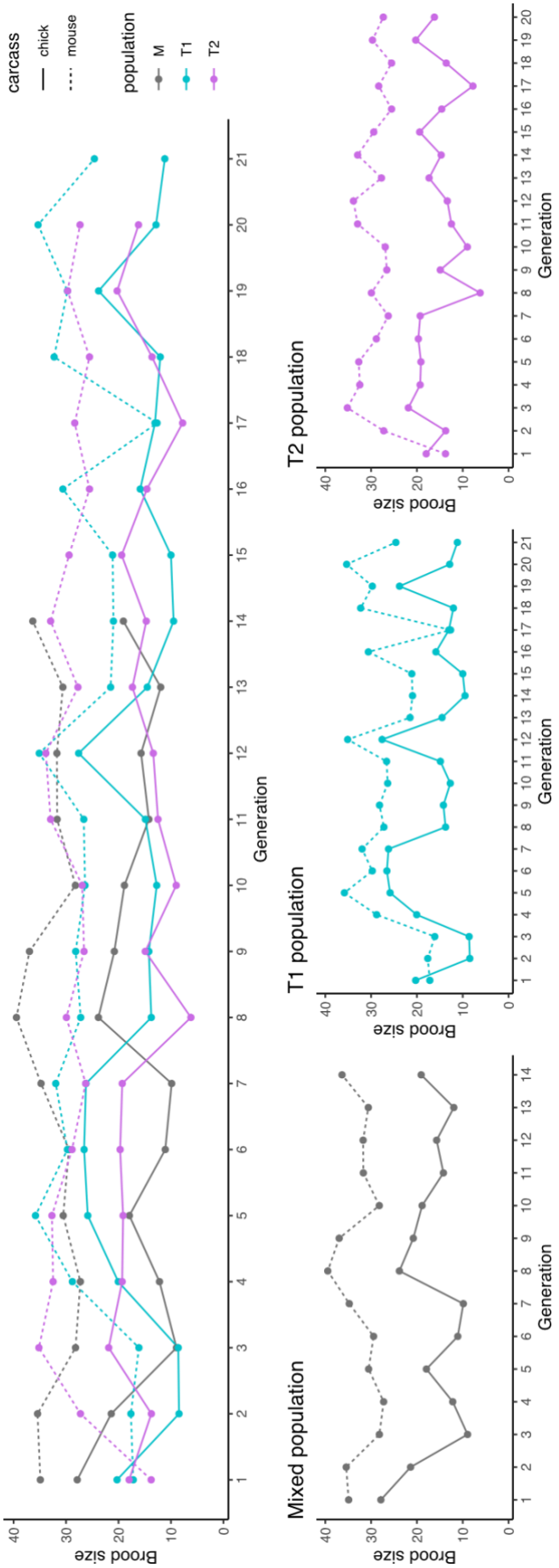
a. Results of ANOVA on the effects of carcass type, generation, population, and their interactions on brood size

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
generation	1.8	1	0.182814
carcass type	4802.8	1	< 2.2e-16 ***
population	234.0	2	< 2.2e-16 ***
generation x carcass type	109.5	1	< 2.2e-16 ***
generation x population	13.5	2	0.001149 **
carcass type x population	120.3	2	< 2.2e-16 ***
generation x carcass type x population	12.8	2	0.001149 **

b. Model summary of the GLM to test the effects of carcass type, generation, population, and their interactions on brood size

Fixed effects:	*Estimate*	*Std. Error*
Intercept	2.998249	0.026722
Generation	-0.018104	0.003144
Carcass Type-Mouse	0.459636	0.032129
Population T1	-0.053272	0.034628
Population T2	-0.098121	0.035096
Generation x Carcass Type-Mouse	0.021693	0.003751
Generation x Population T1	0.007647	0.003614
Generation x Population T2	0.008984	0.003631
Carcass Type-Mouse x Population T1	-0.215752	0.042449
Carcass Type-Mouse x Population T2	0.003854	0.042723
Generation x Carcass Type-Mouse x Population T1	-0.003051	0.004343
Generation x Carcass Type-Mouse x Population T2	-0.012582	0.004370

Figure 2: Summary plot of brood sizes of T1, T2 and M populations breeding on chick and mice carrion over the time course of the experiment. The y-axis indicates the number of larvae per brood while the generations are on the x-axis. Populations are differentiated by colour. Populations that evolved on chicks are represented by a solid line while those that evolved on mice are given by a dotted line. Dots represent averages for each population at each generation, and they are connected by trendlines.

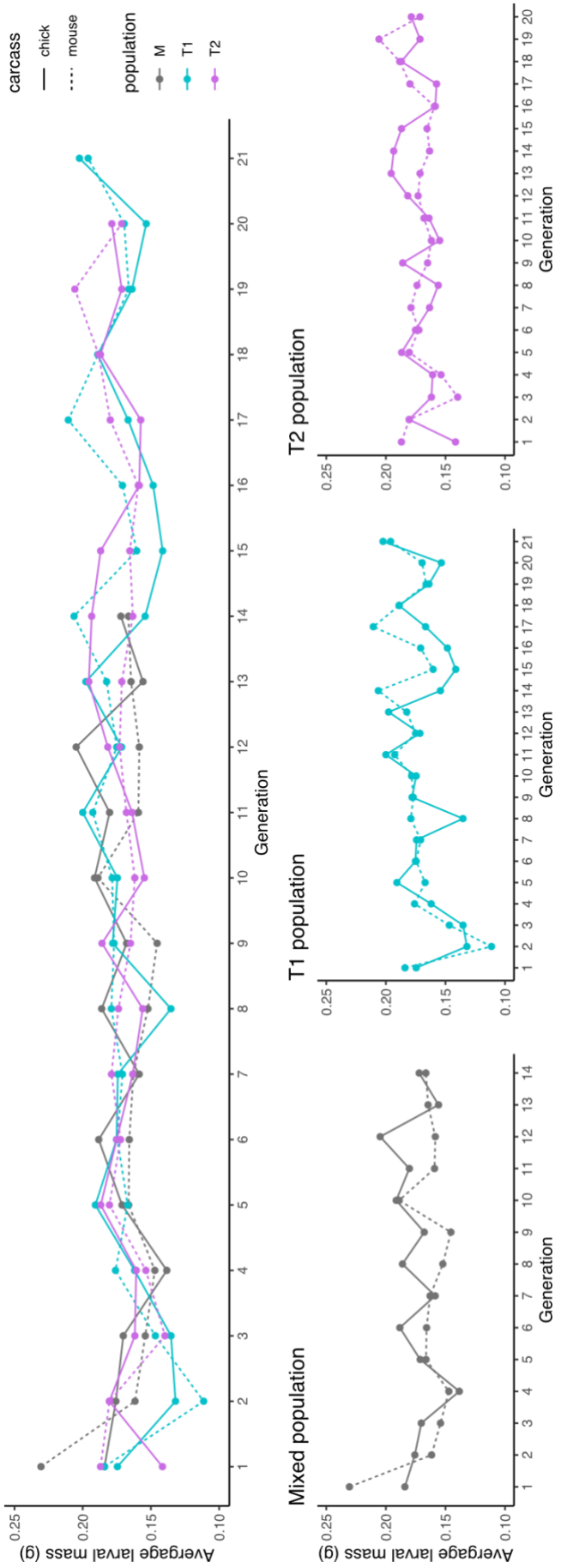


Average larval mass varied in all populations over time (Figure 3, Table 4). Our results indicate a significant 3-way interaction between the generation of selection experiment, carcass type the beetles were evolving on and population (Table 4). There was also a significant 3-way interaction between the generation of selection experiment, beetle population and brood size (Table 4). Over generational time, populations raised on mice carrion evolved to produce, on average, significantly larger larvae than those produced by populations raised on chicks (Tukey post-hoc comparison: t ratio= -2.476, p-value= 0.0133). Population M larvae raised on mice and chicks did not significantly differ in larval mass (Tukey post-hoc comparison: t ratio= -0.084, p-value= 0.9328). However, larvae raised on chicks were smaller than those on mice for both population T1 (Tukey post-hoc comparison: t ratio= -2.286, p-value= 0.0223) and T2 (Tukey post-hoc comparison: t ratio= -2.774, p-value= 0.0056).

Table 4: Results of ANOVA on the effects of carcass type, generation, population, brood size and their interactions on average larval mass

Factors	*Sum Sq*	*Df*	*Pr(>F)*
generation	0.0718	1	3.095e-06 ***
carcass type	0.0933	1	1.066e-07 ***
population	0.0158	2	0.0910577 .
brood size	0.3978	1	< 2.2e-16 ***
generation x carcass type	0.0170	1	0.0230401 *
generation x population	0.0227	2	0.0318384 *
generation x brood size	0.0378	1	0.0007036 ***
carcass type x brood size	0.2006	1	7.691e-15 ***
generation x carcass type x population	0.0381	2	0.0030852 **
generation x brood size x population	0.0254	2	0.0211339 *

Figure 3: Summary plot of average larval mass of T1, T2 and M populations breeding on chick and mice carrion over the time course of the experiment. The y-axis indicates the average larval mass (g) per brood while the generations are on the x-axis. Populations are differentiated by colour. Populations that evolved on chicks are represented by a solid line while those that evolved on mice are given by a dotted line. Dots represent averages for each population at each generation, and they are connected by trendlines.



We found variation in the efficiency with which beetles utilised carrion resources over the generations of selection imposed (Figure 4, Table 5). There was a significant interaction between the carrion type beetles evolved on and population on the efficiency of carrion use (Table 5), though all populations utilising mice carrion significantly more efficiently than chick carrion (Tukey post-hoc comparisons| Population M: t ratio= -17.010, p-value <.0001; Population T1: t ratio= -16.885, p-value <.0001, Population T2: t ratio= -17.233, p-value <.0001). All three populations utilised chick carrion with equivalent efficiency. However, population M beetles utilised mice carrion significantly more efficiently than T1 beetles (Tukey post-hoc comparison: t ratio= -0.437, p-value= 0.0265). The generation of selection also interacted significantly with both carcass type and population (Table 5). Over time, beetles evolved to utilise chick carrion less efficiently than mice carrion (Tukey post-hoc comparison: t ratio= -29.797, p-value= <.0001).

Table 5: Results of ANOVA on the effects of carcass type, generation, population, and their interactions on carrion use efficiency

Factors	*Sum Sq*	*Df*	*Pr(>F)*
generation	499	1	0.007225 **
carcass type	61181	1	< 2.2e-16 ***
population	732	2	0.005022 **
generation x carcass type	1200	1	3.142e-05 ***
generation x population	919	2	0.001301 **
carcass type x population	462	2	0.035358 *

b. Model summary of the LM to test the effects of carcass type, generation, population, and their interactions on carrion use efficiency

Fixed effects:	*Estimate*	*Std. Error*
Intercept	16.82066	0.75670
Generation	-0.44287	0.07890
Carcass Type-Mouse	7.99305	0.72686
Population T1	-2.39991	0.93169
Population T2	-2.61916	0.94681
Generation x Carcass Type-Mouse	0.22798	0.05468

Generation x Population T1	0.28814	0.08329
Generation x Population T2	0.28385	0.08421
Carcass Type-Mouse x Population T1	-2.01733	0.79374
Carcass Type-Mouse x Population T2	-1.54881	0.80347
Generation x Carcass Type-Mouse x Population T1	0.27257	0.16774
Generation x Carcass Type-Mouse x Population T2	0.09847	0.16931

There was variation in larval density on carrion over the generations of selection imposed (Figure 5, Table 6). There was a significant interaction between beetle population and carrion type though in general, all populations had a greater density of larvae on mice carrion compared to chicks (Tukey post-hoc comparisons| Population M: t ratio= -19.693, p-value <.0001; Population T1: t ratio= -17.113, p-value <.0001, Population T2: t ratio= -18.181, p-value <.0001). All three populations had similar densities of larvae on chick carrion. However, population M beetles had greater larval density on mice carrion than T1 beetles (Tukey post-hoc comparison: t ratio= 4.997, p-value <.0001) and T2 beetles (Tukey post-hoc comparison: t ratio= 4.312, p-value <.0001). The generation of experimental evolution also interacted significantly with carcass type: over the generations, larval density on chicks evolved to be significantly lower than that on mice (Tukey post-hoc comparison: t ratio= -32.215, p-value <.0001).

Table 6:

a. Results of ANOVA on the effects of carcass type, generation, population, and their interactions on larval density

Factors	*Sum Sq*	*Df*	*Pr(>F)*
generation	8.21	1	1.563e-08 ***
carcass type	259.82	1	< 2.2e-16 ***
population	2.38	2	0.009475 **
generation x carcass type	2.77	1	0.001009 **
carcass type x population	5.25	2	3.566e-05 ***

b. Model summary of the LM to test the effects of carcass type, generation, population, and their interactions on larval density

Fixed effects:	*Estimate*	*Std. Error*
Intercept	0.880845	0.033051
Generation	-0.015324	0.002448
Carcass Type-Mouse	0.615774	0.044191
Population T1	0.055240	0.036114
Population T2	0.025244	0.036754
Generation x Carcass Type-Mouse	0.010938	0.003324
Generation x Population T1	0.002734	0.005066
Generation x Population T2	0.005864	0.005122
Carcass Type-Mouse x Population T1	-0.215272	0.048270
Carcass Type-Mouse x Population T2	-0.164069	0.048860
Generation x Carcass Type-Mouse x Population T1	-0.003865	0.010208
Generation x Carcass Type-Mouse x Population T2	-0.002768	0.010303

Figure 4: Summary plot of carrion use efficiency by T1, T2 and M populations breeding on chick and mice carrion over the time course of the experiment. The y-axis indicates the average carrion use efficiency (%) per brood while the generations are on the x-axis. Populations are differentiated by colour. Populations that evolved on chicks are represented by a solid line while those that evolved on mice are given by a dotted line. Dots represent averages for each population at each generation, and they are connected by trendlines.

