Consistent shift in nutritional ecology of ants reveals trophic flexibility across alpine tree-line ecotones

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Abstract. 1. Studying the feeding ecology of ants can reveal their trophic position and allow inferences on interactions with other organisms. We investigated the nutritional ecology of ants at the Alpine tree line (from subalpine forests to alpine grasslands), testing the hypothesis that changing food availability reflects upon ant feeding preference and trophic position. Five slopes with five sites each were sampled using a combined experimental (baiting) and chemical (stable isotope) approach.

2. Sugar resources were most preferred by the whole ant community in the alpine environment and on the tree line and were therefore likely limiting in these habitats. This shift was not detected in the two dominant ant taxa occurring over the investigated gradient, viz. the slave ant *Formica lemani* and mound-building wood ants (*Formica* s. str.).

3. Yet, stable carbon (C) and nitrogen (N) isotope signatures of both these ant species likewise revealed a shift in resource use over the ecotone, from a lower trophic position in subalpine forests to a more carnivore-dominated lifestyle in alpine grassland. Moreover, wood ants were found to occupy a higher trophic position compared to *F. lemani*.

4. In conclusion, energy resources turned out to be more limiting for ants in the alpine environment, but less so in subalpine forests. The low trophic position of these ant species in the forest is considered to be a result of abundant trophobiotic associations with honeydew-producing homopterans that occur in far larger numbers on conifer trees in the forest.

Key words. Alpine ecology, ants, ecotone, feeding ecology, stable isotopes, trophic level.

Introduction

In most terrestrial ecosystems, ants exert a significant influence on ecosystem processes such as nutrient fluxes or soil perturbation (Hölldobler & Wilson, 1990; Agosti et al., 2000; Lach et al., 2009), thereby providing essential ecosystem functions and services (del Toro et al., 2012; Wills & Landis, 2017). The most prominent ecological impact of ants is related to their nutritional ecology (Lach et al., 2009). Most ant species in central Europe are considered to be generalist feeders (Fiedler et al., 2007; Seifert, 2018). Yet, determining more exactly the relative contributions of food sources used by ants may provide insight into environmental constraints acting on these insects, with further repercussions on community composition and on the ecosystem effects of the entire ant community (Kaspari et al., 2012; Peters et al., 2014; Orivel et al., 2018).

The resource range of generalist ant species is composed of different types of plant- and animal-derived diets (Blüthgen & Feldhaar, 2009). The main sources of nutrients for European ants are prey organisms (delivering mainly amino acids, lipids, and sodium; Kaspari et al., 2008) and plant resources (mainly carbohydrates, retrieved directly from flowers and extra-floral nectaries, or indirectly through trophobiosis:...
a mutualistic interaction with other insects that deliver nutrients to ants in return for protection; Fiedler et al., 2007; Stadler & Dixon, 2008). Their generalist feeding habits might allow ants to plastically tune usage of different nutrient sources to cope with changing environments or needs (Portha et al., 2002; Csata et al., 2020). On genus or community level, ants were found to change their trophic position, probably as a response to alterations in resource availability (Gibb & Cunningham, 2011). Nevertheless, on the species level, ants seem to maintain a functionally homeostatic stable isotope composition (except for invasive species; Roeder & Kaspari, 2017; Balzani et al., 2021), despite variation in nutrient availabilities (Gibb & Cunningham, 2011; Woodcock et al., 2013; Tanaka et al., 2019). Recently, the question arouse if not all naturally occurring ant species maintain a functionally homeostatic stable isotope composition across the range of ecosystems they inhabit (Tanaka et al., 2019), though a very recent study found evidence of shifting trophic positions in ant species between forests and plantations (Tsang et al., 2020).

Elevational gradients are often chosen as model systems in ecology because here massive variation in important life shaping processes can be observed over small distances (Geraghty et al., 2007; Dunn et al., 2009). Those processes are linked to constraints on net primary production and physiological activity of any organism, both related to the stark decrease of temperatures at high elevations (Körner, 2007). The altitudinal limit of tree growth represents the most obvious effect of increasing environmental stress in high mountain ecosystems (Mayor et al., 2007; Dunn et al., 2009). Those processes are linked to constraints on net primary production and physiological activity of any organism, both related to the stark decrease of temperatures at high elevations (Körner, 2007). The altitudinal limit of tree growth represents the most obvious effect of increasing environmental stress in high mountain ecosystems (Mayor et al., 2007; Dunn et al., 2009). Hence, the tree line ecotone offers the potential of gaining deeper insight into the ways how environmental factors shape ant nutrition and their impact on the ecosystem.

