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Research

Community Ecology

# High Dietary Niche Overlap Between Non-native and Native Ant Species in Natural Ecosystems

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## Abstract

Ants represent a highly diverse and ecologically important group of insects found in almost all terrestrial ecosystems. A subset of ant species have been widely transported around the globe and invade many natural ecosystems, often out-competing native counterparts and causing varying impacts on recipient ecosystems. Decisions to control non-native ant populations require an understanding of their interactions and related impacts on native communities. We employed stable isotope analysis and metabarcoding techniques to identify potential dietary niche overlap and identify gut contents of 10 ant species found in natural ecosystems in Aotearoa New Zealand. Additionally, we looked at co-occurrence to identify potential competitive interactions among native and non-native ant species. Ants fed mainly across two trophic levels, with high dietary overlap. Relative to other ant species sampled, two non-native ant species, *Linepithema humile* and *Technomyrmex jocosus*, were found to feed at the lowest trophic level. The largest isotopic niche overlap was observed between the native *Monomorium antarcticum* and the invasive *Ochetellus glaber*, with analyses revealing a negative co-occurrence pattern. Sequence data of ant gut content identified 51 molecular operational taxonomic units, representing 22 orders and 34 families, and primarily consisting of arthropod DNA. Although we generally found high dietary overlap among species, negative occurrence between a dominant, non-native species and a ubiquitous native species indicates that species-specific interactions could be negatively impacting native ecosystems. Our research progresses and informs the currently limited knowledge around establishing protocols for metabarcoding to investigate ant diet and interactions between native and non-native ant species.

**Key words:** stable isotope, isotopic niche, metabarcoding, gut content analysis, environmental DNA

## Introduction

Ecological interactions between non-native species and native biodiversity occur both directly and indirectly and in the most severe cases cause ecosystem-wide impacts (O'Dowd et al. 2003). A fundamental way that non-native animals drive ecological change is via dietary-related interactions with their recipient community. Whilst some dietary-related impacts are relatively easy to measure (e.g., direct predation reducing a prey population) due to the complexities of both species' interactions and food webs, impact is often difficult to discern. Furthermore, ecological traits of invasive species, such as habitat preferences, cryptic feeding behavior, and/or feeding strategies, may add further uncertainty to conclusions related to ecological dietary interactions and effects (Cohen 2015).

Although feeding strategies of non-native species range from specialized to opportunistic omnivores, discerning the impact of species with more opportunistic and generalist tendencies can be challenging given the complex interactions of such species (Snyder and Evans 2006). In some cases, the establishment and persistence of generalist species relies on their dietary flexibility: when food availability is less likely to be a limiting factor, their niche opportunities within the invaded environment are increased (Shea and Chesson 2002, Tonella et al. 2018). Such a strategy enables populations to persist, despite variations in food availability (Caut et al. 2008). This may broaden

their potential impact through dietary-related ecological interactions within an ecosystem. For instance, non-native species feeding across multiple trophic levels will act as both consumers and potential competitors at a broader scale, making impact assessment more difficult than if they were dietary specialists (Snyder and Evans 2006). Furthermore, this strategy may confer a competitive advantage, allowing invaders to outcompete, and potentially displace, native species (Snyder and Evans 2006).

Dietary flexibility, in the form of an opportunistic, generalist feeding strategy is typical among ant species that establish outside of their native ranges (McGlynn 1999, Rabitsch 2011). The most invasive ant species appear to commonly exploit carbohydrate-rich resources (e.g., honeydew and nectar) when available (Holway et al. 2002), which in turn enables increased activity and colony sizes (Wittman et al. 2018). Indeed, a key finding of Holway et al. (2002) was that increased colony sizes, which lead to numerical dominance, and heightened aggression, are linked to the invasion success of ants.

The establishment of non-native ants can lead to significant alterations to the recipient local ant community (Goodman and Warren II 2019). In some cases, non-native species may completely exclude native species from their natural habitats (Naughton et al. 2020), although native and non-native ant species will often co-occur to some degree at the local scale (Stringer and Lester 2008, Berman et al. 2013, Arnan et al. 2018). Factors potentially facilitating the co-occurrence of native and non-native ant species may be spatial partitioning within their shared habitat and/or the dietary partitioning of food resources (Ward 2008).

Understanding the diet of ant species can be difficult due to broad feeding habits and foraging behaviors (Tillberg et al. 2006). Stable isotope analysis is a well-developed tool that has been used to assess the trophic structure of highly invasive species and can be used to infer ant diet through carbon and nitrogen isotopic ratios (Tillberg et al. 2007, Menke et al. 2010, Roeder and Kaspari 2017). Isotopes of nitrogen (ratio of $^{15}N$ to $^{14}N$, expressed as $\delta^{15}N$) enable estimates of trophic position. Each trophic level is typically enriched with $^{15}N$ by 3–4‰, resulting in primary consumers having lower $\delta^{15}N$ values than secondary and higher consumers (Post 2002). Isotopes of carbon (ratio of $^{13}C$ to $^{12}C$, expressed as $\delta^{13}C$) can provide information on the dietary sources of carbon to an organism. For example, due to the distinct isotopic signature of $C_3$ versus $C_4$ plants—with $C_3$ plants being more depleted in $^{13}C$ than $C_4$ plants—the relative contribution of these different sources to an organism's diet can be estimated (Post 2002).

Whilst stable isotope analysis is often used to understand impact through invasion for a variety of taxa, it is limited in its ability to accurately describe diet at the finer scale (Roemer et al. 2002, Cucherousset et al. 2012, Karlson et al. 2015, Rakauskas et al. 2018). This is better achieved through genetic-based approaches to identify the DNA of prey items and gut or fecal content (Nielsen et al. 2018). The reduced costs associated with DNA barcoding and high-throughput sequencing methods have made this method more accessible for dietary analysis for many taxa, allowing identification for a wide dietary scope (Bohmann et al. 2011, Connell et al. 2014, Gómez and Kolokotronis 2017, Jedlicka et al. 2017). Advantages of this approach include the ability to more accurately describe the diet of omnivorous species, without laborious observation studies, or sorting samples based on morphological identification (De Barba et al. 2014, Harms-Tuohy et al. 2016, Robeson et al. 2018). Use of metabarcoding is becoming a standard practice for dietary analysis in some taxa [e.g., bats (Bohmann et al. 2011, Razgour et al. 2011, Zeale et al. 2011, Arrizabalaga-Escudero et al. 2018, Czenze et al. 2018)], with methods better developed for vertebrates than invertebrates. Although metabarcoding is becoming more regularly used to investigate the diet of arthropods (Gomez-Polo et al. 2015, Lima et al. 2016, Paula et al. 2016), there are few applications to ants (but see Mollot et al. 2014), a group well known for causing dietary-related impacts where they invade (Holway et al. 2002).