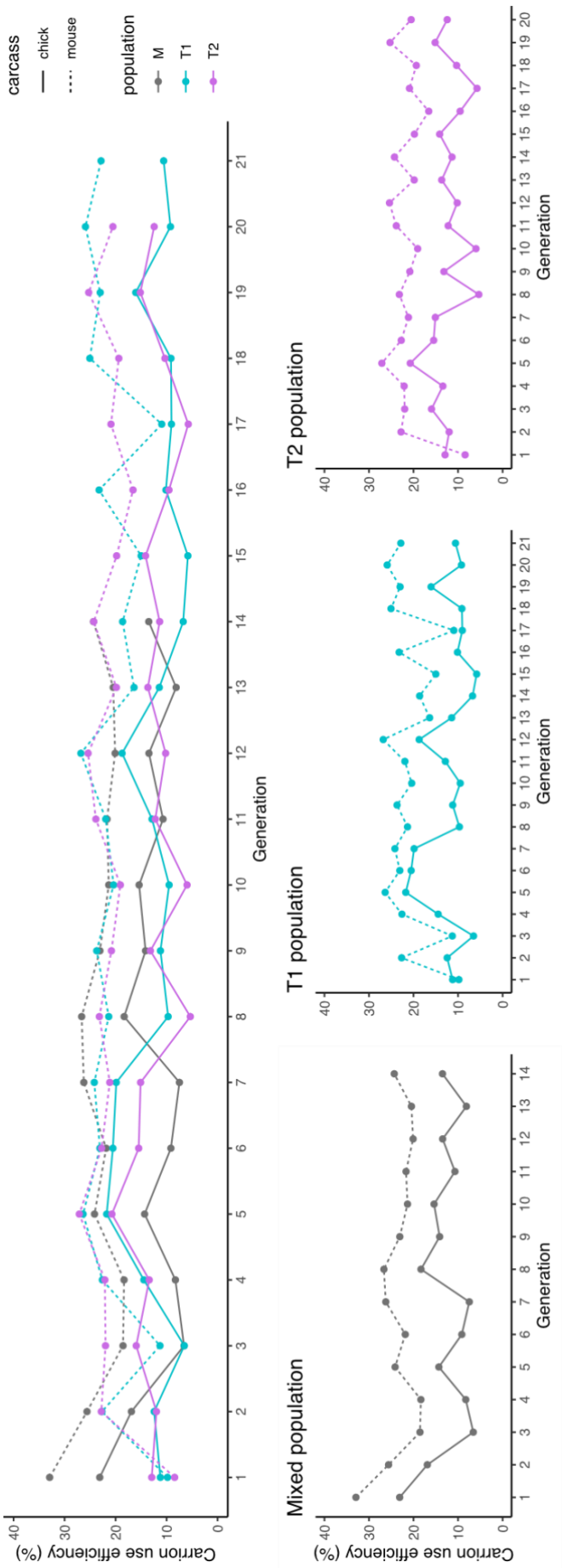
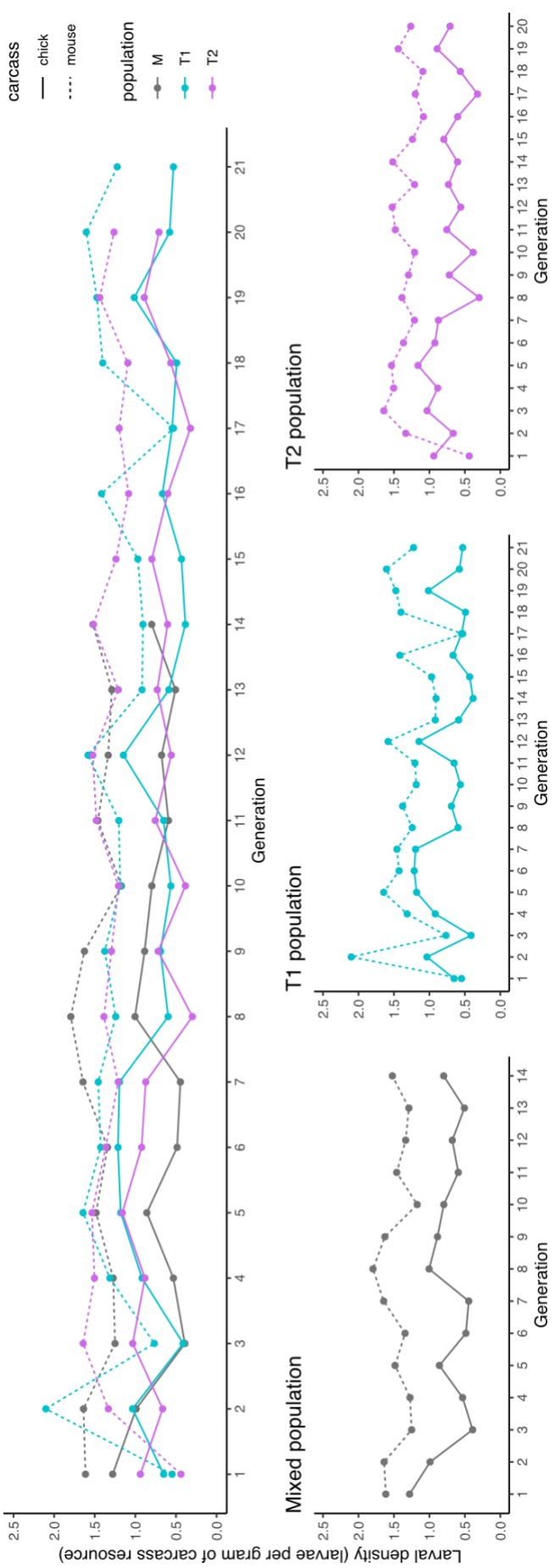


Figure 5: Summary plot of the larval density of broods produced by T1, T2 and M populations breeding on chick and mice carrion over the time course of the experiment. The y-axis indicates the average number of larvae per gram of carcass resource while the generations are on the x-axis. Populations are differentiated by colour. Populations that evolved on chicks are represented by a solid line while those that evolved on mice are given by a dotted line. Dots represent averages for each population at each generation, and they are connected by trendlines.



Question 2: Do beetles have the greatest reproductive success on the carrion type they evolved on?

We found a significant interaction between the type of carrion that a beetle's lineage had evolved upon in the lab, and the type of carrion we gave them to breed upon in this experiment, on the number of larvae that survived to dispersal (Figure 6, Table 7). However, contrary to predictions based on local adaptation, we found that beetles drawn from lineages that had evolved on chicks produced more larvae on mice carcasses than those from lineages that had evolved on mice (Tukey post-hoc comparison: z ratio= 3.586, p-value= 0.0003). Beetles from lineages that had evolved on chicks and mice produced an equivalent number of larvae when they were bred on chicks (Tukey post-hoc comparison: z ratio= 0.450, p-value= 0.6527). Beetles from lineages that had evolved on mice (Tukey post-hoc comparison: z ratio= -5.170, p-value<.0001) and those from lineages that had evolved on chicks (Tukey post-hoc comparison: z ratio= -8.250, p-value<.0001) both produced larger broods on mice carrion than on chick carrion.

Table 7:

a. Results of ANOVA of the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interactions on brood size

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
natal carcass	9.132	1	0.002512 **
carcass environment	92.222	1	< 2.2e-16 ***
natal carcass x carcass environment	3.971	1	0.046292 *

b. Model summary of the GLM to test the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interactions on brood size

Fixed effects:	*Estimate*	*Std. Error*
Intercept	3.11228	0.03516
Natal Carcass-Mouse	-0.02250	0.05000

Carcass environment-Mouse	0.37662	0.04565
Natal Carcass-Mouse x Carcass environment-Mouse	-0.13123	0.06586

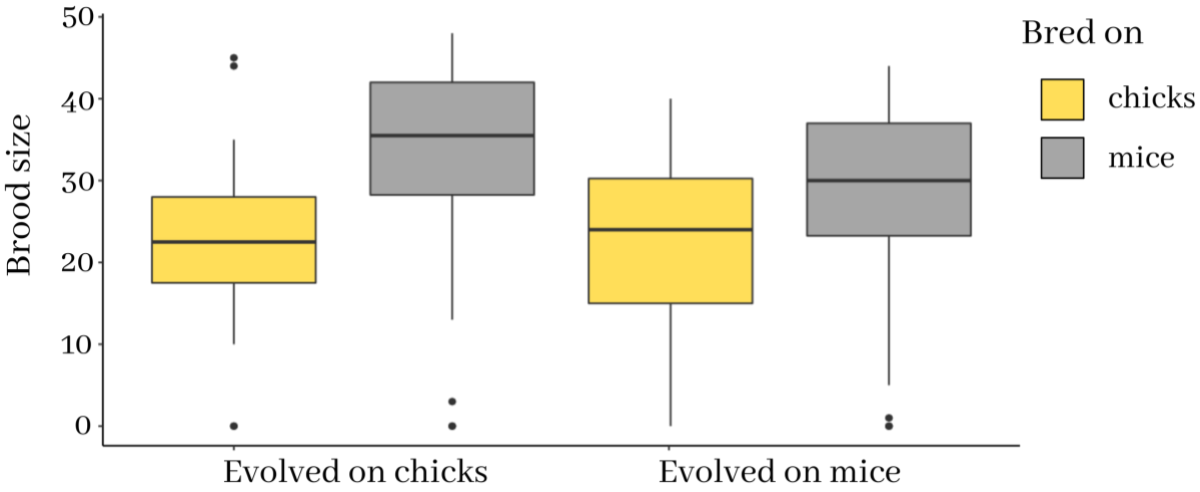


Figure 6: Brood sizes at larval dispersal of Generation 19 T1 beetles, drawn from lineages that had evolved on either chicks or mice, and which were bred for a single generation on either chicks (yellow bars) or mice (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Beetles from lineages that had evolved on chick and mice carrion did not differ significantly in their average larval mass, regardless of the carrion they were given to breed upon in this experiment (Figure 7, Table 8). There was no effect of brood size on average larval mass either (Figure 7, Table 8).

Table 8:

a. Results of ANOVAs of the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interactions on average larval mass

Factors	*Sum Sq*	*Df*	*Pr(>F)*
Dropped/ non-significant terms			
natal carcass	0.00216	1	0.3327

carcass environment	0.00223	1	0.3249
brood size	0.00481	1	0.1487

b. Model summary of the LM to test the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interactions on average larval mass

Fixed effects:	*Estimate*	*Std. Error*
Intercept	0.1379803	0.0106579
Natal Carcass-Mouse	0.0077869	0.0080112
Carcass environment-Mouse	-0.0083488	0.0084501
Brood Size	0.0005019	0.0003456

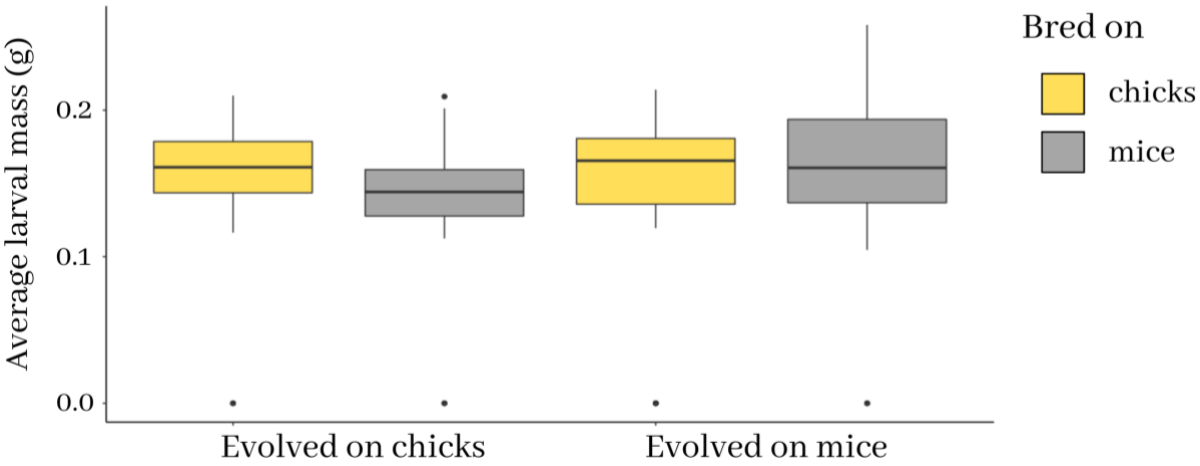


Figure 7: Average larval mass at larval dispersal of Generation 19 T1 beetles, drawn from lineages that had evolved on either chicks or mice, and which were bred for a single generation on either chicks (yellow bars) or mice (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

In general, both T1C and T1M beetles utilised mice carcasses significantly more efficiently than chicks (Figure 8, Table 9).

Table 9:

a. Results of ANOVA on the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interaction on carcass use efficiency

Factors	*Sum sq*	*Df*	*Pr(>F)*
carcass environment	2094.4	1	2.395e-09 ***
Dropped/ non-significant terms			
natal carcass	40.3	1	0.3788
natal carcass x carcass environment	14.1	1	0.6030

b. Model summary of the LM to test the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interaction on carcass use efficiency

Fixed effects:	*Estimate*	*Std. Error*
Intercept	15.477	1.036
Natal Carcass-Mouse	-1.058	1.196
Carcass environment-Mouse	7.627	1.196
Natal Carcass-Mouse x Carcass environment-Mouse	-1.2500	2.3978

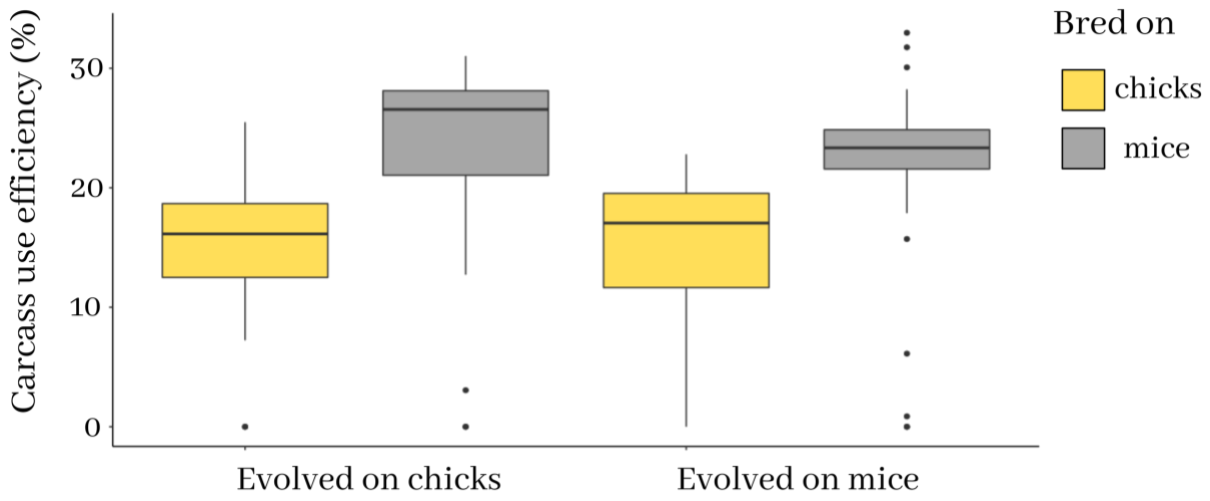


Figure 8: Carcass use efficiency of Generation 20 T1 beetles drawn from lineages that had evolved on either chicks or mice, and which were bred for a single generation on either chicks (yellow bars) or mice (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Beetles produced broods with greater larval densities when they were bred on mice rather than on chicks (Figure 9, Table 10), regardless of whether they came from a lineage that had evolved on chicks or mice.

Table 10:

a. Results of ANOVA on the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interaction on average larval density

Factors	*Sum Sq*	*Df*	*Pr(>F)*
carcass environment	11.658	1	5.518e-09 ***
Dropped/ non-significant terms			
natal carcass	0.654	1	0.1432
natal carcass x carcass environment	0.457	1	0.2201

b. Model summary of the LM to test the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interaction on average larval density

Fixed effects:	*Estimate*	*Std. Error*
Intercept	1.01322	0.07933
Natal Carcass-Mouse	-0.13484	0.09160
Carcass environment-Mouse	0.56906	0.09160
Natal Carcass-Mouse x Carcass environment-Mouse	-0.22523	0.18286

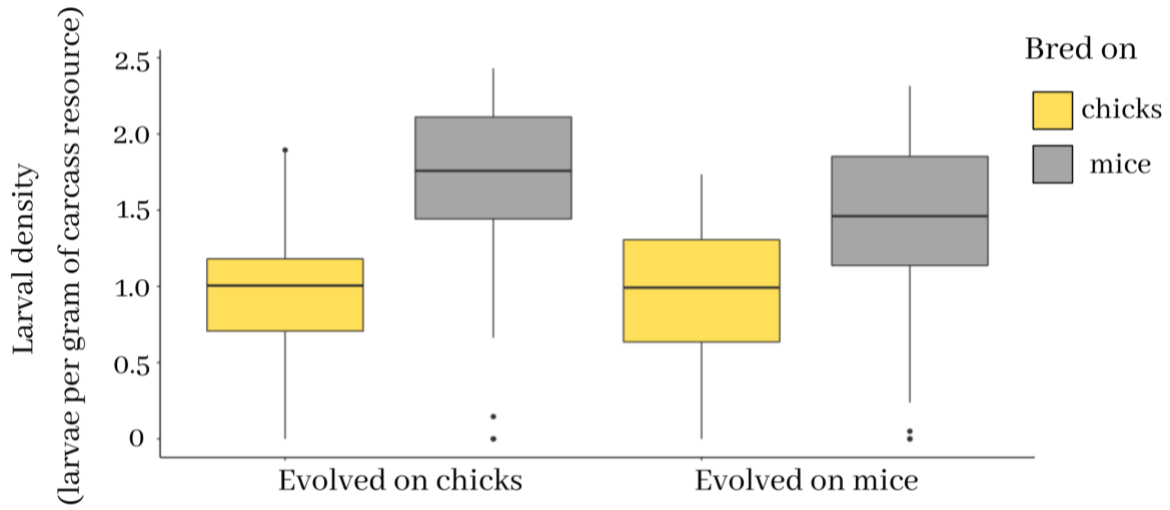


Figure 9: Larval density of broods Generation 19 T1 beetles drawn from lineages that had evolved on either chicks or mice, and which were bred for a single generation on either chicks (yellow bars) or mice (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Question 3: Is differential reproductive performance on carrion a result of differences in clutch size?

We did not find any significant differences in the clutch sizes of beetles from lineages that had evolved on chicks or mice, regardless of the type of carrion they were given to breed upon (Figure 10, Table 11).

Table 11:

a. Results of ANOVA on the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interaction on clutch size.

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
Dropped/ non-significant terms			
natal carcass	2.63230	1	0.1047
carcass environment	0.31759	1	0.5731
natal carcass x carcass environment	1.43381	1	0.2311

b. Model summary of the GLM to test the effects of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interaction on clutch size.