At high elevations, biotic interactions become less important for shaping ant communities, whereas limitation by abiotic factors takes precedence (Stadler et al., 2003; Dunn et al., 2009; Machac et al., 2011; Bishop, 2017). Another crucial aspect is the access to plant-derived carbohydrates (Davidson, 2005; Gibb & Cunningham, 2009), predominantly through trophobiotic partners (Stadler & Dixon, 2008). In Europe, trophobiosis is particularly important for wood ants (genus Formica Linnaeus, 1758) with their huge, long-lived colonies (Domisch et al., 2016 and citations therein). In alpine habitats, trophobiotic homopterans are most common where trees are present, while their density strongly decreases at high elevations (Guariento et al., 2018b).

A typical approach to determine the resource use and preference of ants is to analyse the acceptance of resources offered as standardised baits, either by offering baits with different concentrations of macronutrients (the so called nutritional geometric framework; Raubenheimer et al., 2009) or containing pure resources per bait (Bestelmeyer et al., 2000). The second baiting approach is especially well suited if community-wide resource use is to be investigated in the field. Another way to determine the trophic position of animals in food webs employs stable isotope analysis of carbon and nitrogen (Quinby et al., 2020). By combining stable isotope analysis and experimental baiting, we here aimed to obtain a more complete understanding of the nutritional ecology of alpine ants.

Some studies on resource use of ants along elevational gradients have been conducted in the tropics (Peters et al., 2014; Orivel et al., 2018) and few also on temperate-zone mountains (Fowler et al., 2014; Spotti et al., 2015; Guariento et al., 2018a). Even if all ant species in the alpine zone essentially forage on the same resources, differences in their responses to various baits can be expected. For example, complementarity theory predicts increased visitation of a given resource, the more this resource is limiting in the respective habitat (Kay, 2002). Therefore, from information on bait visitation, it is possible to infer which nutrients are most limiting for a given ant species or community (Kaspars & Yanoviak, 2001) or how limitations might change over environmental gradients (Stadler & Dixon, 2008; Lasmar et al., 2021). The scarcity of trophobiotic partners at high elevations (Stadler et al., 2003) is concordant with a more intense usage of carbohydrates by ants observed in the alpine vegetation zone (Spotti et al., 2015; Guariento et al., 2018a). In contrast, the visitation of carbohydrate baits exposed in forest is expected to be lower, mainly because of the natural abundance of trophobiotic Cinara Curtis, 1835 aphids on conifers, which offer a consistent and reliable supply of carbohydrates for ants (Domisch et al., 2016). Finally, stable isotope analysis can reveal which ant species occupy higher or lower trophic positions (Feldhaar et al., 2010), even within wood ants (Balzani et al., 2021). For the present system, mound-building red wood ants (Formica s. str.) and the black alpine slave ant Formica (Serviformica) lemani Bondroit, 1917 (hereafter referred to as F. lemani) display contrasting positions in a dominance hierarchy (Savolainen & Vepsäläinen, 1988) and a different feeding behaviour (Seifert, 2017), and will be compared in terms of their trophic positions occupied in this system.

In the present study, we therefore investigated the nutritional ecology of ants across the alpine tree line ecotone in the European Central Alps. Rather few ant species occur in upper montane to subalpine forests, and even less species inhabit the low alpine grasslands in Europe. In the Central Alps, for example, less than 20 ant species occur regularly above the tree line, all of which are generalist feeders (Seifert, 2018).

Overall, we expected that the trophic position of the ant community within the food web is lower in the upper montane forests, where due to the abundance of honeydew sources the ants rather attain a ‘herbivore’ role. In contrast, we expected a higher trophic position in alpine grassland, where limited availability of trophobiotics should lead to more opportunistic and predatory feeding habits in ants. These expectations are also supported by a functional trait analysis of species’ nutritional preferences extracted from literature and weighted upon the ant community records in the same system (Guariento & Fiedler, 2021).

Specifically, we tested the following hypotheses:

1 As predicted by compensation theory, the ant community shows a stronger attraction to carbohydrate baits (and higher trophic position) in the alpine grassland and a lower attraction in the forest (where they occupy a lower trophic position) because of variation in the abundance of trophobiotic partners.

2 Resource attraction and stable isotope composition are expected to change over the gradient also intra-specifically in those ant species occurring over the whole gradient.
Materials and methods

Study sites

Sampling was conducted on five slopes of the southern part of the central siliceous Alps, in South Tyrol (Italy). To facilitate comparisons among sites and limit human impact, only south facing slopes within protected areas (Texelgruppe, two slopes; Stilfserjoch, three slopes) were chosen (Fig. 1). Each slope comprised a gradient of vegetation types from subalpine coniferous forest to open alpine grassland interspersed with dwarf shrubs, without major changes in exposition and steepness (Table S1; for more details on environmental parameters and dominant plant species see: Guariento, 2018). The tree line itself was in most cases rather clearly defined because of management that occurred in the past and persists in the form of extensive summer pasturing by cattle (Tasser et al., 2001).