In Aotearoa New Zealand, the native ant fauna is relatively depauperate, consisting of only 11 species, all of which are endemic (Don 2007). In contrast, more than 29 non-native ant species are considered established. Although some previous research has investigated the competitive interactions between New Zealand native and non-native ant species in some contexts (Stringer et al. 2009, Lester et al. 2010), there has been little focus on the dietary characteristics of these species. In this study, we used both stable isotope and high-throughput sequencing methods to investigate the diet of ants inhabiting short-stature indigenous ecosystems, where the ant community is often dominated by non-native species (Probert et al. 2020). Specifically, our aim was to investigate whether dietary differentiation facilitates the co-existence of ant species within natural ecosystems and gain a better understanding of how dietary-related interactions shape invaded ant communities in natural ecosystems. To determine whether dietary niches and the occurrence of dominant non-native and native ant species overlapped, we analyzed co-occurrence patterns and employed stable isotope and DNA metabarcoding to reveal dietary inputs. We predicted that, for non-native ant species, stable isotope and DNA metabarcoding gut content data would reveal a more generalist feeding strategy than for the native ants, which we predicted would be more specialized, predatory feeders (Don 2007).

## Methods

### Sampling Locations

This study was conducted in the Auckland region of New Zealand from October 2016 to March 2017. We sampled ants for both stable isotope and metabarcoding analysis across seven sites ranging from coastal scrub to dune habitats within native ecosystems (Table 1). Maximum vegetation height was around 3 m; foraging ants were collected from the ground and accessible vegetation between 10:00 h and 17:00 h. All seven sites were characterized by their open canopy (low stature vegetation) and were selected as they are vulnerable to invasion by non-native ants, which dominate the local ant communities in these habitats (Probert et al. 2020).

### Community Sampling Methods

For stable isotope samples, sampling at each site was limited to areas <500 m × 500 m to reduce nitrogen baseline variability (Woodcock et al. 2012). Sampling was conducted within the 500 m × 500 m area

**Table 1.** Sampling sites from the greater Auckland area, including information on ecosystem type and global positioning system localities

| Site | Ecosystem type | Locality (latitude, longitude) |
|---|---|---|
| Anawhata | Coastal scrub | −36.926378, 174.458643 |
| Karakare | Coastal scrub | −36.986918, 174.478752 |
| Muriwai | Coastal scrub/sand dunes | −36.827429, 174.427032 |
| Piha | Coastal scrub/sand dunes | −36.937299, 174.460962 |
| Te Henga | Coastal scrub/sand dunes | −36.892398, 174.446596 |
| Wenderholm | Coastal scrub/mangrove | −36.532636, 174.710105 |
| Whatipu | Coastal scrub/sand dunes | −37.043824, 174.507208 |

at each site ($n$ =7, see Suppl Table 2 [online only]) through direct visual searching to maximize the number of ant species collected using an entomological aspirator. At least 50 m was maintained between sampling colonies of the same species. Only ants actively foraging outside of their nests were collected. Top soil type (organic, loam, sand, or clay) and collection locations (ground, plant, or coarse-woody debris) were recorded for each sample. Plant material and other arthropod species broadly classified by their known trophic level [e.g., predators > trophic level (TL)3; and primary consumers TL2] were also sampled in the same 500 m × 500 m area.

Ant samples for DNA dietary analyses were collected both within the 500 m × 500 m plots and the wider surrounding area. Live ants and other arthropods were kept in separate vials and returned to the laboratory and then stored frozen at −20°C until preparation (for stable isotope analyses), or put into 95% ethanol then stored frozen at −80°C (for DNA analyses).

### Sample Preparation

We identified all ants to species level using the key in Don (2007). In preparation for stable isotope analysis, ants and other invertebrates were thawed, rinsed with distilled water to remove any debris, and then placed in a drying oven at 50°C for 48 h. The gaster, petiole, and postpetiole of each ant were removed to ensure that recently ingested food did not affect isotopic values (Tillberg et al. 2006). For other arthropods, individuals were either starved for 72 h prior to freezing, or their digestive tract was dissected out prior to processing. Plant material was rinsed in distilled water, placed in a drying oven for 48 h, and then ground into a fine powder using a Mixer mill MM301 (Retsch, Haan Germany). Only ants collected in March 2017 were prepared for stable isotope analysis (cf. metabarcoding). This sampling period, towards the end of the Austral summer, provides isotopic information for colonies reflecting the temporal assimilation of isotopes for the previous months, when ant colony abundance, and thus potential impact, peaks.

To obtain a sample of sufficient weight for stable isotope processing, we pooled 5–20 ants per colony. A pilot stable isotope analysis determined that the optimal sample weight required for the ant samples was 0.50–0.65 mg. Samples were weighed using an analytical balance (UMX5, Mettler Toldedo, Switzerland, precise to 0.001 mg) into 5 × 9 mm tin capsules (OEA Labs, UK). These were closed and shaped into tight balls using sterilized forceps, placed into individual wells in a 96-well plate, and kept in a desiccator prior to analysis.

All stable isotope analyses were carried out at the National Institute of Water and Atmospheric Research (NIWA) Environmental and Ecological Stable Isotope Facility in Wellington, New Zealand. Analyses were performed with a MAS200 autosampler connected to a Flash 2000 elemental analyzer coupled with a DELTA V Plus (Thermo-Fisher Scientific, Bremen, Germany) continuous flow, isotope ratio mass spectrometer (IRMS). $CO_2$ (calibrated against NBS19-calcite referenced to Vienna Pee Dee Belemnite and corrected for $^{17}O$) and $N_2$ (calibrated against atmospheric air) reference gas standards were introduced to the IRMS with every sample analysis. Carbon isotope data were corrected via a two-point normalization process (Paul et al. 2007) using NIST 8573 (USGS40 l-glutamic acid; certified $\delta^{13}C$ = −26.39 ± 0.09‰) and NIST 8542 (IAEA-CH-6 sucrose; certified $\delta^{13}C$ = −10.45 ± 0.07 ‰). A two-point normalization process using NIST 8573 (USGS40 l-glutamic acid; certified $\delta^{15}N$ = −4.52 ± 0.12‰) and IAEA-N-2 (ammonium sulfate: certified $\delta^{15}N$ = +20.41 ± 0.20‰) was applied to $\delta^{15}N$ data. dl-Leucine (dl-2-amino-4-methylpentanoic acid, $C_6H_{13}NO_2$, Lot 127H1084, Sigma, Australia) was run every 10 samples to check analytical precision and enable drift corrections to be made if necessary. Additional international standards NIST 8574 (USGS41 l-glutamic acid; certified $\delta^{13}C$ = +37.63 ± 0.10‰ and $\delta^{15}N$ = +47.57 ± 0.22‰), NIST 8547 (IAEA-N1 ammonium sulphate; certified $\delta^{15}N$ = +0.43 ± 0.04‰) were run daily to check isotopic accuracy. Repeat analysis of standards produced data accurate to within 0.25‰ for both $\delta^{15}N$ and $\delta^{13}C$, and a precision of better than 0.32‰ for $\delta^{15}N$ and 0.24‰ for $\delta^{13}C$.