Fixed effects:	*Estimate*	*Std. Error*
Intercept	3.71844	0.03484
Natal Carcass-Mouse	0.09597	0.04813
Carcass environment-Mouse	0.02280	0.04899
Natal Carcass-Mouse x Carcass environment-Mouse	-0.08184	0.06835

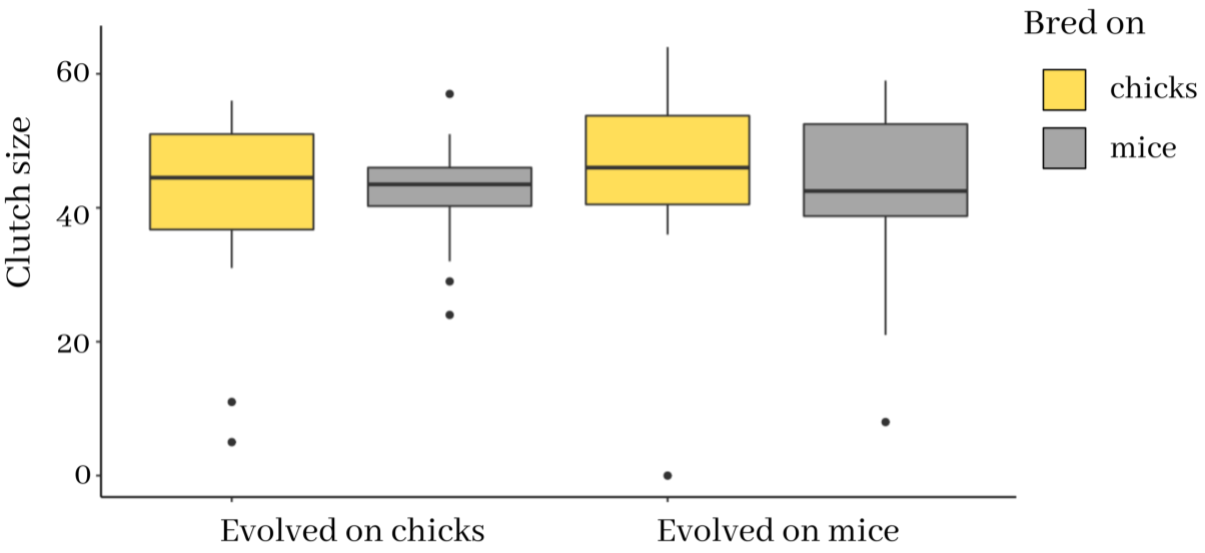


Figure 10: Clutch sizes of Generation 20 T1 beetles drawn from lineages that had evolved on either chicks or mice, and which were bred for a single generation on either chicks (yellow bars) or mice (grey bars). The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.

Question 4: Is differential reproductive performance on carrion associated with life history trade-offs such as increased lifespan?

There was considerable variation in the post-breeding lifespan of different populations (Figure 11, Table 12). We found significant interaction between the type of carrion the beetle's lineage had been bred upon and whether they came from the T or M populations on beetle lifespan (Table 12). We found no significant difference in the post-breeding lifespan of T1 beetles drawn from lineages that had evolved on chick and mice (Tukey post-hoc comparison: z ratio= -1.748, p-value= 0.0804). However, beetles of populations M (Tukey post-hoc comparison: z ratio= -4.085, p-value<.0001) and T2 (Tukey post-hoc comparison: z ratio= -7.536, p-value<.0001), that had evolved on chick carrion, had significantly shorter post-breeding lifespans compared to their mice-line counterparts.

We also found a significant interaction between population and sex on post-reproductive lifespan (Table 12). There were no sex-related differences in the post-breeding lifespans of T1 (Tukey post-hoc comparison: z ratio= -0.795, p-value= 0.4268) and T2 (Tukey post-hoc comparison: z ratio= -0.815, p-value= 0.4148) beetles. However, females of population M lived significantly longer than males after breeding (Tukey post-hoc comparison: z ratio= 2.296, p-value= 0.0217).

Exploring the sex x population interactions further, we found that T2 females and males survived for significantly longer post-breeding than population M females (Tukey post-hoc comparison: z ratio= -5.300, p-value<.0001) and males (Tukey post-hoc comparison: z ratio= -8.408, p-value<.0001), respectively. T2 males and females also lived significantly longer than T1 females (Tukey post-hoc comparison: z ratio= -8.343, p-value<.0001) and males (Tukey post-hoc comparison: z ratio= -8.391, p-value<.0001). T1 females also had a shorter lifespan than M females (Tukey post-hoc comparison: z ratio= 3.071, p-value= 0.0060).

Table 12:

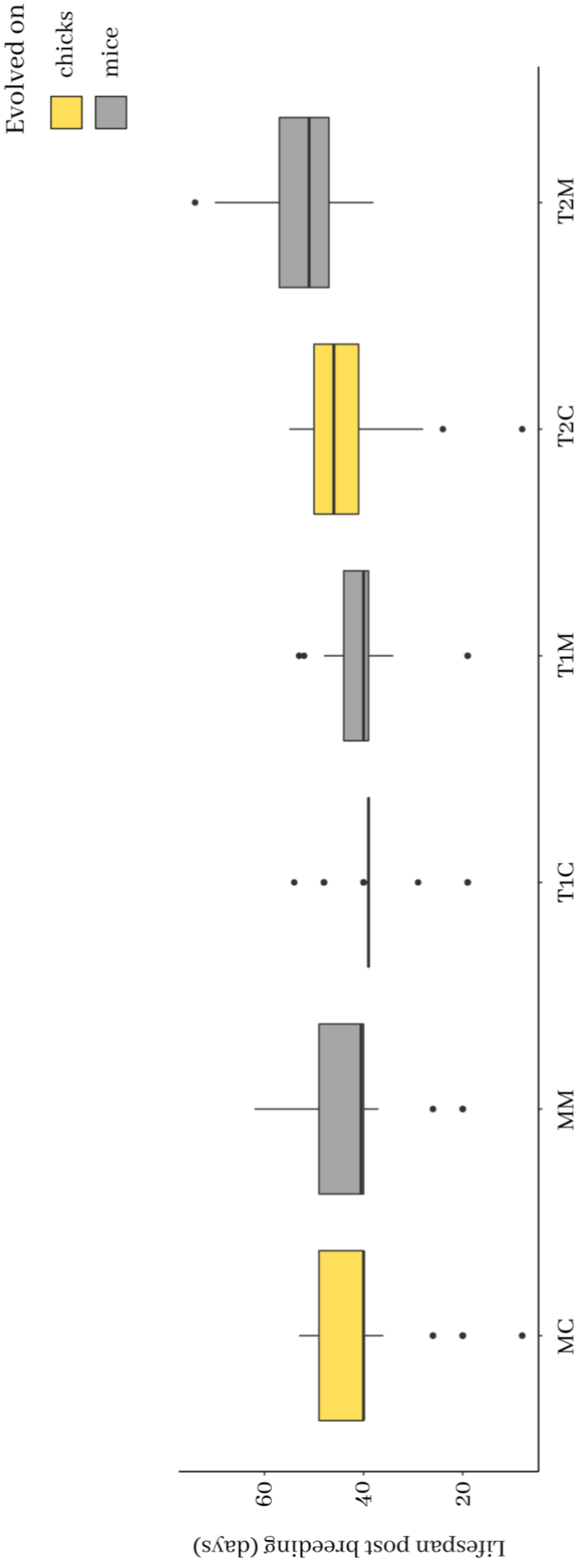
a. Results of an ANOVA on the effect of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interaction on post-reproductive lifespan.

Factors	*Chisq*	*Df*	*Pr(>Chisq)*
population	167.646	2	< 2.2e-16 ***
natal carcass	62.461	1	2.717e-15 ***
sex	0.127	1	0.7216907
natal carcass x population	14.346	2	0.0007669 ***
population x sex	6.444	2	0.0398769 *

b. Model summary of the GLM to test the effect of the lineage from which the beetle was drawn ('natal carcass'), the carcass type on which it was bred for this experiment ('carcass environment'), and their interaction on post-reproductive lifespan.

Fixed effects:	*Estimate*	*Std. Error*
Intercept	3.69470	0.02267
Population-T1	-0.05030	0.03244
Population-T2	0.09451	0.03128
Natal Carcass-Mouse	0.10614	0.02598
Sex-Male	-0.05960	0.02596
Population-T1 x Natal Carcass-Mouse	-0.05982	0.03711
Population-T2 x Natal Carcass-Mouse	0.07455	0.03536
Population-T1 x Sex-Male	0.08066	0.03709
Population-T2 x Sex-Male	0.07907	0.03526

Figure 11: Summary plot showing the post-breeding lifespan of T1, T2 and M populations that had evolved on chicks (yellow bars) versus and mice (grey bars). The y-axis indicates the number of days for which the beetle survived after breeding while the populations are on the x-axis: the suffix to the population code denotes the type of carrion the population was bred upon at each generation. The box bounds represent the inter-quartile range (IQR), the whiskers represent 1.5 * IQR, the central horizontal line is the median, and the single points are outliers in the data.



Question 5: Does beetle preference for their natal carcass increase after several generations of evolving on it?

We found no significant variation in the beetles' inclination to prepare natal carrion over an alternative across the generations of experimental evolution (Figures 12 a, 12b and Table 13). The type of carrion the beetle's lineage had evolved upon, beetle population (T1, T2 or M) and the sex of the beetles did not significantly predict their inclination to prepare natal carrion (Table 13) either.

Table 13:

a. Results of the analysis of deviance on the effect of the lineage from which the beetle was drawn ('natal carcass'), population (M, T1 or T2), sex, generation and their interaction on the preference for natal carrion

Factors	*Deviance resid.*	*Df*	*Pr(>F)*
generation	2.7423	1	0.09772 .
natal carcass type	0.06785	1	0.79449
population	1.3067	2	0.52029
sex	0.8836	1	0.34722

b. Model summary of the GLM to test the effect of the lineage from which the beetle was drawn ('natal carcass'), population (M, T1 or T2), sex, generation and their interaction on the preference for natal carrion

Fixed effects:	*Estimate*	*Std. Error*
Intercept	1.28293	0.309
Generation	0.10099	-0.220
Natal Carcass-Mouse	2.53727	1.516
Population-T1	1.83495	0.270
Population-T2	2.85977	-1.054
Sex-Male	1.88417	0.082

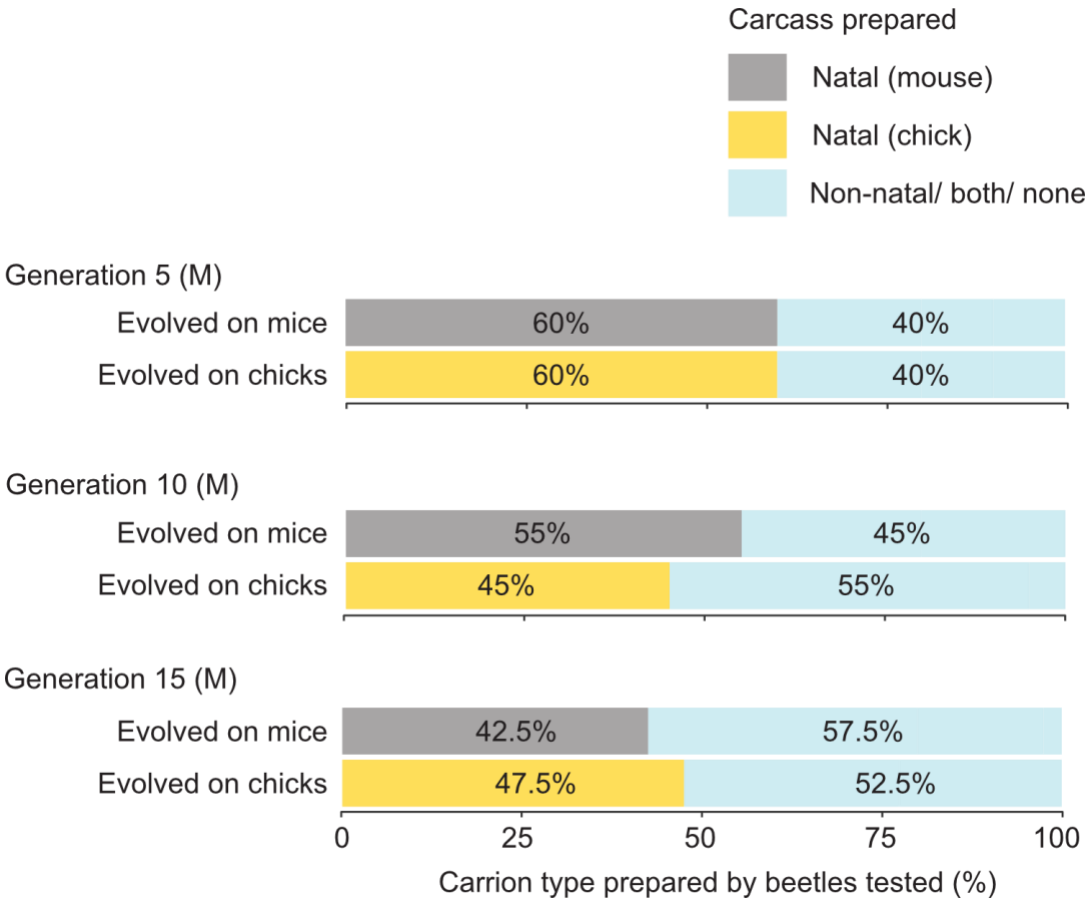


Figure 12 a: Carrion preference of Generations 5, 10 and 15 of population M beetles. The generation and population are indicated by the y-axis. The x-axis indicates the percentage of beetles that prepare a carrion type

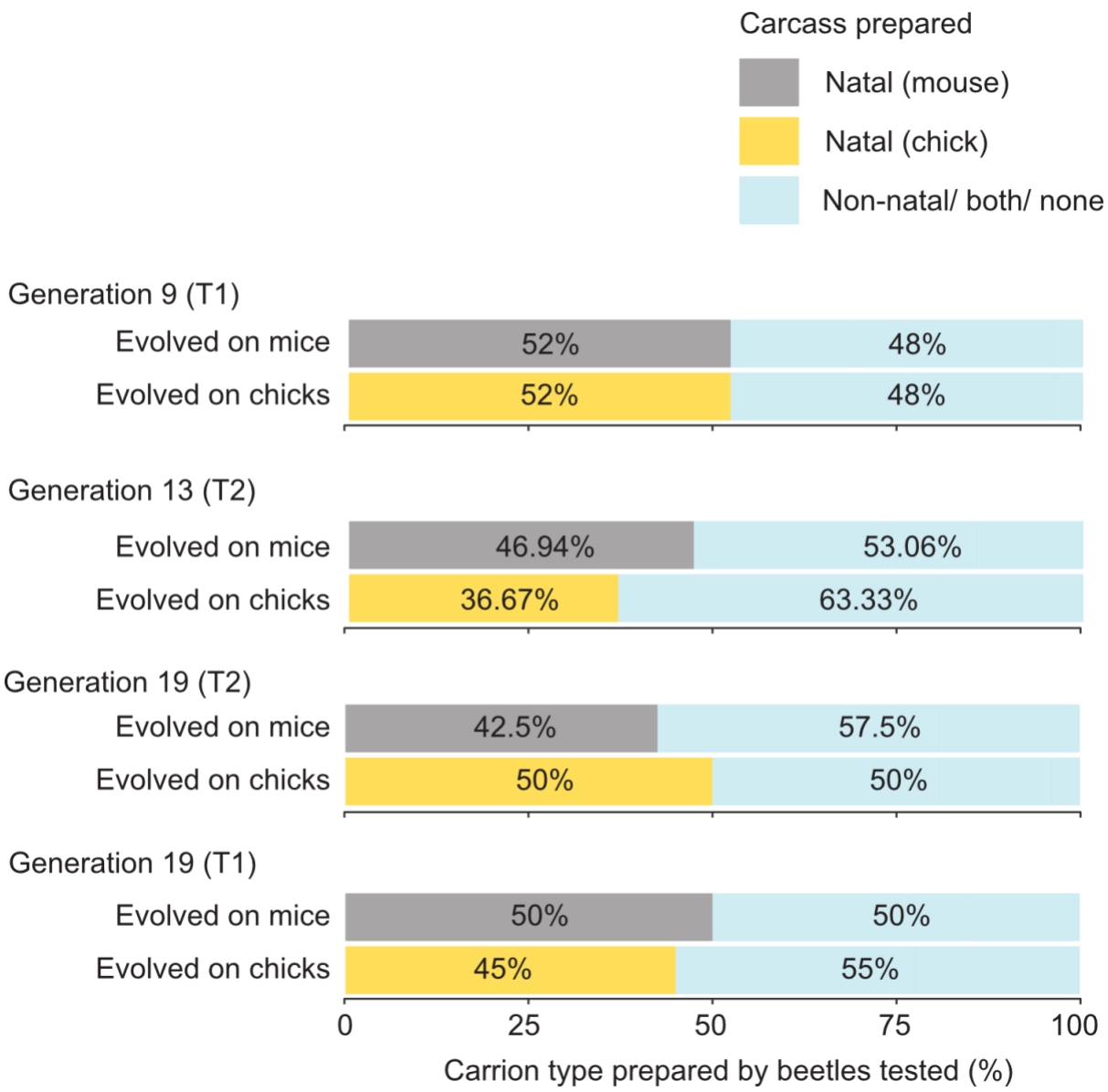


Figure 12 b: Carrion preference of Generations 9 and 19 of population T1 beetles and Generations 13 and 19 of population T2 beetles. The generation and population are indicated by the x-axis. The y-axis indicates the percentage of beetles that prepare a carrion type.