On each slope, five sites of 800 m² area each (40 m × 20 m rectangles, set up orthogonal to the slope) were established. Sites were selected in reference to the tree line with one placed directly on the ecotone itself (tree line elevation ranged between 2090–2300 m), two above in the alpine zone, and two below in subalpine forest. Sites were spaced by 50 elevation meters from each other, thereby covering roughly 200 m of elevation per slope and 470 m in total from 1935 m (the lowest site) to 2405 m (the highest site). Sampling took place in July and August 2016 and 2017.

According to Spotti et al. (2015) the highest ant activity outside the nests occurs in the Alps between 10 and 12 am and ant activity starts to decline significantly after 14.00. Bait exposure was therefore set to occur within this rather narrow time frame, but never started before 08:30 and usually ended before 16.00.

Baits

Baits with six different resource types were exposed following a well-established protocol (Kaspari et al., 2012; Fowler et al., 2014; Peters et al., 2014; Spotti et al., 2015). In summer 2016, five replicates of every resource, grouped in transects with one bait per resource, were placed simultaneously on the ground at each site. All baits were separated by a distance of 2
to 3 meters from the next one. Individual bait transect lines were separated from another line by at least 5 m. In summer 2017, additional baiting took place with two transects on grassland and forest sites and four transects on ecotone sites. In total, 1110 individual baits were offered.

Baits were offered on the ground in 50 ml plastic centrifuge tubes (Microboss HighTech SG), so that the opening was leaning on the ground. The amount of liquid bait was between 10 and 20 ml per tube, applied to a cotton ball to prevent from spilling over and to maintain moist conditions throughout the exposure time. Bait resources were: tap water, NaCl (20 g table salt L$^{-1}$ H$_2$O), sugar (200 g sucrose L$^{-1}$ H$_2$O), lipids (commercially available pure virgin olive oil as offered in: Fowler et al., 2014; Peters et al., 2014; Spotti et al., 2015), one amino acid (200 g L-glutamine L$^{-1}$ H$_2$O; as offered in: Fowler et al., 2014; Peters et al., 2014), and a sugar-amino acid mix (100 g sucrose + 100 g glutamine L$^{-1}$ H$_2$O). Mixtures of carbohydrates and amino acids were found to attract more ants compared to individual baits in some studies (Blüthgen & Fiedler, 2004; Kaspari et al., 2012).

The usage of just one amino acid and one sugar type may not be optimal since it has been demonstrated that some ants prefer a mixture of different sugars and amino acids (Blüthgen & Fiedler, 2004), but for practical reasons and because several studies already deployed baits with exactly the same resource types and concentrations (Kaspari et al., 2012; Fowler et al., 2014; Peters et al., 2014), we decided to run the sampling the same way.

At the beginning of bait exposure, the air temperature was recorded at 1 m height aboveground in the shade. The time of sampling on each site varied randomly within the aforementioned time frame, starting between 9 and 12 am. The exposure time of baits per site ranged between three and four hours. Exceptionally, the retrieval of 144 baits (13%) occurred after this time frame until 6 pm because of remoteness of the sampling locations; exactly, these same baits were also exposed for longer time periods (up to 6 hours). At the end of exposure, each bait was closed with a screw cap and taken to the lab, all ants present were counted and tentatively subdivided into morphospecies. Voucher specimens of each morphospecies per bait were subsequently identified to species level using the keys in Seifert (2018) and Wagner et al. (2017), with the help of a stereo-microscope (90-fold magnification) connected to a computer.

**Stable isotope analysis**

Stable isotope analysis ($^{13}$C and $^{15}$N) was performed to obtain insights into the trophic position occupied by the ant species within the community (Fiedler et al., 2007) and whether this position changes across the tree line ecotone. The material was collected in summer 2017 by hand picking worker ants directly from several nests within the sites. Only samples of *F. lemani* and wood ants (mound-building red wood ants belonging to two species: *Formica lugubris* Zetterstedt, 1838 and *Formica aquilonia* Yarrow, 1955) were analysed, because judged by the visitation intensity on baits these species emerged as by far the most ecologically important ones. Mature foliage (fully expanded leaves with no signs of senescence) of representative plant species (divided into woody and herbaceous plants, combined into a mixture of three to five species per site for isotope analysis) were also collected to provide a site-specific isotopic baseline of the food chain. Several leaves from the more abundant plant species (at least representing three woody species and five herbaceous species) were collected at each site. Haphazardly collected herbivores (‘homopterans’, Orthoptera and Lepidoptera larvae) expected to feed on these plants were also collected. Specifically, at least three individuals of two different insect orders were sampled per site. For the isotope analysis, a reduced design with only 15 plots (including for each of the five slopes an alpine, a tree line, and a forest site) was chosen.