Stable isotope ratios were expressed as delta values (δ) (per mil units (‰)), which represent the ratios of heavy to light isotopes within a sample ($R_{sample}$), relative to the ratio in an international standard ($R_{standard}$) as

$$\delta = \left(\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right) \times 1{,}000$$

Trophic position (TP) was determined as

$$TP = \lambda + \frac{\delta^{15}N_{ant} - \delta^{15}N_{base}}{\Delta N}$$

where λ represents the basal food source trophic level (e.g., primary producer = 1), $\delta^{15}N_{ant}$ represents the individual sample, and $\delta^{15}N$ represents the nitrogen baseline obtained from vegetation. The value of ΔN was the standard enrichment per trophic level, which is typically 3.40‰ (Post 2002). To obtain the nitrogen baseline, we regressed the $\delta^{15}N$ values of plant material against site and found that only the Piha site was significantly different from the other sites. Therefore, we applied corrections to ant and invertebrate samples from the Piha site using the formula:

$$\delta^{15}N_{corrected} = \delta^{15}N_{measured} + (\delta^{15}N_{sites} - \delta^{15}N_{piha})$$

where $\delta^{15}N_{sites}$ was the mean nitrogen value for all sites excluding Piha, and $\delta^{15}N_{piha}$ was the mean nitrogen value for the Piha site (Pfeiffer et al. 2014). The $\delta^{15}N_{sites}$ value was used as the nitrogen baseline for trophic positioning, and in all cases we analyzed and reported the corrected $\delta^{15}N$ values.

We processed 156 ant samples for stable isotope analysis, comprising 13 species (9 non-native, 4 native) (Table 2). Nitrogen isotope values could only be obtained for 150 samples.

## DNA Analysis of Ant Diet

### Sample Preparation

Each sample consisted of 10 workers from a colony, which had been stored in 95% ethanol at −80°C. Individual samples were first surface sterilized in a sterile petri dish containing 5% bleach for 1 min, and then rinsed in molecular-grade water three times (sensu Łukasik et al. 2017). The gut contents of each individual ant was then dissected out onto UV sterilized Kimwipes to: 1) reduce the amount of ant DNA in each sample; and 2) reduce potential PCR inhibition related to the crop structure that has been found for some ant species (Penn et al. 2016). Between dissections for each colony, forceps were soaked for 15 min in 10% bleach, washed with sterile water, and then placed under ultra-violet light for 15 min.

DNA from the Kimwipes with the gut contents of ants were extracted whole. DNA extraction was conducted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Ltd., Crawley, UK), with samples first digested at 56°C overnight with all other steps following the manufacturer's instructions for animal tissues.

**Table 2.** Summary of information including stable isotope values for ant species and the primary consumer and predator trophic levels

| Taxa/trophic level | *N* | Native/non-native | $\delta^{13}C$ (‰) | $\delta^{15}N$ (‰) |
|---|---|---|---|---|
| Dolichoderinae | | | | |
| *Iridomyrmex suchieri* (Forel, 1907) | 29(28) | Non-native | −22.80 ± 0.39 | 5.26 ± 0.31 |
| *Linepithema humile* (Mayr, 1868) | 14 | Non-native | −23.85 ± 0.26 | 3.88 ± 0.24 |
| *Ochetellus glaber* (Mayr, 1862) | 25 | Non-native | −24.43 ± 0.40 | 5.11 ± 0.27 |
| *Technomyrmex jocosus* (Forel, 1910) | 6 | Non-native | −24.54 ± 0.33 | 3.20 ± 0.23 |
| Ectatomminae | | | | |
| *Rhytidoponera chalybaea* (Emery, 1901) | 5 | Non-native | −22.67 ± 0.66 | 6.21 ± 1.17 |
| Formicinae | | | | |
| *Nylanderia* sp. | 24(22) | Non-native | −22.76 ± 0.56 | 5.20 ± 0.28 |
| Myrmicinae | | | | |
| ***Huberia striata*** (Smith, F., 1876) | 1 | Native | −25.90 | 4.89 |
| ***Monomorium smithii*** (Forel, 1892) | 3 | Native | −24.92 ± 0.89 | 5.76 ± 0.11 |
| ***Monomorium antarcticum*** (Smith, F., 1858) | 20 | Native | −24.30 ± 0.52 | 5.10 ± 0.31 |
| *Pheidole rugosula* (Forel, 1902) | 8(6) | Non-native | −20.51 ± 0.76 | 6.50 ± 0.08 |
| *Solenopsis* sp. | 2(0) | Non-native | −26.30 ± 0.65 | – |
| *Tetramorium grassii* (Emery, 1895) | 15 | Non-native | −24.60 ± 0.54 | 5.69 ± 0.48 |
| Ponerinae | | | | |
| ***Austroponera castanea*** (Mayr, 1865) | 4 | Native | −23.27 ± 1.58 | 6.77 ± 0.60 |
| Trophic level | | | | |
| Primary consumer | 32 | | −26.55 ± 0.43 | 0.52 ± 0.39 |
| Predator | 42 | | −23.24 ± 0.43 | 6.34 ± 0.29 |

*N* represents the number of colonies where reliable $\delta^{13}C$ and $\delta^{15}N$ data were obtained. In some cases, nitrogen data were below the analytical detection limit. In these cases, the number of reliable $\delta^{15}N$ values are indicated in brackets. Isotope values shown are averages ± 1 standard error. Species in bold are native to New Zealand.

Initially, we tested several primer pairs to amplify animal and plant material (see Supp information [online only]). We found the primer pair mlCOIinfF and HCO2198 (Folmer et al. 1994, Leray et al. 2013) produced the most consistent amplicons and used these in our final protocol in samples sent for sequencing. The final PCR was performed in a 25 µl reaction volume consisting of 5.50 µl nuclease free water, 12.50 µl Kapa Hifi Hotstart ReadyMix, 1 µl of each primer (at 10 µM), and 5 µl DNA template, with the optimal PCR cycle for these primers. PCR products were examined by electrophoresis at 90 V for 40 min in a 1.50% agarose gel in 0.50% TBE buffer with SYBR Safe (Invitrogen, Australia); a 1 kb plus DNA ladder (Invitrogen) was run alongside the samples to estimate the size of the PCR products. Every PCR cycle included a positive and negative control. The PCR cycles that resulted in optimum amplification for primers mlCOIinfF and HCO2198 were: 95°C for 3 min for initial denaturation, followed by 15 cycles of 95°C 30 s, 63°C 20 s (using 'touchdown' PCR—decreasing 1°C every cycle), and 72°C 60 s, then for 35 cycles of 95°C for 30 s, 48°C for 20 s, 72°C for 60 s and 72°C at 10 min for the final extension.