Discussion

We used experimental evolution to test whether in principle, individuals within natural beetle populations could become divergently and locally adapted to breed on different types of carrion. Our analyses of these evolving populations focused on five specific questions. The first two considered whether there was any evidence for local adaptation in our experimental populations, after 20 generations of evolution on different types of carrion.

Question 1: Does beetle performance on a carcass type improve after several generations of evolving on it?

We found that reproductive success on chick and mice carrion varied significantly across generations for all three populations. However, we did not find any evidence to suggest that beetle performance on either type of carrion improved over the course of our selection experiment. We found that, in general, chick carrion was a poorer quality resource for the beetles in terms of brood size (Figure 2, Table 3), larval mass (Figure 3, Table 4), carrion use efficiency (Figure 4, Table 5) as well as larval density on a resource (Figure 5, Table 6) compared to mice, though this was not immediately evident from our experiments on wild beetles (Chapter 3).

We can think of three explanations for this set of results, which are not mutually exclusive. Two we discuss here, the third is below. The first possibility is that the design of the experiment did not impose selection on breeding performance on each type of carrion. Our experimental design selected against individuals that failed to breed on each carrion type, or that produced larvae that never became sexually mature adults. But it probably did not impose more nuanced selection than that. The results from Chapter 3 suggest that wild beetles are capable of breeding relatively well on chicks and mice in the lab. Therefore, it is possible that we did not impose sufficiently strong selection on our experimental populations to become better breeders on their respective types of carrion and this is why we could not detect any improvements in their performance over the duration of the experiment.

The second explanation is that there was no standing genetic variation in our founding populations upon which selection might have acted to improve breeding performance on mice or on chicks. Perhaps wild populations are already well-adapted to breeding on mice, and so there is little scope for improving breeding performance on this type of carrion. And perhaps they have no adaptations

at all for breeding on something as unnatural as a domestic chick, and so there was little capacity for selecting for improved breeding performance on this type of carrion.

Our analyses of the performance of the different populations can offer some insights into the importance of genetic variation in influencing breeding performance on the two types of carrion. We found that, on mice carrion, M population beetles had greater brood sizes that the Thetford populations (Figure 2, Table 3). They also utilised mice carrion more efficiently (Figure 4, Table 5) and had greater larval density on mice (Figure 5, Table 6) compared to Thetford populations. We also found that beetles from the M population were better able to overcome the poor quality of the resources available on chick carrion to produce larvae of equivalent mass compared to their counterparts evolving on mice (Figure 3, Table 4). It is possible that the greater genetic variation of the founding population of M beetles caused these differences (Reed et al. 2003, Agashe 2009, Agashe et al. 2011).

Question 2: Do beetles have the greatest reproductive success on the carrion type they evolved on?

Again, we found no evidence that the experimentally evolving populations became locally adapted to different types of carrion. Rather, our results further suggest that chick carrion is a poor nutritional resource for breeding beetles. Beetles utilised mice carrion more efficiently (Figure 8, Table 9) and had greater larval density (Figure 9, Table 10) on mice carrion compared to chicks, regardless of the carrion type their lineage was bred on. A single generation of breeding on a better-quality resource, had a rescue effect on the fitness of T1C beetles and, in fact, T1C beetles had significantly larger broods on mice carrion compared to T1M beetles (Figure 6, Table 7). An explanation for these results, which is also a third explanation for the patterns discussed above, is that these populations cryptically adapted to breed effectively on chicks and that these adaptations remained hidden due to harsh breeding environment offered by chicks. It was only when the beetles from the chick lineages were allowed to breed in the more benign mouse environment that the previously cryptic adaptations became apparent.

Question 3: Is differential reproductive performance on carrion a result of differences in clutch size?

We did not find any evidence that the differential reproductive performance on mice and chick carrion is due to the beetles laying fewer eggs on chick carrion (Figure 10, Table 11). Personal observations of failed broods indicate that the differences we observe are instead due to a greater number of unhatched eggs and poorer survival of first-instar larvae on chick carrion. There is some evidence to suggest that maternal investment in egg size, rather than egg number, could help mitigate the effects of a poor post-hatching environment (Rollinson & Hutchings 2013, Schrader et al. 2015). Cryptic adaptations like these may have partially compensated for the disadvantage experienced by larvae developed on carrion and could explain why the chick lineages performed so well when allowed to breed on mice (see above). Therefore, studying the evolution of egg size differences between chick- and mice- bred beetle lineages could be a profitable future avenue of investigation.

Question 4: Is differential reproductive performance on carrion associated with life history trade-offs such as increased lifespan?

Carrion type did not seem to have a significant impact on the lifespan on T1 beetles. However, we observed that T2 and M beetles that evolved on chick carrion had a significantly lower lifespan compared to those that had evolved, and bred, on mice (Figure 11, Table 12). This could be due to a negative genetic correlation between traits required for breeding in a poor environment, such as egg size, and longevity. Or it could be due to the fact that individuals reared on chick carrion are intrinsically inferior to those raised on mouse carrion, all else being equal. A fully factorial cross-fostering experiment could help in future work in disentangling the direct impact of breeding on the carcass from lineage-associated effects.

We did not find any sex differences in lifespan in Thetford beetles, but females of population M tended to have a greater lifespan than males (Figure 11, Table 12). This this may be due to population related differences in maternal versus paternal investment that are beyond the scope of our current study (Kilner et al. 2015).

Question 5: Does beetle preference for their natal carcass increase after several generations of evolving on it?

We did not find any evidence to suggest that beetles were more likely to prepare the same carrion as the type they were raised upon, nor that the likelihood of them doing so increased after several generations of experimental evolution (Figures 12 a & 12 b, Table 13). Instead, we found that beetles prepared carrion at random. Population or sex did not seem to impact carrion preference either. Perhaps the experimental set up we used, where beetles did not have to compete and search for carrion, ultimately reduced selection on loci that could aid distinguishing between different carrion types and lead to a loss in genetic diversity, ultimately affecting the ability of beetles to identify suitable carrion for reproduction.

In summary, we did not find strong evidence with these experiments to suggest that, in principle, beetles could become locally adapted within natural populations to specialise on different types of carrion – at least not within 20 generations. It is possible that this could nevertheless happen in natural populations, and we simply failed to replicate that natural process with our experimental set-up. And it is possible that there was some local adaptation, but in traits that we did not measure – such as egg size or mate preference. In the next chapter (Chapter 6), we consider the latter possibility, with experiments that test whether beetles evolving on different carrion differ in their CHCs, which act as contact pheromones during sexual reproduction for several insect species (Blomquist & Bagnères 2010, Ingleby 2015).

Chapter 6

Chemical basis for differential resource use

Introduction

The initiation and persistence of resource-associated differentiation depends on an individual's ability to locate and respond appropriately to resource-specific cues (Miller & Strickler 1984, Hansson & Wicher 2016, McBride 2016). The goal of this chapter is to investigate whether such resource-specific cues exist for burying beetles.

Generalist *Drosophila* species are attracted to odours emitted from a variety of rotting fruit while specialists such as *D. sechellia* and *D. erecta* are attracted to novel host fruits, *Morinda citrifolia* and *Pandanus candelabrum*, respectively (Rio et al. 1983, Louis & David 1986). Host specialisation in *Drosophila* has been associated with rapid changes in genes associated with olfaction and taste (McBride 2007, McBride & Arguello 2007). Comparative studies with bees and mosquitoes have revealed large-scale extensive changes in the chemosensory system, particularly in the olfactory and gustatory receptors (Robertson et al. 2003, Robertson & Wanner 2006, Amrein & Thorne 2005, McBride & Arguello 2007). Therefore, differential resource use in insects is associated with adaptations that enhance their ability to respond to resource-associated chemical stimuli.

Most insects that use olfactory cues while seeking resources rely on a class of chemical compounds emitted by the host known as volatile organic compounds (VOCs). In terrestrial environments, the enzymatic and microbial decomposition of dead matter is associated with the emission of a wide variety of VOCs, that attract different necrophagous insects at different stages of decay (Catts & Goff 1992, Anderson & van Laerhoven 1996, Hoermann et al. 2013, Tomberlin et al. 2016, Poldy 2020).

Blowflies and carrion beetles are among the first of these necrophagous insects to arrive on a cadaver (Stensmyr et al. 2002, Kalinova et al. 2009, Merritt & De Jong 2015, Tomberlin et al. 2016). For burying beetles, securing a small vertebrate carcass at a stage where it has not yet been heavily decomposed or infested with competing species has immense fitness benefits (Rozen et al. 2008, Kalinova et al. 2009, Duarte et al. 2018). Studies suggest that burying beetles are able to detect

carrion that is several kilometres away as early as one day post-mortem (Petruška 1975, Smith & Heese 1995, Hoermann et al. 2013).

Nicrophorus beetles have specialised large club-like antennae with chemosensory receptors which they use to locate odour plumes originating from carrion (Abbot 1927, Boeckh 1962, Heinzel & Böhm 1989, Kalinova et al. 2009). Wilson & Knollenberg (1984) found that carrion preference in burying beetles depends on the size of the carcass and the life stage of the beetles. Younger beetles with immature ovaries aggregate on large carcasses which are in the later stages of decomposition in order to access an abundant food source that would aid ovarian development (Pukowski 1933, Wilson & Knollenberg 1984, Kentner & Streit 1990, Dekeirsschieter et al. 2011). Hoermann et al. (2013) established that newly eclosed females are able to discriminate between odorants associated with various stages of decomposition of carrion. They favourably respond to large carrion in more advanced stages of decay as a likely means of avoiding competition and contests with mature females on carcasses that are suitable for burial and breeding (Hoermann et al. 2013).

Beetles with mature ovaries avoid large carcasses in favour of fresher small vertebrate carrion for reproduction (Pukowski 1933, Wilson & Knollenberg 1984, Kentner & Streit 1990). Kalinova et al. (2009) identified sulphur containing organic volatile compounds (S-VOCs) as the major components of the odour bouquets of mice carrion that attract beetles using electroantennography and behavioural observations. Both sexes of burying beetles (species *N. vespillo* and *N. vespilloides*) seem equally responsive to odour cues (Kalinova et al. 2009). Trumbo & Steiger (2020) recently demonstrated that the specific S-VOCs that may act as attractants towards a carcass can vary depending on the burying beetle species as well as whether the individual discovers the carcass by flying or walking.

Once beetles discover a carcass, they tend to move it to an appropriate spot for burial. While burying the carcass, the beetles will strip it of all hair and feathers, roll it up into a ball and cover it with oral and anal fluids (Pukowski 1933, Milne & Milne 1976, Scott 1998). This series of behaviours is thought to have evolved as a means of managing microbial communities present on the carcass as well preventing competitors from finding the carcass by masking- and disrupting the production of- carrion-associated VOCs (Woodard 2006, Kalinova et al. 2009, Duarte et al. 2018).

Studies of burying beetle responses to carcass VOCs have exclusively focussed on odour bouquets from mammal cadavers such as mice, rodents or pigs. Therefore, we know little about the cues that beetles exhibiting differential responses to avian or mammalian resources (Chapters 2 and 5) might be experiencing and responding to. If wild populations of beetles were to be composed of generalist and specialist (Chapter 2 & 4), then we would expect only the specialised individuals to show strong responses to carrion-specific cues. The first step in identifying such putative adaptations is to understand whether different types of carrion produce distinctive volatiles. This is the aim of the work described in this chapter. We focus on S-VOCs and the amine indole in different carrion as these compounds are well-known attractants of beetles (Kalinova et al. 2009, Hoermann et al. 2013, Tomberlin et al. 2016).

Differential resource use can be driven by assortative mating as well as by oviposition preference (Table 1, Chapter 4). This is especially likely when mating occurs on the resource, as is often the case with burying beetles. Upon finding a carcass that is suitable for reproduction, males use pheromones to attract females (Barlett 1987, Scott 1998, Haberer et al. 2008). Though mating can occur off the carcass as well, the beetles tend to mate at a high frequency while preparing the carcass for reproduction (Pukowski 1933, Müller & Eggert 1989). If variation in the chemical cues used to attract mates is correlated with variation in the type of carrion those adults were reared upon as larvae, this could help reinforce divergent carrion specialisation within a population.

Cuticular compounds act as contact pheromones in *Nicrophorus* beetles and they are an important means by which beetles recognise conspecifics and distinguish between the sexes (Steiger et al. 2007, Steiger et al. 2008). In addition, beetles raising a brood on a buried carcass are able to rely on specific 'breeding status' related cuticular hydrocarbon (CHC) signatures to distinguish between their nestmate and an intruding conspecific (Müller et al. 2007, Steiger et al. 2007, Steiger et al. 2008). Steiger et al. (2007) found that beetles maintained on a diet of insects versus vertebrate carrion differed significantly in their cuticular signatures. Cuticular hydrocarbons have been known to differ based on dietary resources in several insect species and can facilitate differential mating (Liang & Silverman 2000, Buczkowski et al. 2005, Ferveur 2005, Chung & Carroll 2015).

However, it is unclear whether or not the burying beetle's cuticular hydrocarbons vary according to the type of carrion that adults were raised upon. It is possible that they do not, since beetles commonly feed on resources in early adulthood, between eclosion and attaining sexual maturity, that are different from their natal carrion (Wilson & Knollenberg 1984, Hoermann et al. 2013).

Here we test whether the type of carrion resource that *N. vespilloides* beetles were trapped upon in wild populations, was associated with routine differences in their cuticular hydrocarbons.

Since the carrion resources available to burying beetles for feeding and reproduction vary considerably across the entire field season, it is possible that these seasonal variations in resource use are also manifested in their cuticular hydrocarbons (Haberl & Krystufek 2003, Chase et al. 2005, Clapham 2011). We test that idea too.

If beetles do exhibit divergent CHCs as a consequence of breeding on different types of carrion, then two mechanisms could potentially underpin that divergence. One possibility is that CHCs are simply set at each generation and vary only in relation to the resources that burying beetles consume as larvae and/or after eclosion. The other possibility is that specialisation on a particular resource involves adaptations that cause beetles to be better able to advertise their divergent resource use. If that suggestion is true then beetles that come from a lineage that has faithfully used one type of carrion to breed upon should be more likely to bear a distinct and corresponding CHC signature. We used the experimental laboratory populations of *N. vespillo*, established and bred exclusively on one type of carrion for multiple generations (Chapter 5), to distinguish between these two different mechanisms.

This chapter investigates whether any differential resource by burying beetles can be attributed to chemical cues, by addressing the following specific questions:

1. Do different types of carrion produce distinctive volatiles?

2.a. Do *N. vespilloides* that are attracted to different types of carrion also differ predictably in their CHCs?
2.b. Does seasonality affect *N. vespilloides* CHCs?

3. Do *N. vespillo* that have evolved experimentally in the lab by breeding exclusively on one type of carrion differ accordingly in their CHCs?

Materials and methods

Carrion volatile experiment

We compared volatiles released during the decomposition of mammalian and avian carcasses. The carrion used (Table 1) was obtained frozen and shipped on dry ice to Prof. Patrizia d'Ettorre's lab at Université Paris, where it arrived in a frozen state for volatile analysis.

Table 1: Sourcing information for carrion used in volatile sampling

Carrion	Sourced from	Notes
Coturnix coturnix (common quail; chick)	Kiezebrink UK Ltd	Day old chicks, frozen immediately after culling
Gallus gallus domesticus (domestic chicken; chick)	Livefoods Direct Ltd	Day old chicks, frozen immediately after culling
Mus musculus (House mouse; adult)	Livefoods Direct Ltd	Adult mice, frozen immediately after culling
Passer domesticus (House sparrow; adult)	Dr Julia Schroder, Imperial College London	Adults kept in semi-outdoor aviaries, frozen when found dead (within 24-48 h of mortality)

Volatile sample collection

We sampled the volatiles by solid-phase micro-extraction (SPME) using a CAR/PDMS (Carboxen/Polydimethylsiloxane) fibre. All four carcass types were extracted from the freezer, weighed and placed inside thoroughly washed and sterilised 1000 ml borosilicate glass bottles to thaw at ambient temperature (23 ± 2°C). Immediately after placing the frozen carcasses into the bottles, we created a baseline for the volatiles by SPME sampling at 0 h. This was done by placing the SPME holder containing the CAR/PDMS fibre into the air surrounding carcass for 15 min and then immediately analysing the sample using gas chromatography–mass spectrometry (GC-MS). Volatile sampling was then repeated at 4 h while carrying out the pilots for the experiment using only chick and mice carcasses.