All sampled organisms destined for isotope analysis were directly killed and stored in 96% ethanol in case of animal specimens, or immediately dried in zip-lock bags with silica gel in case of plant material. Using ethanol as storing liquid is not regarded as the optimal conservation method for isotope analysis (see Tillberg et al., 2006) since over 6 months it may alter ant $^{13}$C (by $\sim$ 0.6‰ due to delipidification) while it does not affect ant $^{15}$N. Since the target ants belonged to the same genus, we expected that if there was any effect on their stable C isotope composition, this will be similar for all the collected specimens, hence not influencing the main outcome.

Mixed samples consisted of five to eight fully sclerotised worker ants aggregated from three to five nests of the same species per location. Their abdomens were removed to prevent food stored in the crop to affect isotopic measurements (Blüthgen et al., 2003). For the herbivores, whole Lepidoptera larvae (or several individual homopterans) or the legs of grasshoppers were taken in mixed samples (comprising 2 to 6 individual insects). All samples where dried in an oven at 50 °C for 46 h and homogenised using a ball mill (Retsch MM2000). Ground samples were weighed into tin capsules before being analysed (0.5–0.8 mg for animals; 1.5–2 mg for plant materials). Carbon and N contents and C and N isotope signatures were determined with a continuous-flow isotope mass spectrometer (IRMS) system. The system consisted of an elemental analyser (EA 1110, CE Instruments, Milan, Italy), which was connected by a ConFlo III interface to the IRMS (DeltaPLUS, Thermo Scientific, Bremen, Germany). Reference gases (high purity CO$_2$ and N$_2$ gas, Air Liquide, Vienna, Austria) were calibrated to the Vienna-Pee Dee Belemnite (V-PDB) standard (at-air) using IAEA-NO$_3$, IAEA-N$_1$, and -2 reference materials (International Atomic Energy Agency, Vienna, Austria). Nitrogen and C isotope abundances of each sample were determined in a single run, and N$_2$ and CO$_2$ reference gases were run with each analysis. The natural abundance of $^{15}$N and $^{13}$C was calculated as follows:

$$\delta^{15}N [\% v s. at-air] = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1000$$

$$\delta^{13}C [\% v s. V-PDB] = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 1000$$

where $R$ is the ratio of $^{15}$N/$^{14}$N for nitrogen and $^{13}$C/$^{12}$C for carbon isotope abundance. The standard deviation of repeated
measurements of a laboratory standard was $<0.15\%$ for $\delta^{15}N$, and $<0.10\%$ for $\delta^{13}C$.

**Statistical analysis**

For the baiting experiment, only the presence or absence (i.e. 0/1 incidences) of ants on each bait was used for statistical analysis. Generalised linear mixed models (GLMM) were computed with the package lme4 (function glmer(); Bates et al., 2015) in the R environment (R Core Team, 2020) using a binomial error structure to test which resource was preferred and if there was an interaction with habitat type. Total exposure time and air temperature were tested and included as covariates if they had a significant effect. The bait offering tap water, serving as control, was modelled as intercept. Year, mountain slope identity, site and transect ID were modelled as nested random factors. A model selection was run to implement in each final model only the random factors that resulted to have a significant effect. For the two commonest ant groups, viz. *F. lemani* and wood ants, separate GLMMs were built. These two ant groups reflect strong differences in behaviour (subordinate vs. dominant, *F. lemani* vs. wood ants) and were expected to differ in their nutritional ecology. The two species of mound-building red wood ants, on the contrary, were described to be nearly identical in their nutritional niche (along with other features; Seifert, 2017). They are also closely related (Stockan & Robinson, 2016). Because of these reasons and because these highly dominant species tend to exclude each other locally (since they defend actively a territory), we decided to pool the scores of both *Formica* s. str. species together for both the baiting and the isotope analysis.

The design of our baiting experiment harbours the potential issue of pseudoreplication on the colony level (viz. workers of the same colony may have been foraging on more than one bait). Therefore, observed differences in resource visitation might to some extent reflect colony-specific variation in demands for nutrients rather than differences among species. While we cannot rule this conjecture out completely, we consider this effect to be of minor importance. The foraging distance of wood ants may exceed 100 m (Domisch et al., 2016). Placing baits that far from another to avoid this potential issue would augment variation in other environmental conditions (such as in the vegetation or topographic structure of the rugged alpine terrain). Moreover, the focus of our study was explicitly to investigate a short environmental gradient, spanning just 200 elevation meters. We partially accounted for the issue of pseudoreplication by treating transect, site, and slope identity as random factors. For practicability reasons, we avoided performing aggression tests to differentiate the colony of origin of worker ants at baits. This issue of pseudoreplication is a common feature of baiting studies in the field. Often times, following the rule of thumb and placing individual baits at 10 m distance to another (Fowler et al., 2014; Peters et al., 2014; or even less: Spotti et al., 2015) will not be enough, especially for bigger ant species and/or those with polydomous colony structure. We consider the potentially confounding effect ant colony identity to be of minor relevance because of the robustness of our outcome over five different slopes as true replicates and a broad temporal sampling period.