PCR products were individually purified using ZR-96 DNA Clean-up kits (Zymo Research), then quantified using a Qubit double-stranded DNA (dsDNA) HS assay kit (Life Technologies). The amplified material was then sent to Auckland Genomics, at the University of Auckland (Auckland, New Zealand) for sequencing on an Illumina MiSeq instrument using 2 × 300 bp chemistry. Before sequencing, the sequencing provider attached a unique combination of Nextera XT dual indices (Illumina Inc.) to the DNA from each sample, to allow for multiplex sequencing.

### Bioinformatics Methods

We quality-filtered the sequence data and picked de novo molecular operational taxonomic units (MOTUs) using USEARCH v 7.0 (30). The first 26 bp were trimmed off the start of both the forward and reverse reads to remove primer sequences, before using the fastq_mergepairs command to merge the reads. Any reads with a quality score (Q score) of <3 were truncated at the first position, and we set the minimum length of the merged read to 200 bp. Reads with >1 expected error were discarded. Sequence data were dereplicated (-derep_fulllength), singletons were removed (-sortbysize), and then clustered into MOTUs at 97% sequence similarity, using the UPARSE-OTU algorithm, which also removes chimeras (Edgar 2013).

Taxonomic classification was done using BLAST (Altschul et al. 1990) against the partially nonredundant National Center for Biotechnology Information nucleotide database (downloaded on 21 February 2018). Only one sequence match per MOTU was allowed, and a minimum similarity threshold of 80% was used for the BLAST search. The resulting BLAST file was imported into Megan6 (Huson et al. 2007) to obtain taxonomic paths for each MOTU. Bacterial and fungal sequences were removed from the dataset, as these were likely the result of unspecific primer binding. Additionally, all sequences matching ants (family: Formicidae) were removed for each sample. This resulted in a large range of sequences per sample (1-3386 reads per sample, with from 1 to 8 MOTUs per sample). To achieve a standard sequencing depth across all samples, we rarefied to 60 reads per sample. Although this is a very low rarefaction threshold, rarefaction curves indicate that this adequately sampled the diversity present (Supp Fig. 1 [online only]); we present only descriptive data for nonrarefied samples.

## Statistical Analyses

All statistical analyses were performed in R version 3.4.2 (R Core Team 2017); we report significance at the $P < 0.05$ level.

### Stable Isotope Analysis of Ant Diet

To investigate differences in $\delta^{15}N$ and $\delta^{13}C$ values between species, we used two linear mixed-effect models using the 'lme4' package (Bates et al. 2015), with $\delta^{15}N$ and $\delta^{13}C$ as the response variables.

Top soil type (organic, loam, sand, or clay) and collection locations (ground, plant, or coarse-woody debris) were included in the model as explanatory variables, and site was included as a random effect. Residuals were checked to confirm models met their assumptions; one outlier was removed. We conducted model selection by comparing models using maximum likelihood tests and Akaike's information criterion corrected for small sample size (ΔAICc) to obtain our final model. We then conducted pairwise comparisons between different ant species using the 'pairs' function in the 'emmeans' package (Lenth et al. 2018), using the 'fdr' *P*-adjustment correction (Benjamini and Hochberg 1995).

We also compared isotopic niche width using a Bayesian approach that uses multivariate ellipse-based metrics using the 'SIBER' package (Jackson et al. 2011) for species where we had >3 data points for both $\delta^{15}N$ and $\delta^{13}C$. This approach allows for comparisons between different species among and between different communities, even when sample sizes differ, by creating standard ellipse areas (SEAs), which are comparable to standard deviation in univariate cases (Jackson et al. 2011). $SEA_C$ ($_C$ denotes that the SEA was corrected for small sample size), contains the core aspects of a population's niche and is less sensitive to outliers and small sample sizes. However, this analysis is sensitive to non-normality so shapiro-wilk tests were used and outliers removed to ensure we met this assumption. Additionally, any sample that did not have both the $\delta^{15}N$ and $\delta^{13}C$ values had to be removed. We calculated the differences between species' $SEA_C$ using Bayesian inference ($SEA_B$), using two chains of 100,000 iterations, with a burn-in of 1,000 and thinning of 10.

### Co-occurrence Patterns

To assess species associations, we used occurrence data at baiting stations for the species sampled within open-canopy ecosystems (i.e., short-stature, without a continuous canopy) (see Probert et al. 2020 for sampling protocol). This dataset is largely representative of ant species we collected for dietary analysis, with the exception of *Linepithema humile*. There is strong evidence of *L. humile* suppressing populations of other ant species where it occurs (Human and Gordon 1996, Suarez et al. 1998, Sanders et al. 2001), and at the collection sites where *L. humile* was present, we only observed other species co-occurring with *L. humile* at the periphery of the invaded area, suggesting possible negative associations between *L. humile* and other ant species at these sites.

We used a probabilistic model approach using the 'cooccur' package (Griffith et al. 2016). This analysis allows for comparison of the 'observed co-occurrence' to the 'expected co-occurrence', where the latter is the product of the two species' probability multiplied by the number of sampling sites, or baiting stations in this case: $E(N_{1,2}) = P(1) \times P(2) \times N$ (Veech 2013). Overall patterns of co-occurrence can then be tested between species pairs, calculating the probability that the observed co-occurrence is greater than the expected frequency (a positive association), less than the expected frequency (a negative association), or random. Of the total pair combinations ($n$ = 120), 81.6% had an expected co-occurrence of <1 so were removed from analysis and 22 pairs were analyzed (Veech 2013).

## Results

### Stable Isotope Analyses of Ant Diet

A wide variability of isotopic values were detected for ants, ranging from −28.6‰ (*Nylanderia* sp.) to −17.0‰ (*Tetramorium grassii*) for carbon and 1.4‰ (*Iridomyrmex suchieri*) to 10.0‰ (*Rhytidoponera chalybaea*) for nitrogen (Table 2). Overall, ants fed mainly across two trophic levels as primary and secondary consumers; however, there was large overlap among species (Figure 1). Based on the best model (Table 3), effects of vegetation cover on $\delta^{13}C$ values were negligible (estimate: 0.006, se = 0.01, $t$ = 1.025), although $\delta^{13}C$ was affected by sampling location.