Since the 0 h and 4 h reading did not yield any discernible volatile compounds, the carcasses used for the final experiment were samples at 0 h and then every 24 h for 6 days (i.e., at 24 h, 48 h, 72 h, 96 h, 120 h and 144 h). We had 3 replicates each for our 24 h, 48 h and 72 h sampling. Due to logistical constraints, we only had 2 replicates for 96 h, 120 h and 144 h per carcass type.

Before taking any of the samples, the CAR/PDMS fibre was desorbed for 15 min in the GC injection port at 200°C.

Volatile analysis and characterisation

Prof. Patrizia d'Ettorre's lab analysed the samples by GC-MS using an Agilent Technologies 7890A gas chromatograph equipped with a capillary column (30 m × 0.25 mm × 0.25 μm), with helium as carrier gas at 1mL/min. The GC was coupled to an Agilent 5975 C mass spectrometer (70 eV electron impact ionization). The oven temperature was programmed at 40 °C for 1 min, increased to 250 °C at 6°C/min, kept at 250 °C for 5 min, then increased to 320°C at 120°C/min.
The S-VOCs and indole were identified based on mass spectra. Peak analysis was carried out using the Enhanced 02.01.1177 version of the MSD ChemStation software by Agilent Technologies, Inc.

Cuticular hydrocarbon work

Beetle collection

Wild-caught beetles

N. vespilloides beetles used for this experiment were trapped during the field experiments we carried out in 2017 at Thetford Forest (Chapter 2) where we measured beetle preference for chick and mice carrion across the season of beetle activity. After removing the mites from the body of the beetles, we isolated up to two female beetles from each trap individually in a glass vial for 15-20 min before storing them in a -80 °C freezer until they were processed for CHC extraction.

For this experiment, we sampled a total of 63 individuals; 32 females trapped on chicks and 31 females trapped on mice. 40 of these females were collected on 23 May 2017 (20 on chicks and 20 on mice). 6 females were collected on 14 June 2017 (3 on chicks and 3 on mice). 17 of the total females were collected on 4 September 2017 (9 on chicks and 8 on mice).

Beetles that had evolved on different carrion

We used *Nicrophorus vespillo* beetles that had been evolved on chick and mice carrion for seven generations by Darren Rebar, an alumnus of the Kilner group, to test whether beetles that evolved on different carrion differed in their CHCs. Early behavioural experiments (generations 1 to 4) carried out on these beetles demonstrated that as adults they preferred the carrion type on which they had developed as larvae. They also preferred mates that had been raised on the same carcass type as them.

N. vespillo beetles were bred by Darren using the protocol described in Chapter 5 for *N. vespilloides*: after the larvae developed on the carcass for 8 days, they were put into 5x5 grid boxes with some peat until eclosion. The eclosed *N. vespillo* females were placed in individual boxes containing a mixture of sand and peat. They were fed ~ 1g of minced beef every week for two weeks, until they were sexually mature. The adults were then placed in glass vials for 15-20 min before being frozen at -80 °C till it was time for CHC extraction.

We used a total of 30 females whose lineage had evolved on chick carcasses for this experiment: 15 from the C1 replicate population and the other 15 from the C2 replicate population. We used a total of 29 females whose lineage had evolved on mice carcasses for this experiment: 14 from the M1 replicate population and the other 15 from the M2 replicate population.

The methodology we used to extract and analyse CHCs was adapted from well-established protocols used to study burying beetle cuticular compounds (Steiger et al. 2007, Steiger et al. 2008).

Processing beetles to extract CHCs

We took the beetles out of the freezer and allowed them to thaw at room temperature for 30 min. We then soaked them in 4 ml of high-performance liquid chromatography (HPLC) grade hexane (99%) for 20 mins. We transferred the extract obtained to a clean vial and allowed it to evaporate completely in a fume hood under nitrogen gas. We then sealed the vials and shipped them to Prof. Patrizia d'Ettorre's lab at Université Paris.

CHC analysis and characterisation

Prof. d'Ettorre et al. resuspended the extract in 400 µl of pentane (HPLC grade). They added an internal standard (C18, Octadecane) to each extract. The internal standard was used to determine the absolute amount of cuticular compounds present in each sample. They then analysed 2µl of

the extracts using GC-MS using an Agilent Technologies 7890A gas-chromatograph coupled to a 5975C Agilent Mass Spectrometer operated at 70 eV in the electron impact ionization mode. The carrier gas used was helium at 1 ml/min. The column oven was programmed as follows: an initial hold of 1 min at 70°C, then increased to 200°C at 35°C/min, to 320°C at 4°C/min (held for 20 min).

We identified cuticular hydrocarbons on the basis of their retention times (compared to standards) and fragmentation patterns. We manually integrated the chromatograms and converted the peak areas of the total hydrocarbon fraction using the MSD ChemStation software by Agilent Technologies, Inc.

Data visualisation and statistical analysis

To analyse the chemical profile of both sets of beetles, we selected 18 most commonly occurring GC-MS peaks. These represented the hydrocarbons we had identified and integrated using the MSD ChemStation software.

We carried out the clustering analyses and visualisation of the data using *gplots*, *cluster* and *dendextend* packages in R (RStudio version 1.3.959).

We log-normalised the peak areas within each sample using the following formula (Aitchinson 1986):

$$Z_{ij} = \ln\left[\frac{Y_{ij}}{g(Y_j)}\right]$$

where Z_{ij} is the transformed area of peak *i* for beetle *j*; Y_{ij} is the area of the peak *i* for beetle *j*; and $g(Y_j)$ is the geometric mean of the areas of all peaks for beetle *j*.

For the clustering analysis, we used the divisive analysis (DIANA) technique. In this approach, all our samples are assumed to be in a single cluster at the beginning of the analysis (Seber 1984). They are then divided into two clusters with the least similarity and this process is repeated iteratively until each observation is placed in one cluster. This top-down hierarchical clustering approach is considered better for identifying large clusters in the data, such as broad-scale differences in resource use and preference (Seber 1984, Theodoridis & Koutroubas 2008).

Cluster validation of our data indicated one outlier each in each of the *N. vespilloides* (Sample M13E) and *N. vespillo* (Sample C15) datasets. We confirmed this visually using a 2-dimensional scatterplot before removing the outliers. We then repeated our clustering analysis using DIANA. Clusters which are demarcated in the dendrograms and principal component analysis (PCA) plot, are based on the integer vector, with group memberships derived using the 'cutree' function of the *dendextend* package.

We scaled and centred the density plot in the enhanced heatmaps such that the sum of the peak areas of all compounds within a sample was equal to 1. This allowed us to compare the entire sample set on the same scale.

Results

1. Do different types of carrion produce distinctive volatiles?

Our data (Table 1) do not indicate any prominent qualitative differences between carrion volatiles derived from avian and mammalian cadavers. However, we found that the release of volatiles from the dead mice seemed to begin considerably faster than chick decomposition, even though similar compounds were detected in both cases (Figure 1, Table 1).

House sparrows (*Passer domesticus*) seem to be the only clear outgroup in our data. Since these individuals died a natural death and were frozen after some time post-mortem, the most likely explanation for our findings is that they had undergone considerable decomposition pre-sampling.

Table 1: VOCs found in different carrion types

Carrion	24 h	48 h	72 h	96 h	120 h	144 h
Coturnix coturnix (common quail; chick)	No compounds	No compounds	No compounds	Dimethyl trisulphide (DMTS), dimethyl tetrasulphide (DMQS), indole	DMTS, DMQS, indole	DMTS, DMQS; Only in replicate Q3: dimethyl pentasulphide (DMPES)
Gallus gallus domesticus (domestic chicken; chick)	No compounds	No compounds	Indole	DMTS, DMQS, indole	DMTS, DMQS, indole	DMTS, DMQS, DMPES; traces of indole
Mus musculus (House mouse; adult)	No compounds	No compounds	DMTS, DMQS, indole	DMTS, DMQS, indole	DMTS, DMQS; traces of DMPES & indole	DMTS, DMQS, DMPES; traces of indole

Passer domesticus (House sparrow; adult)	S1: Indole; S3 & S2: no compounds	S1 & S2: Indole; S3: no compounds	S1, S2 & S3: traces of indole	No compounds	No compounds	No compounds

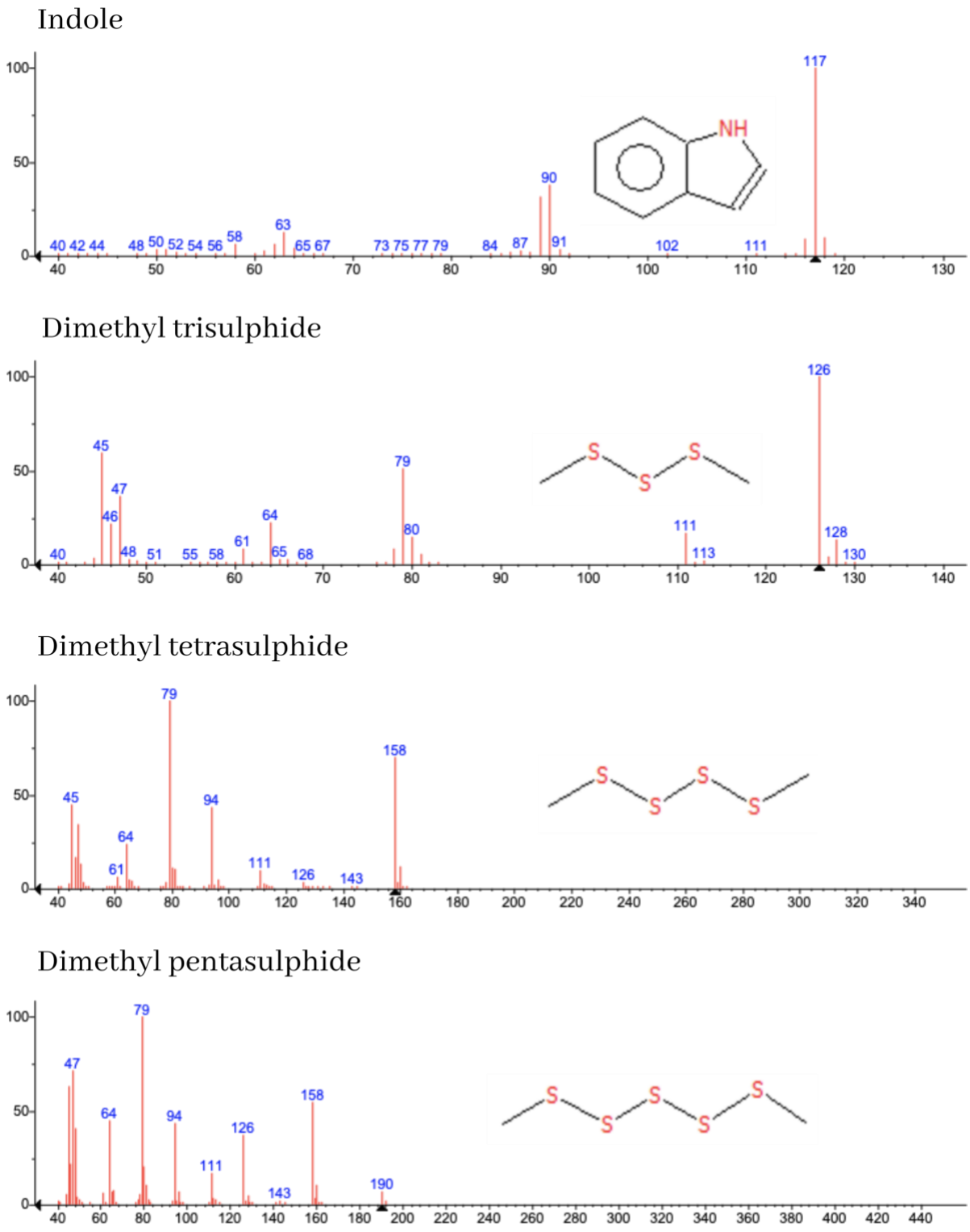


Figure 1: Structure and spectral signatures of the main volatile compounds that we found in all our samples. The x-axis of the chromatogram represents the retention time (in mins) at which the

compounds are volatilised. The y-axis indicates the intensity (or abundance) of the signal. (Source: NIST Mass spectra library Version 2.2, build Jun 10 2014).

2.a. Do *N. vespilloides* beetles that prefer different carrion differ in their CHCs?

Our findings suggest low divergence between the CHC profiles of beetles attracted to different carrion (Figure 2, Table 2).

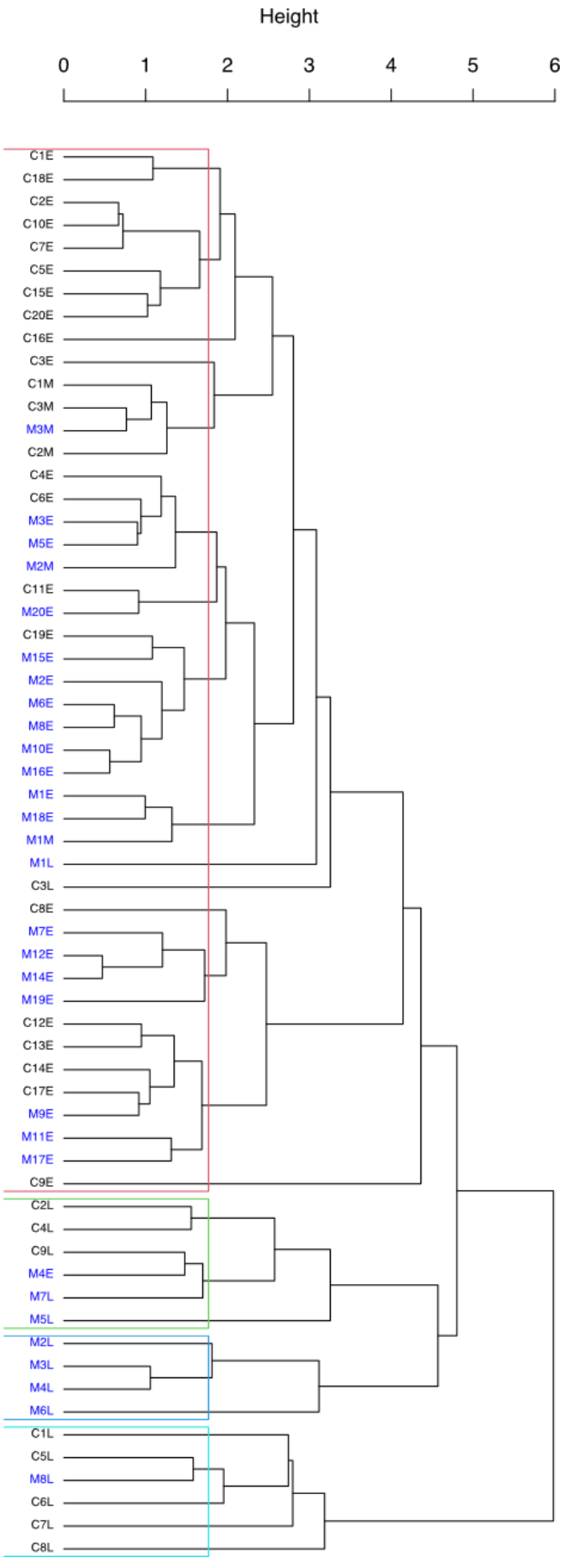
The first cluster in our analysis (Figure 2a) seems to encompass a majority of the beetles, of which an equivalent number were trapped on chicks and mice. These results are similar across the other clusters. Within these clusters, beetles trapped on the same carcass appear to diverge from the same branch. However, there are no consistent patterns of divergence, and we do not find any evidence to suggest that beetles preferring the same carrion type have similar cuticular hydrocarbon profiles. These results persist even when we increase the number of clusters or analyse the early and late season beetle CHCs separately.

Table 2: Carrion preferences of field beetles within clusters differentiated by CHC profile

Total beetles in cluster	Number trapped on mice	Number trapped on chicks
Cluster 1 (46)	22	24
Cluster 2 (6)	3	3
Cluster 3 (4)	4	0
Cluster 4 (6)	1	5

From the heatmap (Figure 2b), we observe that some compounds such as nC25, C25:1, nC23 and 3MeC25, are highly variable among different samples while other compounds, such as 5MeC25, nC22, 3MeC27 and nC27 appear to occur in similar volumes across all samples. The compounds with greater variability between samples are more likely to be contributing to the pattern of clustering we see.