This renders it very unlikely that clear statistical patterns in resource attraction would have only emerged due to possible differences among individual ant colonies in their resource visitation preferences.

Isotope signatures of animal samples were corrected with reference to the isotopic baseline of the food web, that is, the plant nitrogen isotopic composition for the same sites (as done routinely in ecological studies involving stable isotopes; Quinby et al., 2020), to allow a comparison of ant trophic position across the different habitats and slopes. The correction was based on computing the deviation of plant isotopic signatures of each site from the grand mean plant isotopic signature (across all plant measurements) and subtracting this site-specific deviation from the isotopic signatures of ants (and herbivores) on the very same site. The resulting data for ants were analysed using linear mixed models (package nlme; Pinheiro et al., 2020), again with slope identity modelled as random factor.

For both GLMMs and LMMs, AICc (AICc $< 2$), $R^2$ (partitioned into marginal and conditional $R^2$; Nakagawa & Schielzeth, 2013), and Akaike weights (Wagenmakers & Farrell, 2004) were computed to determine the variable combinations best explaining the data. Zero inflation was excluded for all models. A null model was always implemented and rejected because of worse fit to the data than the optimal model. Residual distributions were inspected using the performance (Lüdecke et al., 2020) package with default function; residuals met the assumptions (of normality and homogeneity of variances) in all cases. Visualisation of results was done using the package ggplot2 (Wickham, 2016).

**Results**

**Bait experiment**

In total, 2406 individual worker ants were recorded at the baits representing 10 different species (Table S2). Only 321 baits (28.9%) were ever visited by ants. This value continually declined from the alpine (37.4%) across the tree line sites (33.7%) to the forest, with just 17.4% of baits attracting ants (Fig. 2). This rather low fraction of visited baits is in line with results from similar studies applying the same procedure in different habitats (Fowler et al., 2014; Peters et al., 2014; Spotti et al., 2015). The main visitor was *F. lemani*, which was responsible for 77.0% (247 incidences on 22 sites) of total visitations. Wood ants accounted for 29.0% (93 incidences on 20 sites) of visited baits (of which 82%, on 18 sites, occurred by *F. lugubris* and 18%, on four sites, by *F. aquilonia*). Ants of the Myrmicinae subfamily accounted for just 10.6% (35 incidences on 15 sites) of all bait visits, with 5.0% (16 incidences on 5 sites) due to *Manica rubida* (Lateille, 1802) alone. Note that the overall visitation pattern of the entire ant community is therefore largely driven by the high visitation of *F. lemani*.

Visitation incidence also varied among the offered resources. The mixture of sugar and glutamine was visited in 30.8% of the cases (99 incidences), followed closely by pure sucrose baits 29.6% (95 incidences). Salt (16.2%; 52 incidences) and glutamine (15.0%; 48 incidences) were visited equally often, while water (7.2%; 23 incidences) and lipids (just 1.3%;
4 incidences) were mostly ignored (Fig. 2). On 65 baits more than one ant species were found simultaneously. All co-occurrences at individual baits (reason why sum of % of group visitation incidences exceed 100%) included the subdominant ant species *F. lemani*, 35 times together with wood ants and 31 times with various Myrmicinae species.

All resource types differed significantly in visitation from the control bait offering tap water, except for lipids that were significantly less visited (Table 1). Also, habitat scored significantly, thus a change in overall visitation intensity occurred between habitats (Table S3). This interaction between habitat and bait depicts a change in the resource attraction over the gradient (Table S3). This interaction between habitat and resource type resulted as significant only in the case of sugar baits (Table 1). Further, exposure duration influenced the total visitation (i.e. we noted a tendency of more occurrences of ants with longer exposure) and was therefore kept in the final model, although no effect on resource attraction could be detected, meaning that overall resource attraction was not affected by a longer exposure time. Finally, air temperature did not significantly affect overall bait visitation.

Bait visitation was also analysed separately for *F. lemani*, for wood ants, and for combined Myrmicinae species (Fig. 3). Because of the very low visitation of the latter group only a graphical representation is informative. In GLMMs for both *F. lemani* (Table S4) and wood ants (Table S5) habitat scored as a significant factor, with a low visitation incidence in the forest for *F. lemani* and the opposite for wood ants. However, for both groups the interaction between habitat and resource type was non-significant, meaning that along the ecotone the resource attraction did not change. Longer exposition time increased the attraction to baits, although only for *F. lemani* it also increased the visitation on sugar, salt, and the amino acid. Resource visitation by wood ants was higher on sugar, mixture, and salt baits compared to the control bait.