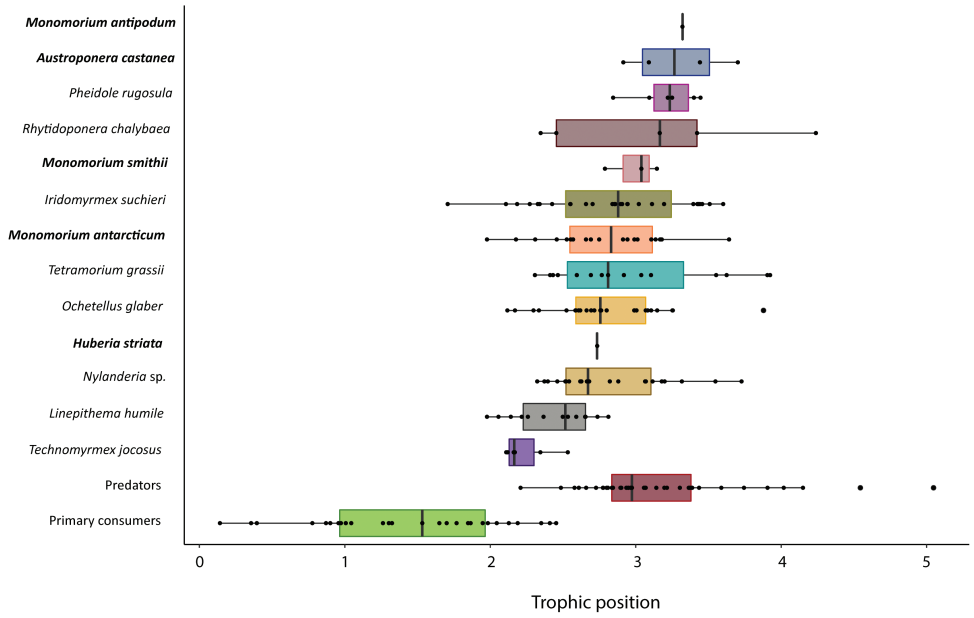


**Figure 1.** Interquartile range box plots for trophic position estimates of ants, primary consumers and predacious arthropods, sampled across open-canopy (short-stature vegetation without a continuous tree canopy) sites in Auckland, New Zealand. In the box plots, the boundary of the box closest to zero represents the 25th percentile, the black line within the box represents the median and the boundary farthest from zero represents the 75th percentile. Points left and right of the whiskers indicate outliers of the 10th and 90th percentiles. Trophic position represents a continuous score, with lower numbers reflecting lower trophic position (i.e., primary consumers). Species native to New Zealand are in bold.

**Table 3.** Linear mixed-effect models examining the effect of environment and species on the $\delta^{13}C$ and $\delta^{15}N$ values of ant species collected in short-stature indigenous ecosystems in Auckland, New Zealand, with site included as a random effect

| Response variable | Model | $AIC_c$ | $\Delta i$ | $w_i$ | acc $w_i$ |
|---|---|---|---|---|---|
| $\delta^{13}C$ | **Veg + Spp + Wo + (1\|Site)** | 609.98 | 0.00 | 0.93 | 0.93 |
| | Veg + Spp + Wo + Soil + (1\|Site) | 615.59 | 5.61 | 0.06 | 0.99 |
| | Wo + (1\|Site) | 618.61 | 8.63 | 0.02 | 0.99 |
| | Spp + Wo + (1\|Site) | 624.29 | 14.32 | 0.00 | 0.99 |
| | Spp + Wo + Soil + (1\|Site) | 629.56 | 19.59 | 0.00 | 1.00 |
| $\delta^{15}N$ | **Veg + Spp + (1\|Site)** | 518.42 | 0.00 | 0.80 | 0.80 |
| | Veg + (1\|Site) | 521.96 | 3.54 | 0.14 | 0.93 |
| | Veg + Spp + Soil + (1\|Site) | 523.51 | 5.09 | 0.06 | 0.99 |
| | Veg + Spp + Wo + Soil + (1\|Site) | 527.88 | 9.46 | 0.01 | 1.00 |

Models are in order, based on their $AIC_c$ values. $\Delta i$ is the difference in the $AIC_c$ value of each model compared to that for the top model (in bold); $w_i$ is the Akaike weight for each model; acc $w_i$ is the cumulative Akaike weight.

Veg, percentage vegetation in 2 m radius from ant collection location; Spp, ant species; Wo, sampling location; Site, sampling locality.

**Table 4.** Percentage of isotopic niche area overlap, as determined through standard ellipse area (SEAc), between different ant species using the Bayesian SIBER model (Jackson et al. 2011). Species in bold are native to New Zealand

| | Isuc | Lhum | Nyl | Ogla | Tgra | Tjos | Rcha | Prug | **Acas** | **Mant** | **Msmi** | Prim |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Isuc | | | | | | | | | | | | |
| Lhum | 18 | | | | | | | | | | | |
| Nyl | 66 | 12 | | | | | | | | | | |
| Ogla | 33 | 11 | 39 | | | | | | | | | |
| Tgra | 21 | 8 | 22 | 39 | | | | | | | | |
| Tjos | 1 | 22 | 0 | 0 | 2 | | | | | | | |
| Rcha | 40 | 4 | 32 | 15 | 21 | 0 | | | | | | |
| Prug | 21 | 0 | 17 | 4 | 4 | 0 | 17 | | | | | |
| **Acas** | 10 | 0 | 4 | 13 | 27 | 0 | 24 | 17 | | | | |
| **Mant** | 39 | 10 | 43 | 82 | 38 | 0 | 18 | 8 | 12 | | | |
| **Msmi** | 4 | 0 | 1 | 19 | 10 | 0 | 4 | 3 | 14 | 16 | | |
| Prim | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | |
| Pred | 34 | 2 | 29 | 32 | 38 | 0 | 45 | 22 | 50 | 34 | 10 | 0 |

Isuc, *Iridomyrmex sulchieri*; Lhum, *Linepithema humile*; Nyl, *Nylanderia* sp; Ogla, *Ochetellus glaber*; Tgra, *Tetramorium grassii*; Tjos, *Technomyrmex jocosus*; Rcha, *Rhytidoponera chalybaea*; Prug, *Pheidole rugosula*; **Acas, *Austoponera castanea*; Mant, *Monomorium antarcticum*; Msmi, *Monomorium smithii***; Prim, Primary consumers; Pred, Predators. Native species are in bold.