Figure 2(a): Dendrogram indicating hierarchical clustering of the CHC profiles of *N. vespilloides* females. Distance between two different samples is a measure of how similar their CHC composition is, with adjacent samples within a clade being the most similar. Each cluster indicated by our analysis is represented using a line in a different colour. Beetles trapped on chicks are denoted by a 'C' (n= 32) and those trapped on mice are denoted by an 'M' (n= 31). The last letter in the sample name indicates the season of trapping: 'E' for early ('CE' & 'ME'), 'M' for mid ('CM' & 'MM') and 'L' for late ('CL' & 'ML') season.



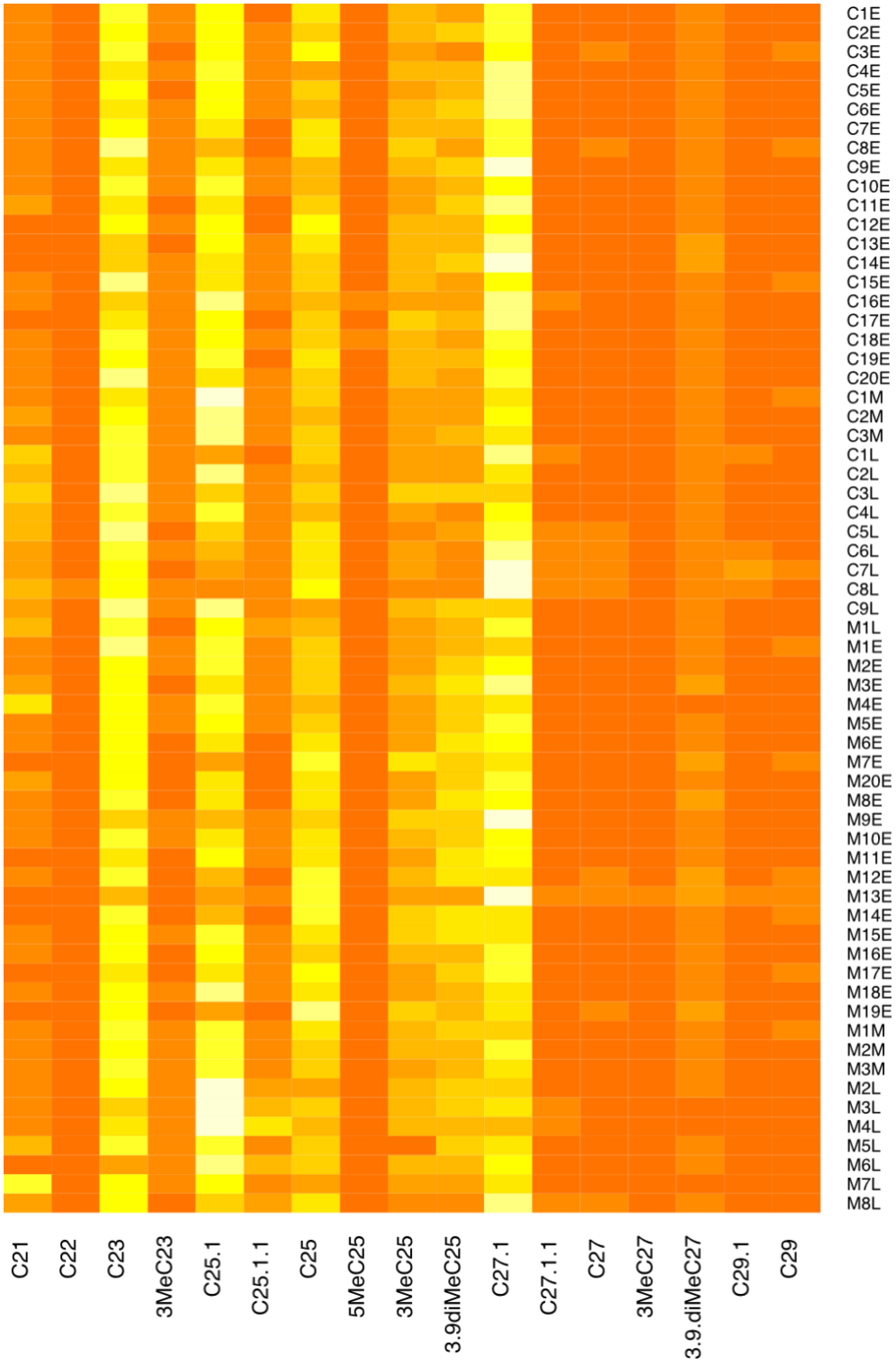


Figure 2(b): Heatmap showing relative quantities of cuticular hydrocarbons present in samples of *N. vespilloides* females trapped on chicks (denoted by 'C', n= 32) and mice (denoted by 'M', n= 31) during the early ('CE' & 'ME'), mid ('CM' & 'MM') and late ('CL' & 'ML') field seasons. The y-axis indicates sample/beetle identity, and the x-axis shows the compound identity. The darker shades (oranges) indicate a greater quantity of the compound compared to the lighter shades (yellows).

2.b. Does seasonality affect *N. vespilloides* CHCs?

Our data seem to suggest divergence in the CHC profiles of beetles trapped at different time points in the field season, that is greater than the divergence between beetles trapped on different types of carrion (Figure 3, Table 3).

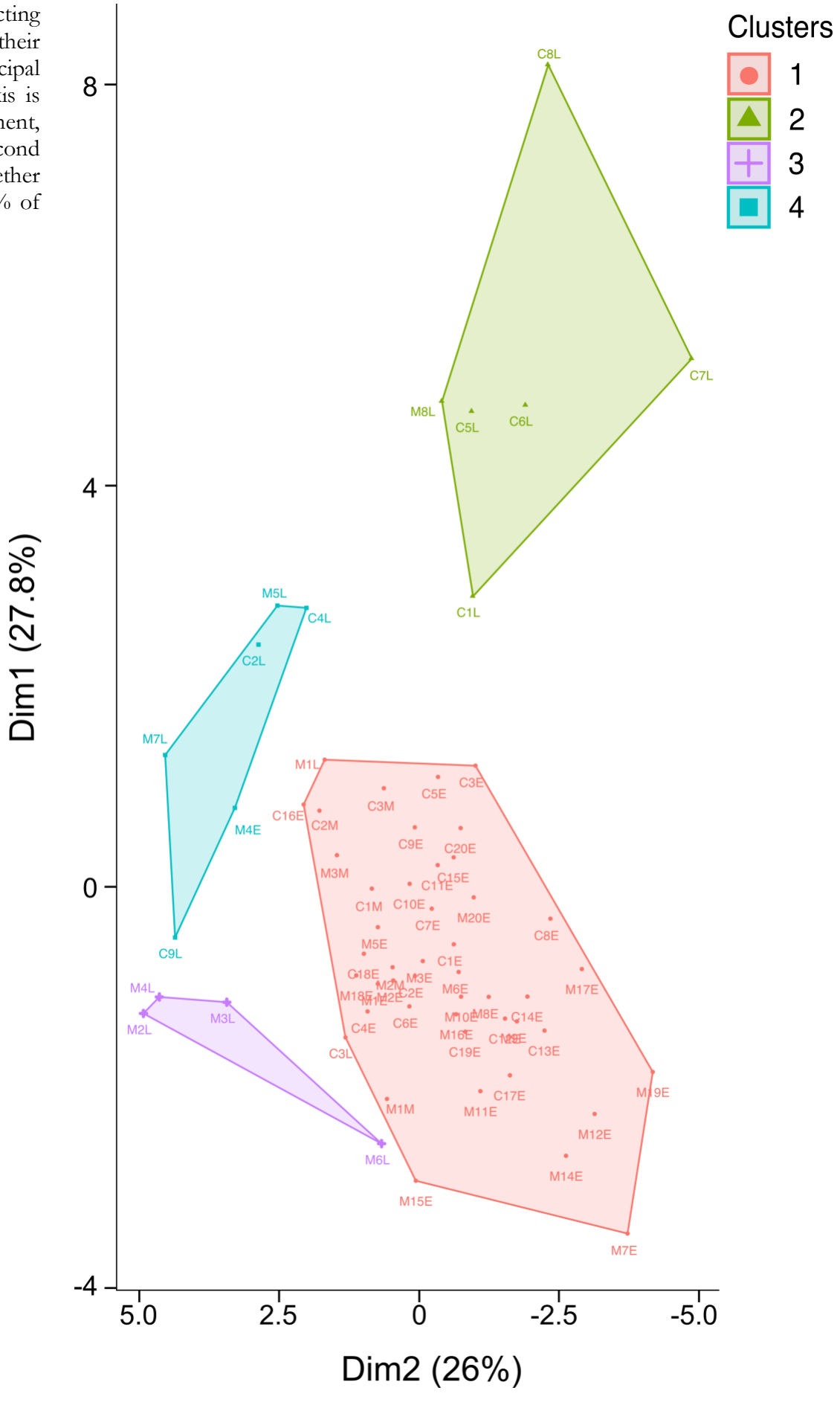
Table 3: Activity season of field beetles within clusters differentiated by CHC profile

Total beetles in cluster	Number trapped during early season	Number trapped mid-season	Number trapped during late season
Cluster 1 (46)	38	6	2
Cluster 2 (6)	0	0	6
Cluster 3 (4)	0	0	4
Cluster 4 (6)	1	0	5

From the four major clusters in our data, the first one is the largest- composed of 46 beetles. All 6 of the mid-season beetles are within this cluster, along with 38 early-season beetles and 2 late-season beetles. Only one early season beetle lies outside of the first cluster. The clusters 2, 3 and 4 are much smaller clusters and they are mainly composed of late season beetles.

Our findings indicate greatest divergence between the CHC profiles of early and late season beetles (Figures 2b and 3). However, the clustering we observe (Figure 3) explains only 53.8% of the variation in our CHC data.

Figure 3: Scatter plot depicting field beetles clustered by their CHC profile along two principal component axes. The y-axis is the first principal component, and the x-axis is the second principal component. Together the two axes explain 53.8% of the variation in the data



3. Do *N. vespillo* beetles from lineages that have evolved on different carrion differ in their CHCs?

Our findings suggest low levels of divergence between the CHC profiles of beetles from lineages evolving on different types of carrion (Figure 4, Table 4).

Table 4: Development resource of *N. vespillo* within clusters differentiated by CHC profile

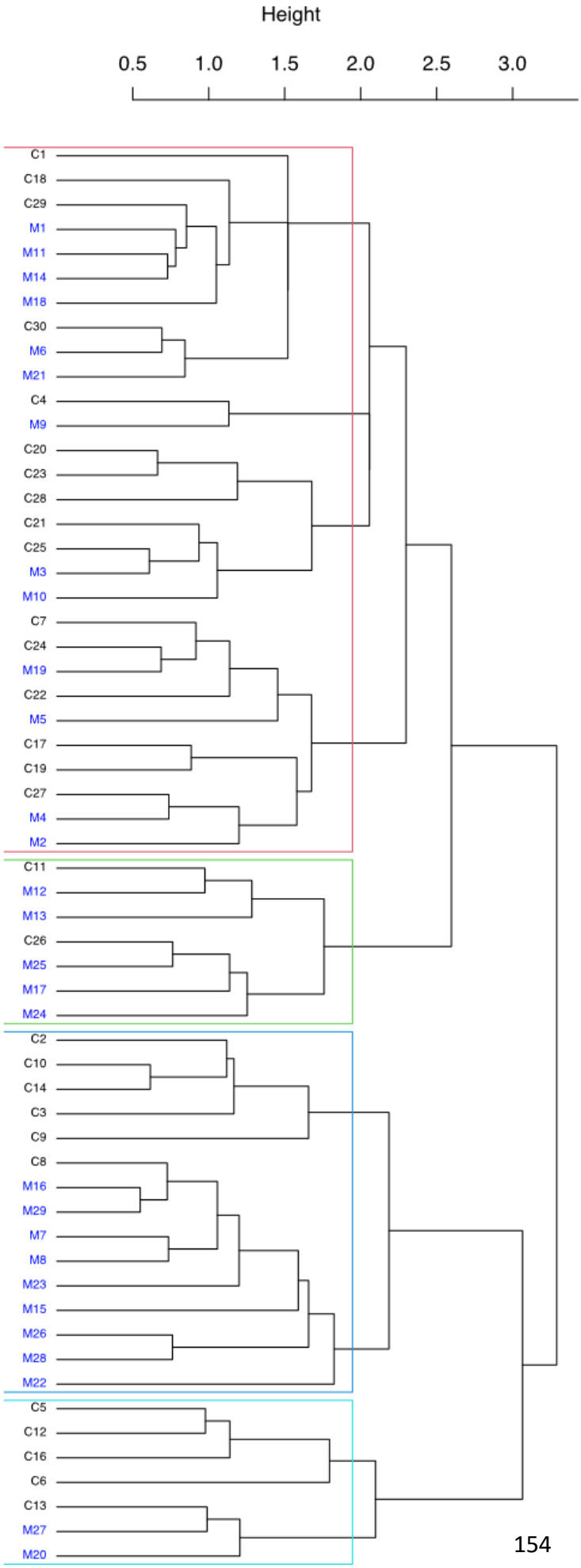
Total beetles in cluster	Number evolved on mice	Number evolved on chicks
Cluster 1 (29)	13	16
Cluster 2 (7)	5	2
Cluster 3 (15)	9	6
Cluster 4 (6)	2	4

The first cluster has 29 *N. vespillo* beetles, with 13 mouse-bred and 16 chick-bred ones (Figure 4a, Table 4). The other three clusters are smaller, with 7 (5 mouse-bred, 2 chick-bred) beetles in the second cluster, 15 (9 mouse-bred, 6 chick-bride) in the third and 6 beetles (2 mouse-bred, 4 chick-bred) in the fourth. Within these clusters, beetles evolving on the same carcass appear to diverge from the same branch. However, this is not consistent, and we do not find any evidence to suggest that beetles evolving on the same carrion type have similar cuticular hydrocarbon profiles.

From the heatmap (Figure 4b), we can see that certain cuticular compounds, such as nC25, C25:1, C27:1 and 3MeC25, vary more between different samples and are, therefore, contributing more to

the clustering of the data than other CHCs. A few compounds seem to be occurring in similar volumes in all our samples (such as 5MeC25, nC24 and 3MeC23).

Figure 4(a): Dendrogram indicating hierarchical clustering of the CHC profiles of *N. vespillo* females. Distance between two different samples is a measure of how similar their CHC composition is, with adjacent samples within a clade being the most similar. Each cluster indicated by our analysis is represented using a line in a different colour. Beetles bred on chicks are denoted by a 'C' (n= 30) and those bred on mice are denoted by an 'M' (n= 29).