**Table 1.** Contributions of the fixed factors to the best GLMM model based on asymptotic Wald tests (see model selection in Table S3).

| Factor                          | Estimate | SE  | z     | P      |
|--------------------------------|----------|-----|-------|--------|
| (Intercept)/water              | −2.04    | 0.34| −6.00 | <0.001 |
| Habitat                        | 0.28     | 0.27| 1.02  | 0.307  |
| Glutamine                      | 0.93     | 0.29| 3.18  | 0.002  |
| Mixture                        | 2.30     | 0.29| 8.03  | <0.001 |
| Lipids                         | −2.54    | 0.90| −2.81 | 0.004  |
| Salt                           | 1.07     | 0.29| 3.70  | <0.001 |
| Sucrose                        | 2.18     | 0.29| 7.55  | <0.001 |
| Exposition time                | 0.29     | 0.24| 1.23  | 0.220  |
| Glutamine × habitat            | 0.31     | 0.30| 1.02  | 0.309  |
| Mixture × habitat              | 0.52     | 0.29| 1.79  | 0.074  |
| Lipids × habitat               | 1.17     | 0.87| 1.35  | 0.177  |
| Salt × habitat                 | 0.21     | 0.30| 0.70  | 0.487  |
| Sucrose × habitat              | 0.82     | 0.30| 2.76  | 0.006  |

**Stable isotope analysis**

The trophic level enrichment (TLE) from plants to herbivores was on average 1.25‰ for δ¹⁵N and 0.78‰ for δ¹³C (in the special case of plant sucking insects only 0.62‰ for δ¹⁵N and 0.46‰ for δ¹³C). For ants, the TLE calculated between ants and plants was on average 4.82‰ in δ¹⁵N and 2.66‰ in δ¹³C for *F. lemani*, and 5.52‰ in δ¹⁸N and 3.63‰ in δ¹³C for wood ants, respectively. As expected, the heavier isotopes of C and N accumulated along the food chain (Table S6). The two ecologically different ant species groups within the genus *Formica*, viz. *F. lemani* and wood ants, showed a weak segregation from each other (Fig. 4), with wood ants occupying a slightly higher trophic level (2.34‰ in δ¹⁵N on average) compared to *F. lemani* (1.39‰ in δ¹⁵N on average; Fig. 4).

The outcome of a linear mixed model analysis for the nitrogen stable isotope signatures revealed a small but significant reduction in the δ¹⁵N signature of forest ants compared to alpine grassland ants. The decline in δ¹⁸N towards forests was 1.11‰ for *F. lemani* and 0.70‰ for wood ants. Also, the difference between wood ants and *F. lemani* was significant and amounted to 0.67‰ in δ¹⁵N on average (Tables 2 and 3; Fig. 4).

**Discussion**

A high attraction to sugar baits in the alpine grassland above the tree line confirms our first expectation based on complementarity theory (Kay, 2002, 2004), namely, that energy resources (i.e. carbohydrates) are more limiting in the alpine environment in comparison to the forest where intense trophobiosis, mostly with *Cinara* aphids, seems to cover this need. Yet, a lower usage of amino acid and salt in the forest (as a consequence of a more vegetarian nutrition style following complementarity theory) was only recorded as a weak and non-significant tendency among wood ants. The expected intraspecific change in resource attraction was not detected for *F. lemani* or wood ants based on bait visitation. However, our results from stable isotope analysis indeed confirmed a parallel change in trophic position in both ant groups over the tree line ecotone.

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Ant nutritional ecology across tree-line ecotones

Fig. 3. Mean incidence (±95% confidence limits) of bait visitation by three ant species groups across habitats. [Colour figure can be viewed at wileyonlinelibrary.com].

(a) (b)

Fig. 4. (a) Double box-plot of C and N isotope signatures (autotrophic signatures corrected by plant baseline) of all analysed samples, grouped as plants (woody and herbaceous plants combined), herbivorous insects (grasshoppers, moth larvae and plant lice combined) and the two main ant players along the elevational gradient, viz. *F. lemani* and wood ants. Also, a lower scoring of *F. lemani* in comparison to wood ants can be seen. (b) Double box-plot of C and N isotope signatures of *F. lemani* (on the left) and of wood ants (on the right; *F. lugubris* and *F. aquilonia* combined, the only *F. aquilonia* sample is marked with a red dot) split by habitat of collection reveals an overall lower trophic position of ants in subalpine forests than in alpine grasslands and on the ecotone. [Colour figure can be viewed at wileyonlinelibrary.com].

Table 2. Results of the selection of a linear mixed model on base-line corrected N isotope signatures of ants.

| Fixed factor | AICc  | Δ AICc | w    | R² marginal (in %) | R² conditional (in %) |
|--------------|-------|--------|------|-------------------|----------------------|
| Null model   | 98.45 | 6.57   | 0.02 | 0                 | 9.64                 |
| Habitat      | 93.89 | 2.01   | 0.24 | 24.55             | 38.58                |
| Habitat + species ID | 91.88 | 0      | 0.66 | 35.56             | 50.21                |
| Habitat × species ID | 96.48 | 4.60   | 0.07 | 34.28             | 48.87                |

Slope was modelled as random factor. AICc, Akaike weights (w) and R² marginal/conditional were used for model selection. The best model is highlighted in bold face.