Pairwise contrasts revealed that ants collected from plants were associated with significantly higher $\delta^{13}C$ values than ants collected from coarse-woody debris (df = 131, *t* ratio = 3.062, *P* = 0.008). Similarly, ants collected from the ground had significantly higher $\delta^{13}C$ values than ants collected from coarse-woody debris (df = 130, *t* ratio = 2.436, *P* = 0.042). Estimated marginal means for $\delta^{13}C$ values were not found to significantly differ between any species pair. Based on the best model (Table 3), effects of vegetation cover on $\delta^{15}N$ values were weak (estimate: −0.009, se = 0.005, *t* = −1.903). Estimated marginal means for $\delta^{15}N$ values were significantly different only between two species pairs: *Austroponera castanea* and *Technomyrmex jocosus* (df = 130, *t* ratio = 3.50, *P* = 0.03) and *R. chalybaea* and *T. jocosus* (df = 132, *t* ratio = 3.52, *P* = 0.02), with *T. jocosus* having lower $\delta^{15}N$ values than either species.

### Isotopic Niche Overlap Between Species

The isotopic niche, which combines $\delta^{13}C$ and $\delta^{15}N$ values and is measured by the standard ellipse area (SEAc) (Newsome et al. 2007, Jackson et al. 2011), overlapped between almost all ant species and the predator trophic level. However, overlap with the predator trophic level was low (≤10%) for *Linepithema humile* and *Monomorium smithii*, and absent for *T. jocosus* (Table 4, Figure 2). In contrast, SEAc values clearly differed between the primary consumer trophic level and all ant species except *T. jocosus*, although the overlap between the primary consumer trophic level and *T. jocosus* was low (4%). The largest overlap in isotope niche was observed between the invasive species *Ochetellus glaber* and the native *Monomorium antarcticum*. These two species had very similar niche breadth for $\delta^{15}N$, although *M. antarcticum* had a wider $\delta^{13}C$ niche breadth (Figure 2). A relatively large overlap was also observed between *Nylanderia* sp. and *I. sucheri* (66%), and *A. castanea* and the predator trophic level (50%); all other overlaps were <50% (Table 4). *Rhytidoponera chalybaea* and *A. castanea* had the largest and *L. humile* and *T. jocosus* had the smallest $SEA_B$ (Supp Fig. 2 [online only]).

### Co-occurrence Patterns

Of the 22 species pairs analyzed, random associations represented the majority (90.9%) of co-occurrences between ant species at bait stations. Two significant, negative associations were detected with the probabilistic modeling; between *O. glaber* and *T. jocosus* (both non-native) and between *O. glaber* and the native species, *M. antarcticum*.

## DNA Analysis of Ant Diets

In total, 171 samples were sequenced; as expected, the majority of sequence reads belonged to the host, with 99.7% of sequence reads

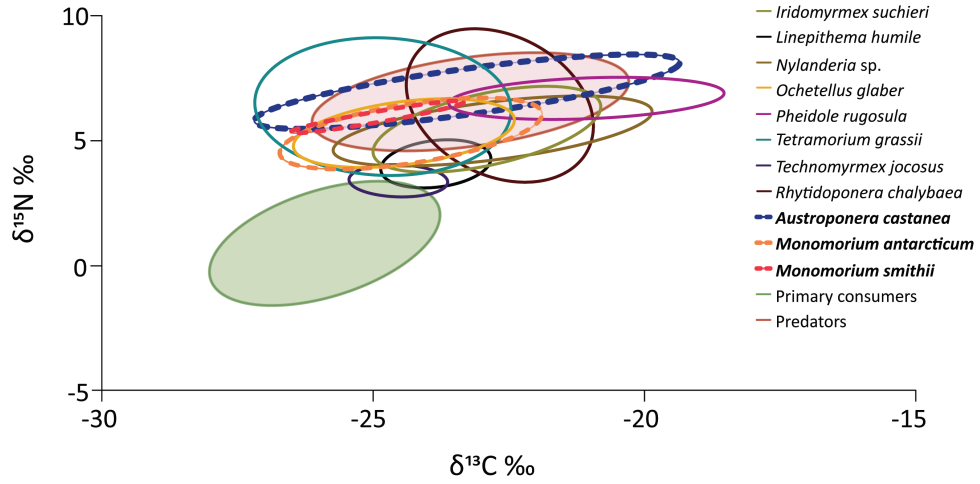


**Figure 2.** Stable isotope biplot illustrating the isotopic niches of different ant species and two functional groups (primary consumers in green and predators in red are shown with shaded ellipses) in Auckland, New Zealand. Ellipses represent isotopic niche widths of 40% (SIBER default) corrected for small sample size (SEAc; Jackson et al 2011). Species native to New Zealand are in bold and shown with dashed ellipses. Percentages of isotopic niche area overlap for each species pair are detailed in Table 4.

corresponding to ant DNA. Once nontarget reads (i.e., bacterial/fungal MOTUs and unassigned MOTUs) were also removed, 69 samples yielded data (51 distinct MOTUs from 19,988 sequences). Among the 51 MOTUs, 22 orders and 34 families were identified (Table 5). There is relatively poor coverage of native New Zealand arthropods in reference databases (Holdaway et al. 2017); therefore, genus- and species-level resolution for many sequences produces misleading identifications and should, therefore, be interpreted with caution. Thus, we graphically present our results in Figure 3 at order level (or subclass/infraclass where lower resolution was not possible).

Most orders were detected infrequently occurring in few samples (Figure 3); however, of the 69 samples that yielded target DNA data, sequences from Coleoptera were detected in 62% of the samples, Lepidoptera in 26% and Diptera in 21%. After rarefaction, data from 34 colonies across eight ant species allowed comparison; however, as more than half of these colonies were represented by *Nylanderia* sp., comparative species analyses were not feasible. The class Insecta was dominant in the diet across all ant species (Table 5).

## Discussion

### Trophic Breadth of Invasive Ants

We predicted that the isotopic values of dominant invasive ant species would reveal a relatively broad dietary niche. Our findings partially verified this, with *T. grassii* and *R. chalybaea* found to exhibit both broad isotopic niches and variation within trophic position. In contrast, other dominant invasive species showed less variation, particularly within their $\delta^{15}N$ values. Nitrogen isotope ratios for *L. humile* and *T. jocosus* revealed these two species obtain nitrogen from lower trophic levels, indicating that they are more reliant on plant-derived food resources than through scavenging or predation of other animal species. This finding is well supported in the literature, at least for *L. humile*, where they exhibit a dietary shift from obtaining nitrogen from animal-sources in their native range to plant sources in their introduced range (Holway et al. 2002; Tillberg et al., 2007). The utilization of carbohydrate-rich resources has been implicated in the invasion success of ants (Rowles and Silverman 2009), thus the finding that *T. jocosus* exhibits a similar feeding habit to *L. humile*, may justify monitoring this species in pest surveillance programmes. Since species in the genus *Technomyrmex* tend to be arboreal or sub-arboreal (Heterick 2009), the lower trophic position for *T. jocosus*, may reflect their habitat associations with vegetation, with plant-based food sources (nectar) being more accessible. This idea was weakly supported by the finding of a negative relationship between $\delta^{15}N$ values and vegetation coverage. Given nitrogen is a limiting nutrient in plant-based diets (Mattson 1980) an association between the two variables is not unexpected. As many invasive ant species feed opportunistically (Holway et al. 2002), colonies in close proximity to vegetated areas are more likely to have access to honeydew and plant nectar and exploit such resources. Thus, the higher reliance on carbohydrate-rich food resources would be reflected in lower $\delta^{15}N$ values.