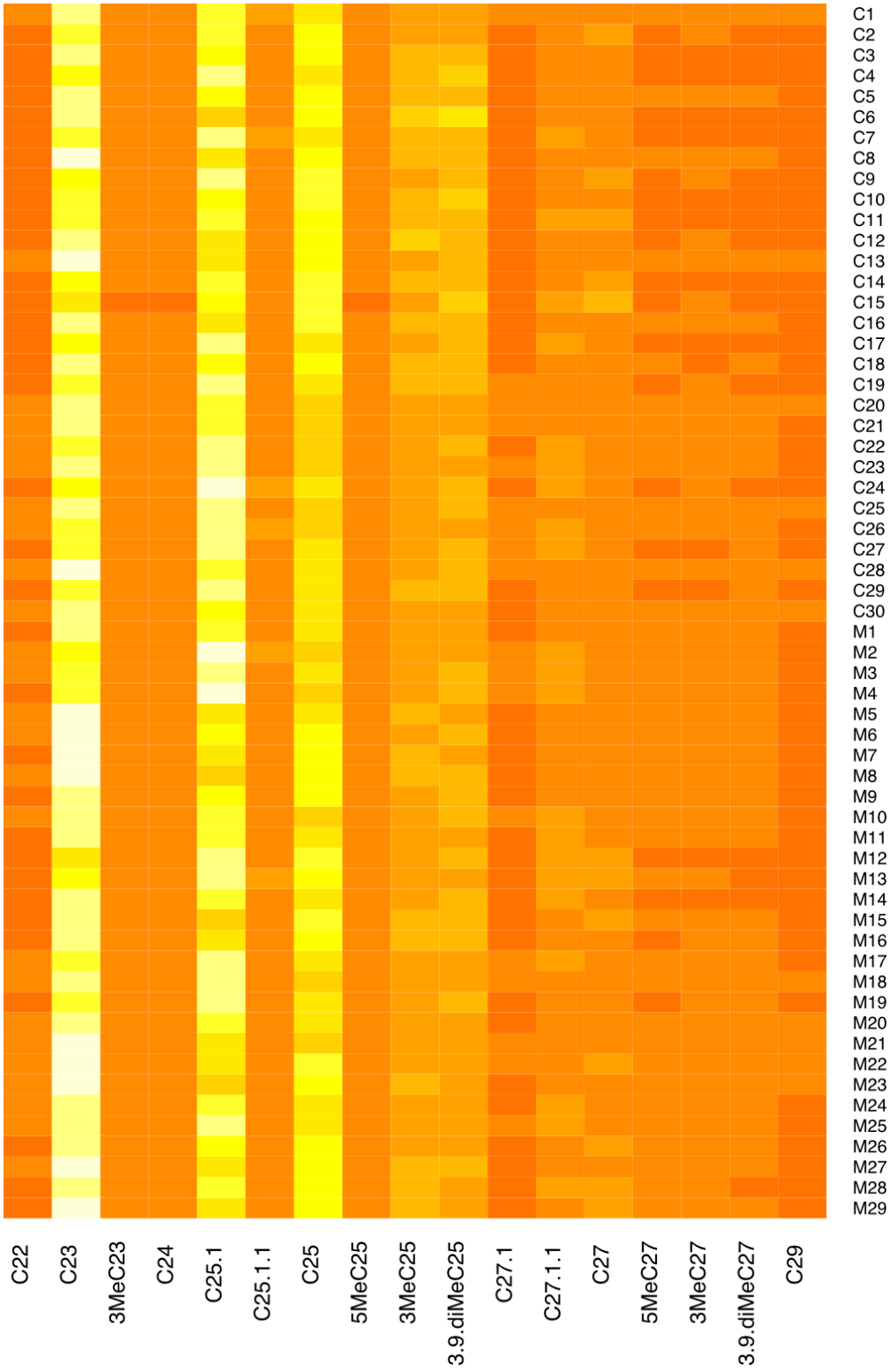


Figure 4(b): Heatmap showing relative quantities of cuticular hydrocarbons present in samples of *N. vespillo* females bred on chick (C1 and C2; n= 30) carrion and mice (M1 and M2; n= 29) carrion for 7 consecutive generations. The y-axis indicates sample/beetle identity, and the x-axis shows retention times, which are a proxy for compound identity. The darker shades (oranges) indicate a greater quantity of the compound compared to the lighter shades (yellows).

Discussion

Volatiles produced by different carrion types

We found that, with the exception of the house sparrow, the decomposition of all the carrion types we sampled seemed to follow a similar pattern. It began with the release of dimethyl trisulphide (DMTS), dimethyl tetrasulphide (DMQS) and some traces of indole. These compounds gradually increased over time. In the later stages of decomposition, we observed the release of DMPES. For reasons that are unclear, we didn't detect dimethyl sulfide (DMS) or dimethyl disulfide (DMDS) in any of the carrion, though this has been reported in other studies (Woodard 2006, Kalinova et al. 2009).

The mouse carcasses we used began releasing S-VOCs and indole much faster than other carrion. This could be because the adult mice are likely to harbour more microbes in their guts and on their bodies. The quail chicks and domestic chicken we used for our experiment are culled one day post-hatching and it is therefore likely that their microbiota are less abundant.

From previous research, it has been established that beetles are able to distinguish carrion size and degree of decomposition using olfactory cues (Wilson & Knollenberg 1984, Dekeirsschieter et al. 2011, Hoermann et al. 2013). Though our results have been inconclusive, this work has been a useful starting point for us in understanding the chemical basis of differential carrion use in *Nicrophorus* beetles. Several studies have identified hundreds of other VOCs that distinguish avian and mammalian cadavers such as aldehydic compounds, ketones and N-containing organic molecules (Forbes & Carter 2015, Poldy 2020). Further work is needed to get a complete picture of how these compounds might play a role in the beetles' ability to locate and identify carcass-specific cues.

CHCs of wild *N. vespilloides* that are attracted to different carrion

Our results did not suggest any differences between the CHC profiles of beetles that were trapped on different types of carrion. Since diet-attributed differences in CHCs occur due to the incorporation of dietary hydrocarbons into cuticular lipids, it is likely that the hydrocarbons beetles derive from birds and mammals may not be different enough to result in a signature of diet on the cuticle (Liang & Silverman 2000, Blomquist 2010, Otte et al. 2014). On the other hand, it may be

that individuals in the field are not sufficiently consistent in their use of carrion for there to be a carcass-use related signature in their CHCs.

However, the CHC profiles of different beetles seem to cluster well according to the time of year when they were trapped. We found greater variation in the CHCs of late season beetles compared to the early season ones. This mirrors the greater variation in reproductive outcomes that we observed later in the season compared to early season beetles (Chapter 3). Populations in late summer are likely to be a mix of adults that have reproduced in late spring or early summer as well as newly eclosed individuals (Pukowski 1933, Urbański & Baraniak 2015). Since the cuticular profiles of the beetle vary according to their reproductive state (Steiger et al. 2007, Scott et al. 2008), it is likely that our results are due to seasonal differences in individual quality, age and breeding status (Chapter 3).

CHCs of beetles that have evolved on different carrion

We did not find any evidence to suggest that beetles evolving on different carrion types differed in their CHCs. Steiger et al. (2007) reported significant differences in the cuticular compounds of *N. vespilloides* that were fed different diets from eclosion until reproductive maturity. The *N. vespillo* evolving on both chicks and mice were maintained on a diet of beef mince as adults before we collected CHCs. This could explain why their cuticular compounds were so similar. Our data suggests that adults do not retain signatures of the carrion type they developed upon as larvae in their cuticular hydrocarbons. However, this could still happen in natural populations if beetles that developed as larvae on different types of carrion then consumed different diets after eclosion and before reaching sexual maturity. The data from wild *N. vespilloides* (above) suggest that explanation is unlikely though, since we found no evidence that beetles attracted to different types of carrion could be distinguished by their CHCs.

While analysing our data, we followed the standard approach of focussing on the most abundant compounds in our samples and excluded peaks that did not occur in all our samples (Liebig et al. 2000, Steiger et al. 2008). For future analyses, we plan to follow a slightly different approach of including all of the peaks we find in our data to be certain that we have not overlooked subtle but significant differences between populations (Steiger et al. 2007)

In summary, we found no evidence to suggest that the carcasses of birds and mammals can be distinguished from the volatiles they produce. However, this maybe because the birds we analysed were depleted in their microbial populations. For future experiments, we will be using carrion derived from mortality in more natural conditions, rather than factory farmed animals, to better understand whether different types of carrion produce different sorts of volatiles that could be detectable by natural populations of burying beetles.

Further, we did not find any evidence for CHC signatures that predict the type of carrion that wild *N. vespilloides* will be attracted to. There is some evidence that CHCs vary seasonally in wild beetles but this is likely to be a function of age and reproductive status. Since pheromones are another important mating signal in insects, future studies could assess whether these long-range compounds exhibit resource-associated differentiation in burying beetles (Haberer et al. 2008, Steiger 2015, Steiger & Stökl 2017).

Chapter 7

Discussion

This thesis set out to investigate the following key questions, with resource partitioning in natural populations driven by competition as its central focus:

1. Is there evidence of divergence in resource use between and within natural populations of burying beetles?
2. Is this divergence adaptive?
3. What mechanisms maintain divergence?

Is there evidence of divergence in resource use between and within natural populations of burying beetles?

To address the first question, we tested for evidence of a bias in the carrion type favoured by *N. vespilloides* within and among three different woodland populations (Chapter 2). Using trapping data, we tested if this bias in preference for avian and mammalian carcasses varied across the burying beetle season (annually from April to October). Our work confirmed that *N. vespilloides* did indeed diverge in their use of resources both over time in the same population as well as between different populations, with similar patterns observed across populations. While there was very high spatial and seasonal variation in the abundance of beetles trapped on chick and mice carcasses, this did not seem to match the relative abundances in the field that we expected due to seasonal variation in the availability of avian and mammalian carcasses in nature described in the literature (Merritt et al. 2001, Chase et al. 2005; Clapham 2011; Capstick 2017). It is still possible that seasonal variation in the availability and abundance of different carrion types in each woodland caused the patterns of resource use that we observed within burying beetle populations.

Beetles from two of the woodlands we sampled (Thetford Forest and Gamlingay Wood) had an overall greater preference for mice, whereas beetles from the third population (Waresley Wood) showed an overall greater preference for chicks. Gamlingay Wood and Waresley Wood are adjacent to each other. Despite their spatial proximity, we found distinct and starkly contrasting patterns of resource use in these two woodlands. While we observed an overall greater preference

for mice in Gamlingay Wood, more beetles were trapped on chicks than on mice in late-June and late-September. In Waresley Wood, more *N. vespilloides* were caught on chicks than on mice overall, but greater numbers were trapped on mice only in mid-June and late-September.

Previous work has demonstrated that burying beetle populations in Gamlingay and Waresley woods have divergently adapted to breed on carrion of different sizes (Sun et al. 2020). Specifically, Gaminglay *N. vespilloides* are specialised to breed on small carrion because they face competition from larger sympatric *Nicrophorus* species, which favour intermediate-size carrion and are more effective at competing for it (Sun et al. 2020). By contrast, Waresley *N. vespilloides* are generalists with respect to carrion size, and more effective at reproducing on larger carrion as well as smaller carcasses – presumably because there are fewer sympatric *Nicrophorus* species to be found in that woodland. Perhaps this means that Waresley *N. vespilloides* are generalists in other senses too, including carrion type as well as carrion size. Perhaps Gamlingay *N. vespilloides* are so attuned to locating small carrion quickly that this has made them favour mice over birds for reproduction.

The high temporal variation in our data is hard to interpret without the ability to track individual beetles. It may be that resources fluctuate in their availability over time, and individual beetles change their behaviour accordingly to track those changes. Or, more likely from the results in Chapter 3 and 6, there is seasonal variation in the quality and reproductive status individual *N. vespilloides* and this is correlated with the type of carrion they can most quickly locate. Exactly how *N. vespilloides* might distinguish between different types of carrion at a distance is unclear, though. We found no evidence that mouse and bird carrion emit different volatiles (Chapter 6) although our results are admittedly somewhat preliminary.

<u>Is this divergence adaptive?</u>

We predicted that the patterns of resource we observed in our field experiments resulted from the adaptive partitioning of resource type within populations. We used a two-pronged approach to address test this prediction: the first using "common garden" breeding experiments in the lab to test whether wild *N. vespilloides* were specialised to breed on the type of carrion they were trapped upon in the field (Chapter 3); and second using an experimental evolution approach in the lab to test whether, in principle, beetles within a natural population could become divergently and locally adapted to specialise on different types of carrion (Chapter 5).

For our "common garden" experiment (Chapter 3), we compared the reproductive performance of *N. vespilloides* collected at Theford Forest in June and August 2017. In June, beetles were more likely to be trapped upon mice-baited traps and, consequently, we expected them to have higher reproductive success on mice carrion. Consistent with our predictions, we found that beetles trapped on dead mice had significantly greater brood sizes on mice compared to all other treatments. In August, *N. vespilloides* was trapped on mice and chick carrion with equal frequencies in the field. When we compared the reproductive success of these beetles on the carrion they were trapped upon, we found that their reproductive performance on chick and mice carrion was similar across all measured parameters of reproductive success (brood size, larval mass, larval density and carcass use efficiency). However, the beetles from June had overall greater reproductive success (larger broods and higher larval density) on both mice and chick carrion compared with those trapped in August. This indicated that our results are not simply due to adaptive resource use and are more likely to have another ecological or life-history related explanation. They might be better explained by phenological variation in individual quality, which could be correlated with differential expression of genes associated with olfaction (Chapter 4).

Results from our replicated experimentally evolving populations of *N. vespilloides*, which were bred on either mice or chicks for ~ 20 generations (Chapter 5), did not provide any evidence to support the possibility that beetles could divergently adapt to the carrion type they had evolved on, to become specialists. In general, we found that chick carcasses were a poorer quality resource for beetles. Compared to those raised on mice, beetles raised on chicks seemed to have smaller brood sizes, lower larval mass, poorer carrion use efficiency as well as lower larval density, though this was not immediately evident from our experiments on wild beetles in Chapter 3. The reproductive performance of beetles did not seem to improve on either carrion type over the course of our selection experiment. Neither did we find any evidence to suggest that beetle preference for a carcass increased after several generations of experimental evolution- the carrion type that beetles chose to prepare for reproduction seemed to be selected at random and was unrelated to the type of carrion they were raised upon as larvae. We have discussed possibilities such as low standing genetic variation in our founding populations and the specific design of our selection experiment that might have contributed to our results (see 'Discussion' Chapter 5). However, at the end of our selection experiment, when we bred chick-evolved beetles from one of our replicate populations on mice carrion, they seemed to have significantly larger broods compared to mice-evolved beetles. This result leads us to speculate whether the chick lineage may have, in fact,

adapted to breed more effectively on its natal carrion in more cryptic ways. This could be investigated in the future, using cross-fostering experiments.

Burying beetles are multivoltine insects that are likely to produce multiple broods in a year (Pukowski 1933, Scott 1998). One drawback of the methodology that we used to measure reproductive success on a carcass was that it only provided us with a single snapshot of breeding performance on the carcass, which might not be an accurate representation of the true fitness of an individual. Therefore, it may be useful to consider other approaches to adequately capture lifetime fitness on different carrion types for future work. Additionally, it is possible that divergent and adaptive changes might have occurred in our evolving population in traits such as egg size and mate preference which we may have missed (Rollinson & Hutchings 2013, Schrader et al. 2015, Matsubayashi et al. 2010, Forister et al. 2012).

What mechanisms maintain divergence?

A heritable mechanism that transmits divergent selection on resource use to a trait which causes reproductive isolation is a necessary component of adaptive host-associated differentiation (Schluter 2000, Kirkpatrick & Ravigné 2002, Verzijden et al. 2012). Through our collaborative work with Dr Michael Sheehan at Cornell University (Chapter 4), we investigated whether there was any evidence of genetic differences between beetles that differed in their preference for carrion in the three woodland population that we sampled. On comparing the genomes of female *N. vespilloides* trapped on dead chicks and mice carrion, we found divergence at ~ 50 loci, several of which were associated with olfaction and sensory system development. For instance, the gene *spineless*, which is involved in olfactory system development, was differentiated between chick- and mouse- trapped beetles in both early and late season Thetford beetles as well as Waresley Wood. As was the case with our trapping data (Chapter 2), we found some spatial variation in loci that diverged between beetles trapped on different carrion, though the Gamlingay population seemed to show the least amount of genetic divergence in this respect. We also found temporal variation in loci that were divergent when comparing beetles trapped in the early- and late- season within Thetford Forest. This result could be attributed to temporal variation in the competition for carrion resources across the season which might influence the strength of divergent selection experienced by the population (Anderson 1982, Scott 1998). If this is true, then it suggests that the temporal variation we report in Chapter 2 might be partly due to differences in individual quality (suggested by Chapter 3) and partly due to genetic divergence within populations. Our

results were a first step in identifying putative specific genetic mechanisms that could be linked to divergent and specialised resource use in nature (Nosil 2012). Nevertheless, on comparing F_{ST} values with other species where host-associated differentiation is indicated (Table 1, Chapter 4) we conclude that there may be lesser resource specialisation within the *N. vespilloides* populations that we sampled than has been observed in other insect species that are known to be divergently specialised on different resources. Perhaps we have identified a more subtle genetic demarcation in resource use within *N. vespilloides* populations, with some individuals tending to specialise and others tending to be generalists but none sticking to hard and fast rules. The balance of these tendencies might vary between populations, according to local ecological conditions such as the extent of competition for carrion (Sun et al. 2020).

In addition, these genetic boundaries associated with carrion use could be further blurred by the beetle's inability to identify different types of carrion accurately at a distance. In Chapter 6, we looked at how burying beetles might distinguish between different types of carrion with an exploratory study using mass spectrometry to characterise volatiles from avian and mammalian carcasses. This work did not yield any evidence to suggest substantial differences in the volatiles emitted by bird and mice carrion. Furthermore, we were unable to find any evidence that beetles bear a signature of carrion use in their cuticular hydorcarbons, and so are unlikely to be to use this short-range cue to mate preferentially with beetles that have bred on the same carrion type as them. When compared the cuticular hydrocarbon (CHC) profiles of *N. vespilloides*, we could find no evidence that a beetle's CHCs predicted the carrion type it was trapped upon in nature. We did, however, find seasonal variation in the cuticular hydrocarbon profiles of wild-caught beetles, which could be related to differences in beetle quality or breeding status (Steiger et al. 2007, Scott et al. 2008) – consistent with the results reported in Chapter 3. In addition, on comparing CHCs of *N. vespillo* beetles that had been evolved on chick- and mouse- carcasses, we found no evidence that adult beetles bore a signature of the carrion type they had developed upon as larvae suggesting that even long-term exposure to the same type of carrion within a lineage did not cause correlated changes in the CHC signature.