Table 3. Linear mixed model outcome of baseline-corrected nitrogen isotope signatures of ants.

|                         | Value | SE  | d.f. | t value | P     |
|-------------------------|-------|-----|------|---------|-------|
| (Intercept)             | 1.94  | 0.32| 24   | 6.14    | <0.001|
| Tree line               | 0.29  | 0.32| 24   | 0.91    | 0.369 |
| Forest                  | −0.94 | 0.32| 24   | −2.93   | 0.007 |
| Wood ants               | 0.67  | 0.26| 24   | 2.53    | 0.018 |

Alpine grassland specimens differed from forest ones, whereas tree line samples did not differ from grassland specimens. Also, a significant difference between *F. lemani* and wood ants was detected. Significant effects (P < 0.05) are highlighted in bold face.
The strong demand of alpine ants for carbohydrates has earlier been found by Spotti et al. (2015) (along a montane to subalpine gradient) and by Guariento et al. (2018a) in alpine grassland. This sugar demand appears to be a consistent characteristic of ant communities of alpine habitats. Our results also match with the changes in the availability of trophobiotic aphids which are far less abundant above the alpine tree line (especially of the genus Cinara; Guariento et al., 2018b). Moreover, a lower carbohydrate visitation by ants in habitats with increased environmental primary production was recently reported by Lasmar et al. (2021) on a biome comparison, a relation that appears also to apply to our study sites. Further, knowing that insects with a more carnivorous lifestyle are more limited in sugar (Kaspari et al., 2012), alpine grassland ants appear to rely more on a carnivorous feeding habit than at lower elevations. Interestingly, the indirect analysis based on functional species traits and community composition in the same system revealed the same outcome, viz. more carnivorous feeding habits in alpine grassland and increased intensity of trophobiosis in subalpine forest (Guariento & Fiedler, 2021). The results of stable isotope analysis largely corroborated this inference and detected this effect on the species and group level where bait visitation scored as non-informative.

Interestingly, δ¹⁵N signatures followed the same response over the tree-line ecotone for the two groups of Formica ants (Fig. 4). The subordinate F. lemani may switch its feeding behaviour towards a more opportunistic life-style and collect smaller prey items in the presence of wood ants in coniferous forests (as found for the closely related species Formica fusca; Savolainen, 1991; Johansson & Gibb, 2016). Consequently, a change in resource attraction over the ecotone (as expected by the change in availability of trophobionts) could have been masked, since wood ants were more abundant in the forest (Guariento & Fiedler, 2021). However, since the isotope data displayed the same trophic change for both ecological groups within the ant genus Formica, we do not expect that competitive interactions among dominant and subordinate ant groups alone explain this pattern.

Higher δ¹⁵N values and therefore a higher trophic position were found for the wood ants than for F. lemani (Fig. 4, Table 3). Two different explanations may apply here: (i) F. lemani may overall feed more on plant resources or (ii) the arthropod prey of F. lemani occupies a generally reduced trophic position. As a further hint, wood ants also feed on ground active spiders (Sanders & Platner, 2007) that due to their predacious habits can be expected to have elevated δ¹⁵N signatures. Moreover, spider abundance was found to negatively correlate with wood ant density on exactly the same locations, indicating the possibility that wood ants indeed predate on ground spiders (Guariento et al., 2018b). Wood ants also feed on arthropods from the brown food web (Robinson et al., 2016), which might further shift their isotopic N signatures towards higher levels.

Tanaka et al. (2019) recently hypothesised that naturally occurring ant species (as opposed to invasive alien species) maintain their isotopic composition, and thus their trophic position, over habitat gradients, despite variation in resource abundance. Our results across the alpine tree line ecotone, in contrast, indicate that a subtle change in their isotopic composition and therefore in their trophic niche does happen also in naturally occurring native ant species, although this change was far smaller than a whole trophic level step. The overall TLE of both groups of Formica ants in our study was similar to the level found for Formica podzolica (Mooney & Tillberg, 2005), a species of North America with a similar ecological role as held by F. lemani in the Alps (Deslipping & Savolainen, 1994).