Our findings also in part supported our prediction that native ant species would exhibit a more specialized, predatory feeding strategy. For most native New Zealand ant species, knowledge of their general ecology and feeding behavior is limited, although our predictions were based on the little information available (e.g., Don, 2007). We found that the trophic position for three of the five native species overlapped with the predator trophic level, supporting our predictions and indicating that native ant species may be more reliant on animal-based sources of nitrogen than nectar and honeydew.

### Competition Through Invasion

The establishment of invasive ant species is often associated with the suppression of other ant species (Holway et al. 2002). Whilst we found mainly random associations between ant species, a significant negative association between the non-native *O. glaber* and both *T. jocosus* and *M. antarcticum*, the latter of which is native, were revealed. Moreover, *O. glaber* and *M. antarcticum* were also found to feed at the same trophic level and had an 82% overlap in their isotopic niches. This dietary overlap, together with the reduced likelihood of co-occurrence, suggest competitive exclusion for the same dietary niche. *Monomorium antarcticum* represents New Zealand's most ubiquitous native ant species (although likely represents a species complex, see Wang and Lester 2004, Dann 2008), occurring across a range of habitats in both the North and South Islands, as well as many offshore islands. This species has been demonstrated to exhibit aggressive behavior toward other ant species, and in some contexts may be able to suppress small colonies of

**Table 5.** List of taxonomic ranks identified in gut contents for all samples sequenced (all ant species combined), as detected through amplification of the COI gene region. Family-level rank was included if present in New Zealand

| Class | Order | Family |
|---|---|---|
| Arachnida | Araneae | Theridiidae |
| | Mesostigmata | Parasitidae |
| | Sarcoptiformes | Acaridae |
| | | Brachypylina |
| | | Ceratoppiidae |
| | Trombidiformes | Eupodidae |
| Chromadorea | Rhabditida | Steinernematidae |
| Clitellata | Haplotaxida | Lumbricidae |
| Collembola | Entomobryomorpha | Entomobryidae |
| | | Isotomidae |
| | Symphypleona | Sminthuridae |
| Diplopoda | Helminthomorpha | Julidae |
| Gastropoda | Stylommatophora | Helicidae |
| Insecta | Archaeognatha | |
| | Coleoptera | Chrysomelidae |
| | | Coccinellidae |
| | | Scarabaeidae |
| | | Staphylinidae |
| | Diptera | Agromyzidae |
| | | Anthomyiidae |
| | | Calliphoridae |
| | | Cecidomyiidae |
| | | Chironomidae |
| | | Culicidae |
| | | Muscidae |
| | | Mydidae |
| | | Sciaridae |
| | | Tachinidae |
| | Hemiptera | Aphididae |
| | Hymenoptera | Apidae |
| | | Braconidae |
| | Lepidoptera | Geometridae |
| | | Tineidae |
| | Megaloptera | Corydalidae |
| | Orthoptera | Gryllidae |
| | Psocodea | |
| Malacostraca | Amphipoda | Talitridae |
| Protura | Sinentomata | |
| Reptilia | Squamata | |

*L. humile*; however, any competitive edge appears to diminish when the *L. humile* colony increases in size beyond a threshold (Sagata and Lester 2009). It is possible that the negative association between *M. antarcticum* and *O. glaber* reflects a similar competitive exclusion of the former species by the latter, which relative to *M. antarcticum*, consists of many more worker ants foraging outside of the nest (A. Probert, pers. obs.).

Interestingly, *O. glaber* and *T. jocosus* were found to have negative co-occurrence and no overlap in isotopic niche. Compared to *T. jocosus*, *O. glaber* was enriched in $^{15}N$ even though *O. glaber* has been found to be a dominant floral visitor, commonly feeding on nectar within the sampled ecosystems (Probert 2019). The data we used to obtain association patterns were largely representative of the non-native ant communities occurring within the ecosystems in which we sampled, with the exception of *L. humile*, which was absent in the occurrence data we used. However, *L. humile* has been widely documented to suppress other ant species in the areas it invades (Holway 2005, Stringer et al. 2009, Inoue et al. 2015), and for this study, was found to only co-occur with other ant species at the very periphery of its invaded habitat (A. Probert, pers. obs).

Three of the most dominant species occurring within open-canopy, short-stature, indigenous ecosystems (*I. sulchieri*, *O. glaber* and *Nylanderia* sp.) (Probert et al. 2020), were found to feed at a similar trophic level, with 66% overlap in the isotopic niche of *I. sulchieri* and *Nylanderia* sp. However, no evidence of negative associations (competitive exclusion) between any of these species was found, even though they co-occurred at some sites. This suggests that an alternative factor to dietary partitioning may enable the co-existence of these dominant non-native species. It is possible that habitat complexity facilitates the co-occurrence of ant species with similar dietary niches (Sarty et al. 2006); indeed, the occurrence of spatial and temporal partitioning in New Zealand ant communities is not well understood.

### DNA Analysis of Ant Diet

We took the approach advocated by Piñol et al. (2014), to obtain prey sequence data without the use of blocking probes for invertebrate prey. Once nontarget DNA was filtered, we were left with 19,000 sequence reads. The majority of ant colonies sampled (59%, $n$ = 102/171) failed to amplify anything other than ant and nontarget DNA (i.e., bacteria, fungi). There are several possible explanations for this; it is possible that the ant guts did not contain amplifiable material because this was either absent or highly degraded. Although other studies have found evidence of PCR inhibition related to the crop structure of ants (Penn et al. 2016), we attempted to reduce this by dissecting out the gut contents from the digestive tract. In some cases, dissections may have still contained parts of the digestive tract; however, in pilot samples, there was no improvement in amplification even with the addition of bovine serum albumin. Whilst we selected primers intended to amplify a broad range of taxa as evidenced through the literature (Brandon-Mong et al. 2015), primers will inherently preferentially bind to sites introducing a bias that is difficult to overcome without adding increasingly specific primer pairs. This highlights the uncertainty in identifying the actual diet of consumers. Nevertheless, metabarcoding provides an invaluable tool to rapidly assess diet within a snapshot of time.