Summarising our main results, we found some evidence for differential carrion use within and among natural populations of burying beetles. This differential use was associated with some genetic differences among individuals within populations. However, as is expected in wild populations, a large component of this interindividual variation could be attributed to seasonal effects and phenotypic variation in individual quality.

We can put these conclusions in a broader context, by considering their ecological and evolutionary consequences on the population as a whole. At the genus level, burying beetles are obligate carrion breeders that collectively utilise a wide variety carrion types for their reproduction (Anderson 1982, Anderson & Peck 1985, Scott 1998, Hocking et al. 2007). Therefore, high interspecific and intrapopulation competition is an intrinsic part of burying beetle ecology (Wilson & Fudge 1984, Trumbo 1994). In burying beetle populations, both genetic polymorphisms as well as phenotypic plasticity could potentially increase the niche breadth of the population and reduce intraspecific competition (Agrawal 2001, Forsman et al. 2008, Hughes et al. 2008, Wennersten & Forsman 2012). The picture that is emerging from our results is that the differential resource use we observe within and among populations (Chapter 2) is most likely a combination of both these effects. Differential resource use in the populations we studied does seem to be have a genetic basis (Chapter 4). However, considering the fluctuating selection pressures that seasonal environments can impose on populations, a large component of differential resource use may be due to phenotypic plasticity (Hallsson & Björklund 2012). Any remaining variation might be due to chance – stochastic variation in resource availability and errors arising from the difficulty of locating particular resource types at a distance (Chapter 6).

Understanding the role that phenotypic plasticity is playing in differential resource use within burying beetle populations is a challenging yet essential part of the puzzle (Chapter 1; Agrawal 2001, Pigliucci 2001, Svanback et al. 2009, Hendry et al. 2011). Studying the evolutionary consequences of phenotypic plasticity- whether it contributes to the release of cryptic genetic variation and the assortment of genotypes or masks existing genetic variation -has emerged as a key future direction from this thesis. Svanback et al. (2009) found that unstable environments are more likely to lead to the evolution and persistence of phenotypic plasticity within populations rather than genetic divergence and speciation. Since *N. vespilloides* populations seem to be experiencing fluctuating selection pressures arising from seasonality, we conclude that the probability of carrion specialists evolving within populations is relatively low and none of our results seem to provide strong evidence in support of their existence. Burying beetle community data indicates (Figure 8, Chapter 2; Anderson 1982, Scott 1998) that *N. vespilloides* populations are likely to experience a high degree of seasonal variation in inter- and intra- specific competition. This means that the strength of divergent selection experienced by the population will fluctuate considerably throughout the season. Seasonality has emerged as the most persistent trend in our

data and our results demonstrate the of suitability of burying beetles as a model system for future studies on the evolutionary impacts of seasonally fluctuating selection (Williams et al. 2017).

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Appendix

Table A.2.1 Geographical coordinates of trapping locations at all three sampling sites

Site	Trap location	Geographical coordinates	
		Latitude	Longitude
Gamlingay Wood (2017)	1	52 09.867'N	0 11.339'W
	2	52 09.749'N	0 11.266'W
	3	52 09.802'N	0 11.426'W
	4	52 09.704'N	0 11.354'W
	5	52 09.749'N	0 11.491'W
Thetford (2017 and 2019)	1	52.3443120	0.5374620
	2	52.3428090	0.5382080
	3	52.3446849	0.5406787
	4	52.3435720	0.5398280
	5	52.3443100	0.5397050
	6	52.3451601	0.5406294
	7	52.3434220	0.5414500
	8	52.3449940	0.5430960
	9	52.3440520	0.5441710
	10	52.3471700	0.5435390
Waresley Wood (2017)	1	52 10.655'N	0 09.508'W
	2	52 10.603'N	0 09.377'W
	3	52 10.586'N	0 09.261'W
	4	52 10.512'N	0 09.427'W
	5	52 10.572'N	0 09.523'W

Table A.2.2 Beetle collection trips at all three sampling sites

Site	Trip #	Date	Trap locations samples
Gamlingay Wood (2017)	1	15/06/2017	1, 2, 3, 4 and 5
	2	29/06/2017	1, 2, 3, 4 and 5
	3	13/07/2017	1, 2, 3, 4 and 5
	4	27/07/2017	1, 2, 3, 4 and 5
	5	10/08/2017	1, 2, 3, 4 and 5
	6	24/08/2017	1, 2, 3, 4 and 5
	7	07/09/2017	1, 2, 3, 4 and 5
	8	21/09/2017	1, 2, 3, 4 and 5
	9	19/10/2017	1, 2, 3, 4 and 5
Thetford Forest (2017)	1	23/05/2017	1, 2 and 3
	2	29/05/2017	1, 2 and 3
	3	04/06/2017	1, 2 and 3
	4	14/06/2017	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	5	28/06/2017	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	6	12/07/2017	1, 2, 3, 7 and 10
	7	21/07/2017	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	8	28/07/2017	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	9	04/08/2017	1, 2, 3, 4 and 5
	10	19/08/2017	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	11	04/09/2017	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	12	13/09/2017	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	13	29/09/2017	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	14	11/10/2017	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
Thetford Forest (2019)	1	16/06/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	2	29/06/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	3	19/07/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	4	23/07/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	5	05/08/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	6	21/08/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	7	27/08/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	8	01/09/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	9	08/09/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	10	20/09/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
	11	09/10/2019	1, 2, 3, 4, 5, 6, 4, 5, 6, 7, 8, 9, 10
Waresley Wood (2017)	1	15/06/2017	1, 2, 3, 4 and 5
	2	29/06/2017	1, 2, 3, 4 and 5
	3	13/07/2017	1, 2, 3, 4 and 5
	4	27/07/2017	1, 2, 3, 4 and 5
	5	10/08/2017	1, 2, 3, 4 and 5
	6	24/08/2017	1, 2, 3, 4 and 5
	7	07/09/2017	1, 2, 3, 4 and 5
	8	21/09/2017	1, 2, 3, 4 and 5
	9	19/10/2017	1, 2, 3, 4 and 5

Table A.4.1 Results from whole genomic sequencing of beetles caught on chick- and mouse-baited traps in Thetford Forest (early and late season), Waresley Wood and Gamlingay Wood. The first column gives information about a.) the site at which the populations are differentiated and the genes that are likely involved; and b.) the number of populations (out of c., d., e. and f.) with an F_{ST} value greater than 0.02 between beetles attracted to chicks and mice for a 5kb window. The populations that are significantly differentiated at a particular site are highlighted in yellow.

a. Site of differentiation /chromosome Genes within (or near window of high F_{ST})	b. Number of populations with F_{ST} > 0.02	c. Early season Thetford on chicks - Thetford on mice	d. Late season Thetford on chicks - Thetford on mice	e. Waresley on chicks - Waresley on mice	f. Gamlingay on chicks - Gamlingay on mice
NW_017095504.1 ncRNA (LOC108556996); transcription factor btd-like (LOC108556993)	2	0.0248	0.0222	-0.0053	0.0010
NW_017095694.1 Upstream of metabotropic glutamate receptor 7 (LOC108560036)	2	0.0232	0.0214	-0.0062	-0.0057
NW_017095698.1 S-adenosylmethionine synthase (LOC108560216)	2	-0.0556	0.0337	0.0250	0.0031
NW_017095703.1 End of scaffold	2	0.0236	-0.0117	0.0204	-0.0060
NW_017095732.1 Cerebellar degeneration-related protein 2-like (LOC108561450); transmembrane protein 65 (LOC108561436)	2	0.0066	0.0236	0.0222	-0.0135
NW_017095764.1 Closest gene (~30kb away) is zinc finger protein Noc (LOC108562666)	2	-0.0068	0.0204	0.0318	0.0018
NW_017095787.1 End of scaffold	2	0.0492	0.0177	-0.0035	0.0413
NW_017095801.1 Rho guanine nucleotide exchange factor 10-like protein (LOC108564560)	2	-0.0026	0.0318	-0.0037	0.0278
NW_017095802.1 No genes on scaffold	2	0.0217	-0.0040	-0.0155	0.0422
NW_017096003.1 No genes on scaffold	2	0.0000	0.0272	-0.0027	0.0222
NW_017096072.1 No genes on scaffold	2	-0.0008	0.0354	0.0008	0.0327
NW_017096093.1 GDP-fucose protein O-fucosyltransferase 2 (LOC108569501); biogenesis of lysosome-related organelles complex 1 subunit 6 (LOC108569502); 26S proteasome non-ATPase regulatory subunit 9 (LOC108569510); dual	2	-0.0092	0.0209	0.0229	-0.0073

specificity protein phosphatase CDC14B-like (LOC108569493); vacuolar protein sorting-associated protein 35 (LOC108569522)					
NW_017096093.1 Neogenin (LOC108569519) may be frazzled in Drosophila, involved in neural development	2	0.0021	0.0450	-0.0041	0.0230
NW_017096128.1 Glutamate-gated chloride channel-like (LOC108569710)	2	0.0059	0.0201	0.0386	-0.0300
NW_017096133.1 Myotubularin-related protein 10-B-like (LOC108569714); myotubularin-related protein 10-B-like (LOC108569719); dolichol kinase (LOC108569721)	2	0.0532	-0.0189	0.0392	-0.0345
NW_017096151.1 No genes on scaffold	2	-0.0096	0.0595	-0.0382	0.0210
NW_017096324.1 No genes on scaffold	2	0.0651	0.0567	0.0112	0.0129
NW_017096372.1 Not close to genes but one of closest is potassium channel subfamily K member 10-like (LOC108556854) is ~40kb away	2	0.0942	-0.0070	-0.0316	0.0239
NW_017096637.1 cAMP-specific 3',5'-cyclic phosphodiesterase (LOC108557663). Annotated as dunce in drosophila, important in learning including olfactory	2	0.0298	-0.0156	0.0281	-0.0056
NW_017096684.1 Glutamate receptor ionotropic, NMDA 2B-like (LOC108557899)	2	0.0327	-0.0143	0.0092	0.0282
NW_017096746.1 Uncharacterized LOC108558040	2	0.0096	-0.0126	0.0266	0.0275
NW_017096990.1 No close genes, nearest is C-type lectin 37Da-like (LOC108558663)	2	-0.0193	0.0285	-0.0182	0.0761
NW_017097091.1 Semaphorin 2a (LOC108558881) includes end of scaffold	2	-0.0074	0.0364	-0.0004	0.0344
NW_017097164.1 No genes on scaffold	3	0.0141	0.0241	0.0213	0.0242
NW_017097262.1 Serine proteinase stubble (LOC108559606)	2	-0.0011	0.0785	-0.0143	0.0484
NW_017097305.1 No genes on scaffold with only tRNA genes	2	0.0293	-0.0229	0.0298	-0.0443
NW_017097361.1	2	0.0273	-0.0180	-0.0011	0.0232

Only gene on scaffold is uncharacterized LOC108559911, ~20 kb away					
NW_017097509.1 C-type lectin 37Db-like (LOC108560296); C-type lectin 37Da-like (LOC108560297); C-type lectin 37Da-like (LOC108560298)	2	-0.0059	-0.0086	0.0318	0.0254
NW_017097588.1 No genes on scaffold	2	0.0080	0.0214	-0.0099	0.0335
NW_017097588.1 No genes on scaffold	3	0.0344	0.0302	0.0611	0.0044
NW_017097707.1 No genes on scaffold	2	0.0223	-0.0068	0.0369	-0.0105
NW_017097793.1 Close to uncharacterized LOC108560991; venom serine protease-like (LOC108560992)	2	-0.0197	0.0224	-0.0177	0.0374
NW_017098369.1 Between putative gustatory receptor 39b (LOC108562264). Closest to this and upstream, possible in promoter region) and anosmin (LOC108562266) and mucin-2-like (LOC108562268)	2	0.0327	0.0309	0.0062	-0.0147
NW_017098369.1 Upstream of aryl hydrocarbon receptor protein 1 (LOC108562270). Best match is *spineless* in *Drosophila*	3	0.0262	0.0208	0.0218	-0.0148
NW_017098425.1 No genes on scaffold	2	0.0409	-0.0395	-0.0838	0.0303
NW_017098544.1 Uncharacterized LOC108562723	2	0.0049	0.0292	-0.0003	0.0211
NW_017098596.1 No genes on scaffold	2	0.0365	-0.0065	0.0009	0.0265
NW_017098718.1 End of scaffold. This region is the promoter of uncharacterized LOC108563043	2	-0.0111	0.0258	0.0275	0.0149
NW_017099114.1 Intron of glutamate receptor ionotropic, kainate 2 (LOC108564059)	2	0.0377	0.0321	-0.0133	-0.0123
NW_017099136.1 No genes on scaffold	2	-0.0122	0.0232	0.0282	0.0091
NW_017099143.1 Upstream of angiopoietin-2 (LOC108564089). Annotation in *Drosophila* is uncharacterized	2	-0.0128	0.0315	0.0279	0.0038
NW_017099143.1 Downstream of centrosome-associated protein 350-like (LOC108564093);	2	0.0283	-0.0015	0.0527	0.0036

upstream of protein ecdysoneless (LOC108564094)					
NW_017099143.1 Downstream of centrosome-associated protein 350-like (LOC108564093); upstream of protein ecdysoneless (LOC108564094)	2	0.0343	-0.0124	0.0665	-0.0130
NW_017099143.1 Downstream of centrosome-associated protein 350-like (LOC108564093); upstream of protein ecdysoneless (LOC108564094)	2	0.0345	0.0091	0.0386	0.0011
NW_017099184.1 Mediator of RNA polymerase II transcription subunit 15 (LOC108564145)	2	0.0248	0.0707	-0.0122	-0.0069
NW_017099374.1 Ion transport peptide (LOC108564407)	2	0.0331	-0.0227	0.0234	-0.0130
NW_017099578.1 Moesin (LOC108564809). Involved in neural development	2	0.0231	0.0172	0.0469	0.0192
NW_017099676.1 Closest gene is cgmp-specific 3',5'-cyclic phosphodiesterase (LOC108565120). Annotated as phosphodiesterase in Dmel	2	-0.0190	0.0252	-0.0138	0.0446
NW_017099682.1 Uncharacterized LOC108565192; Uncharacterized LOC108565191	2	-0.0148	0.0247	0.0201	-0.0290
NW_017099685.1 Inositol-trisphosphate 3-kinase A (LOC108565210)	2	0.0214	0.0464	-0.0240	-0.0224
NW_017099743.1 No genes on scaffold	2	0.0368	-0.0039	-0.0088	0.0421
NW_017099753.1 End of scaffold	2	0.0224	-0.0107	0.0417	-0.0176
NW_017099782.1 Alkaline phosphatase-like (LOC108565566)	2	-0.0173	0.0105	0.0373	0.0345
NW_017099840.1 End of scaffold	2	0.0046	0.0238	-0.0090	0.0214
NW_017100047.1 End of scaffold	2	-0.0053	-0.0333	0.0270	0.0324
NW_017100055.1 No genes on scaffold	2	0.0006	-0.0291	0.0323	0.0261
NW_017100093.1 Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial (LOC108567660)	2	0.0237	-0.0124	0.0224	0.0163
NW_017100102.1	2	0.0180	0.0235	0.0161	0.0479

Mnt (LOC108568128) cell cycle, regulates body size					
NW_017100108.1 Between dual oxidase (LOC108568369) and lipase 3-like (LOC108568326)	2	-0.0153	0.0247	0.0235	-0.0122
NW_017100108.1 Upstream of vacuolar fusion protein CCZ1 homolog (LOC108568333)	2	0.0138	0.0365	0.0233	0.0089
NW_017100110.1 ER lumen protein-retaining receptor-like (LOC108568459); sodium-independent sulphate anion transporter-like (LOC108568458)	2	0.0238	0.0212	-0.0114	0.0052
NW_017100113.1 Between uncharacterized LOC108568666 and ras-associated and pleckstrin homology domains-containing protein 1-like (LOC108568699 which is similar to pico in Dmel, involved in imaginal discs and development	2	-0.0030	-0.0030	0.0256	0.0375
NW_017100114.1 Large intergenic region, closest gene is uncharacterized LOC108568729	2	0.0290	-0.0146	-0.0037	0.0242