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Wet Season Carbon ($\delta^{13}\text{C}$) and Nitrogen ($\delta^{15}\text{N}$) Composition of Modern Plants as Isotopic Framework for Agropastoral and Palaeoecological Studies in Northern Greece

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ABSTRACT

Mediterranean wetlands are one of Europe's most vital and endangered biodiversity hotspots. This study determined the carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope values of modern plants to construct an isotopic framework by which to contextualize agropastoral management in and around past wetland ecosystems. Dispilio is a Neolithic site (5700–3600 cal BCE) on the shore of Lake Kastoria, western Greek Macedonia, where ongoing studies are exploring the nature and scale of livestock management and its implications for understanding how early farming communities adapted to wetland and upland ecosystems. In order better to interpret the stable isotope values of faunal bone collagen from the site in terms of animal diet, habitat use, and feeding strategies, this study examines the carbon and nitrogen isotopic composition of 126 wild plants from 12 localities within the Dispilio catchment area and one proxy location on neighboring Lake Prespa. The plants were collected at the end of the wet season across different habitat types, and the influence of precipitation and altitude was considered. The results indicate a high variability of plant carbon and nitrogen isotope values in the environment. The $\delta^{13}\text{C}$ values differ amongst life forms (trees/shrubs, grasses, and herbs) with implications for distinguishing the feeding habits and anthropogenic management of domestic grazers and browsers. Furthermore, $\delta^{15}\text{N}$ values of terrestrial and wetland habitats differ significantly, demonstrating the potential to distinguish isotopically between the use of these landscapes in the past. This study will serve as the first stable carbon and nitrogen isotopic framework for agropastoral, palaeodietary, and palaeoecological studies in northern Greece.

1 | Introduction

Stable isotope analyses of faunal bone collagen have become a fundamental technique for the study of animal husbandry systems in the past (Balasse et al. 2003; Schulting et al. 2017; Vaiglova et al. 2018; Hamilton et al. 2019; Isaakidou et al. 2019). The isotopic systems (C, N, O, Sr., S) implemented in these studies are closely linked to local and regional environmental factors. Accordingly, to interpret past agropastoral activity securely, it is vital to understand the driving forces of isotopic variability in the local environment. The development of isotopic baselines provides the necessary basis for

palaeoecological, paleo-agropastoral, and palaeodietary studies (Hartman and Danin 2010; Szpak et al. 2013; Ventresca Miller et al. 2019).

Stable carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotopes are commonly used to reconstruct animal diets, the local conditions in which animals lived, and by extension human management practices. The isotopic composition of their bone collagen, tooth dentine, and enamel is most closely related to the foods they consume (Deniro and Epstein 1981; Ambrose and Norr 1993; Lee-Thorp 2008). In the case of herbivores, these foods are plants. To infer the types of plants consumed and habitats in which

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herbivores were feeding, from the stable isotope values of their bone collagen, an interpretative isotopic framework is needed. Because the plant parts consumed by herbivores are rarely preserved in the archaeological record, modern plant isotope values can be a useful proxy for ancient plant isotope values. Although these values cannot be applied directly to the past, their strong correlation with local ecosystem processes can be used to deduce similar mechanisms that drove isotopic variability in the past environment.