Baits containing a glutamine/sucrose mixture were visited similarly as pure sucrose baits, suggesting that sugar was the main cause of ant attraction to this resource mixture. We did not observe an enhanced visitation to this mixed bait (as found by Detrain et al., 2010). Furthermore, ant visitation of baits containing glutamine or salt was overall low. Only mound-building red wood ants in the subalpine forest showed a slight affinity to these two resource types. Blüthgen and Fiedler (2004) and Peters et al. (2014) found a tendency of some tropical ants to reject glutamine as sole amino acid in nectar-mimic baits. This might have been an issue also in the present study, where this amino acid was offered individually in aqueous solution. Nevertheless, usage of glutamine was very similar to the usage of salt solution in our study, most probably indicating the general quest of ants for prey items. Carnivorous arthropods are expected to be limited by lipids, and lipids are regarded as interchangeable to carbohydrates for ants as a carbon source (Rosumek et al., 2017). Indeed, Peters et al. (2014) found support for lipid limitation of ants at the community level with increasing elevation along an alfromontane elevational gradient. In the present study, however, lipids were clearly avoided by all ant species occurring around the alpine tree line and were even visited less frequently than pure water. This rejection of lipids by ants in the European Alps was also observed by Spotti et al. (2015) (using virgin olive oil) and Guariento et al. (2018a) (using peanut butter) and therefore seems to be a consistent characteristic of alpine ants. The rejection of lipid baits might depend on the resource offered (olive oil, i.e. a plant-derived rather than animal-derived source) and less on lipids per se as bait. However, Fowler et al. (2014), Peters et al. (2014), and Lasmar et al. (2021) also used virgin olive oil as bait and observed a higher visitation rate than at other resources. Additional studies in further alpine settings worldwide would be necessary to provide a more comprehensive picture of lipid and sugar limitation of ants in these harsh habitats.

Finally, ants of the subfamily Myrmicinae occurred on the baits only rarely, even though they comprised a rather varied assemblage of species belonging to the genera Tetramorium, Leptothorax, Myrmica, Tetramorium, and Manica. All these ants belong to the subordinate fraction of European ant communities and are usually displaced by the more dominant mass-recruiting Formica ants at resources after some time (Savolainen & Vepsäläinen, 1988; Pisarski & Vepsäläinen, 1989). Due to our limited data on these subordinate ants, we cannot discuss their feeding preferences any further except for noting that sugary baits were clearly preferred (as one would expect considering their rather carnivorous feeding preference [Seifert, 2017]), and their bait visitation activity tended to be high at sites directly situated at the tree line.
Conclusion

The ecological role of the entire ant community changed, although in a subtle manner, from the subalpine forest to the alpine grassland vegetation zone. In the forest, ants were more shifted towards a ‘herbivorous’ life style in comparison to a more ‘carnivorous’ habit in the alpine zone. The most likely main driver of the high prevalence of wood ants (as for their lowered trophic position) in the forest was a high density of trophobiotic aphids, delivering substantial carbon (and so energy) supply in a kind of short-cut from trees. In the alpine zone, on the other hand, trees (as key stone species) were missing, thereby limiting the abundance of trophobionts and driving the ants to follow a more carnivorous feeding style.

The advantage concerning the trophic flexibility of ants over habitat gradients is that more species have the potential to coexist by fine-tuning their realised nutritional niches (e.g. in the presence of dominant species as by the slave ants; Savolainen, 1991) or to persist in marginal habitats (e.g. the mound-building wood ants above the tree line). On the other hand, this variation in the trophic position of individual ant species over a gradient poses the analytical challenge of inferring the trophic position of ant species in functional studies. Hence, it is required to take samples at every site, since extrapolations based on average isotope signatures, even within the same ant species from one site to the next, may be misleading.

Overall, the combination of baiting experiments and stable isotope analyses yielded largely concordant complementary results and proved to be important for a deeper functional understanding of alpine ant assemblages, which are still under-explored in most mountainous regions of the Earth.

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Ethics statement

Sampling permissions were granted by ‘28.4. Amt für Landschaftsökologie’ from the Province of Bozen, Italy.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Site information. MAT (mean annual temperature) and MAP (mean annual precipitation) was derived from (Wessely et al., 2017).

Table S2. Ant species incidence per site and resources visited, summed per habitat. Note that alpine grassland (A) and sub-alpine forest (F) had 10 sites each and tree line (T) just five. Formica lemani was present on every alpine and tree line site both on sucrose and mixture baits.

Table S3. Results of model selection for bait visitation. Only random factors with significant influence were implemented, with year of sampling and site ID nested within year. Slope and transect resulted as non-informative random factors. AICc, Akaike weights (w) and R²marginal/conditional were used for model selection. The model used in Table 1 is highlighted in bold.

Table S4. GLMM output of the best model on bait visitation by the alpine slave ant (Formica lemani). Note that habitat and bait type interaction resulted non-significant. All random factors had a significant influence. Transect was nested in site, nested in slope and nested in year of sampling.

Table S5. GLMM output of the best model on bait visitation by the wood ants (Formica s. str.). Note that habitat and bait interaction resulted non-significant. Only random factors with significant influence were implemented. Site was nested in slope and nested in year of sampling. Transect ID was non informative as random factor.

Table S6. Stable isotope signatures are reported for mixed samples collected at each single site for 15N (Table A) and 13C (Table B). The average and standard deviation per habitat is also reported in Table C. Values of heterotrophs are corrected base on the plant values on each site.

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