An additional problem associated with the metabarcoding was the low comparable sample sizes after rarefying data, which meant we did not conduct comparable analyses among species. Despite this, the sequence data revealed novel information of the diets for several invasive ant species and provided taxa-specific information unobtainable through stable isotope analysis alone. Three of the largest insect orders, such as Coleoptera, Lepidoptera and Diptera, were most frequent among samples. Coleoptera was found in 62% of the samples, with sequences matching to four different families for this order. Lepidoptera was the second most dominant taxa, comprising a relatively high proportion of sequence reads for the diet of *I. sulchieri* and *T. jocosus* colonies. Whilst overall diversity within colonies was low, this was not unexpected due to the foraging behavior of ants. Although it is not possible to distinguish between food sources acquired via predation or scavenging, identifying the arthropods most likely to be consumed by non-native ants is an important first step to assess the ecological risk of species occurring in natural ecosystems. Future work to describe dietary variety among ant species could be improved by pairing behavioral observations with metabarcoding to better understand the arthropods at risk from predatory behaviors.

## Conclusions

A common characteristic for many invasive ant species is their generalist feeding strategy, which is posited to explain, at least in part,

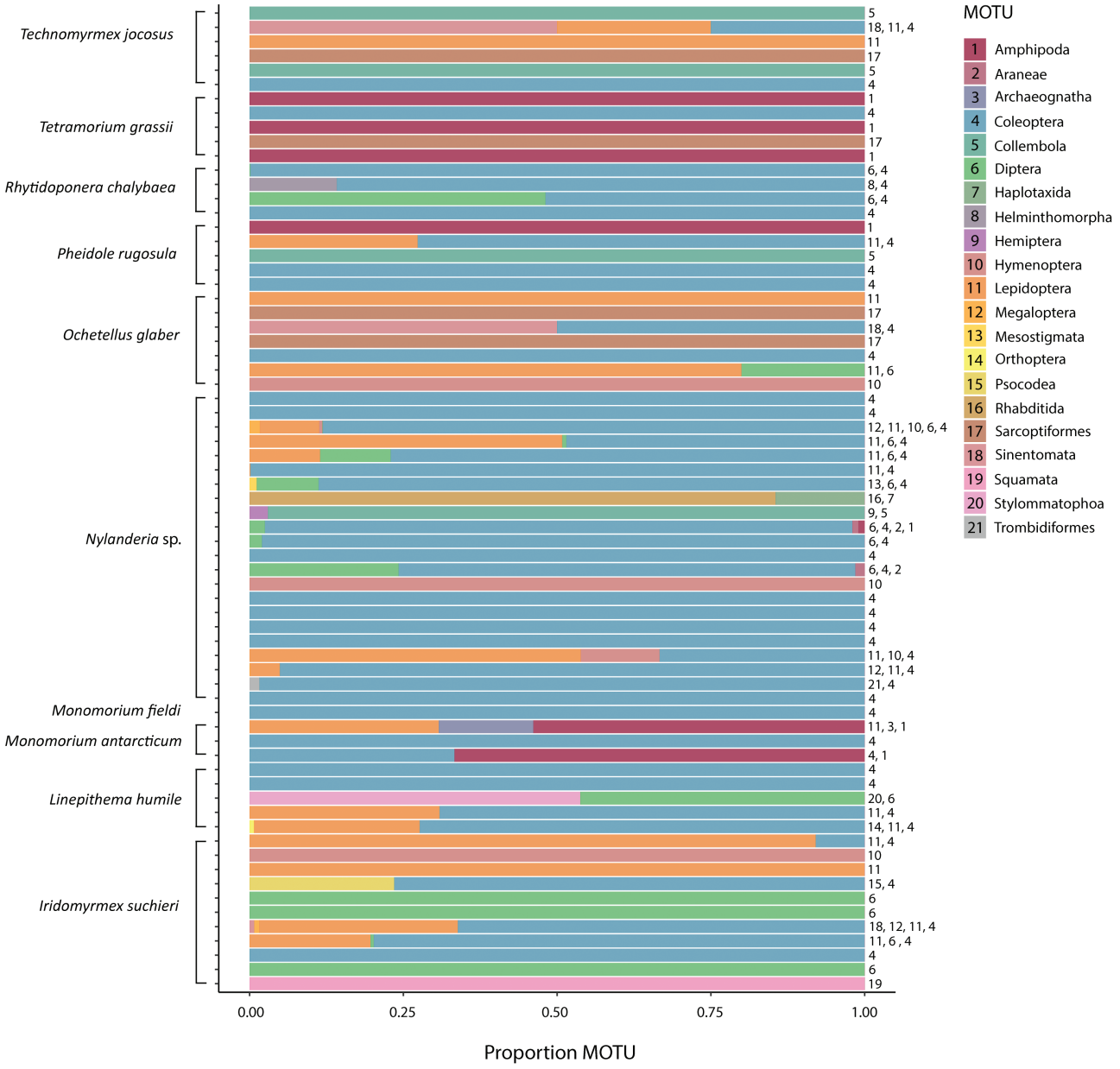


**Figure 3.** MOTU proportions identified at the order level [in two cases, subclass (Collembola) and infraclass (Helminthomorpha)] occurring within the gut contents of different ant species, based on amplification of the COI gene region. Each sample comes from an independent colony consisting of the gut contents for 10 individual worker ants. Numbers correspond to the MOTUs for additional clarity.

the establishment success of species outside of their native ranges (Holway et al. 2002). We used two different methods to investigate the diet of ants commonly occurring within natural ecosystems to understand the potential impact of dietary-related ecological interactions associated with non-native ants. Metabarcoding identified some of the invertebrates at risk of predation impacts from invasive ants. The use of metabarcoding to infer fine-scale variation in diet provides a potential tool for future dietary analyses, although here it proved difficult to obtain sufficient data to compare the diet of native and non-native species. Stable isotope analyses revealed broader-scale variation in the isotopic niche of non-native ant species compared to native ants, yet there was large dietary overlap. A significantly negative co-occurrence pattern was found between a dominant non-native and native ant species, potentially indicating competitive exclusion, however, random co-occurrence patterns were found for most ant species. This may be explained by other factors, not explored here, such as low inter-specific aggression, or temporal partitioning to avoid competition and facilitate occupancy within the same habitat.

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## Conflict of Interest

The authors declare no conflicts of interest.

## Author Contributions

**AFP**, **MCS**, **DFW**, and **JRB**: conceived the project with input from GL. **AFP**: collected and processed the data in the laboratory with help from SJB and much assistance from SMH. **AFP** and **SMH**: analyzed the data. **AFP**: writing of the manuscript with input from all other authors.

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