Dispilio is a Neolithic (c. 5700–3600 cal BCE) (Maczkowski et al. 2024, Vidas-Cardador et al., forthcoming) to Bronze Age (c. 2300–2100 cal BCE) (Facorellis et al. 2014) site on the shore of Lake Kastoria (Orestiada), northern Greece, and is one of several lakeshore pile-dwelling sites in the “lake district” of the Balkans and one of the earliest in Europe (Hafner et al. 2021). The site lies at the intersection of Mediterranean and Alpine biogeographic and subtropical and temperate climatic zones (Pinborg and Larsson 2002). Ongoing studies at the site are using multiproxy approaches to explore climatic, environmental, and agricultural developments during the site occupation (“Exploring the dynamics and causes of prehistoric land use change in the cradle of European farming” (EXPLO) ERC project). A significant component of this research is the nature of livestock management at the site and its implications for understanding how agriculture developed within wetland and upland ecosystems. Establishing an isotopic framework is necessary to understand and contextualize the archaeological faunal carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) values in terms of their diet and the habitats exploited. While other studies in Greece have investigated plant and animal management practices using these isotopic systems, they compared the isotope values of domestic herbivore bone collagen to those of wild fauna or made comparisons between domestic species (Chantzi et al. 2017; Vaiglova et al. 2023). Only in one instance so far was the relevance of modern plants explored for interpreting prehistoric domestic plant and animal datasets from Crete, where, in the absence of local modern plant baselines, those from Hartman and Danin’s (2010) study in Israel were used (Isaakidou et al. 2022). No study thus far has used modern plants to establish an appropriate stable carbon and nitrogen isotopic baseline for Greece. This is particularly crucial given Dispilio’s transitional position between climatic and environmental contact zones.

This study investigates the stable carbon and nitrogen isotope composition of wild plants collected from the Dispilio catchment area to examine the variability of isotopic composition across the local landscape. This study demonstrates the high isotopic variability in the environment and the isotopic distinction between wetland and terrestrial habitats, as well as life forms. These values will serve as a framework for interpreting the isotope values of archaeological faunal remains from the Neolithic site of Dispilio and inform interpretations about animal husbandry practices, land use, and the adaptability of past agriculture within the context of local ecosystems. A greater understanding of the agricultural systems which subsisted here for millennia without this level of ecological impact may inform contemporary discussions of the preservation of ecosystem services, conservation, and rewilding of wetlands

in Greece and the Mediterranean (Zogaris, Skoulikidis and Dimitriou 2017; Taylor et al. 2021).

2 | Study Site

Dispilio is located in northern Greece near its borders with Albania and North Macedonia (Figure 1). The site lies at an altitude of about 630 m. on the shore of Lake Kastoria, a small, shallow, naturally eutrophic lake extending over c. 30 km² with an average depth of 4 m and a maximum depth of 9 m. The lake is hydrologically open with inflow mainly from ground-water springs and streams and is surrounded by the Verno, Askio, Korisos, and Vigla mountains, which form part of the Pindus range.

The Kastoria basin encompasses a mosaic landscape with diverse habitats. Around 30% of contemporary land use is dedicated to agriculture and pasture and 2% to urban infrastructure (Demertzi et al. 2019). The flora is dominated by grassland areas in the lowlands, deciduous oak forests, and shrubby vegetation in the uplands and beech and coniferous forests at higher elevations. The wetland landscape by the lake is populated by willows, poplars, elms, and aquatic species (Karkanas et al. 2011; Papanikolaou and Panitsa 2020). The climate in Dispilio is continental to sub-Mediterranean, characterized by temperate weather with cold winters and warm and dry summers. The mean annual temperature is 12°C, while the mean annual precipitation is c. 700 mm and increases with altitude. The region experiences a wet season from October to May (mean temp. c. 6°C, mean precipitation 64 mm per month) and a dry season from June to September (mean temp. c. 21°C, mean precipitation 29 mm per month). The climate classification is Cfb in the lowlands and Dfb in the uplands based on the revised Köppen Geiger classification system (Peel et al. 2007).

3 | Theoretical Background

Plant stable carbon isotope ($\delta^{13}\text{C}$) values express the ratio of ¹³C relative to ¹²C and reflect the carbon isotope discrimination that occurs when plants incorporate atmospheric carbon dioxide (CO₂) into their tissues during photosynthesis (Farquhar et al. 1989). In the Mediterranean, most plants follow a C₃ (Calvin cycle) photosynthetic pathway and tend to have $\delta^{13}\text{C}$ values ranging from −22‰ to −34‰ (O’Leary 1988; Farquhar et al. 1989). The $\delta^{13}\text{C}$ values in C₃ plants are related to stomatal conductance—the rate of passage of CO₂ through stomata—which is influenced by water availability (Farquhar et al. 1989). During periods of limited water availability, stomata close to reduce water loss, also reducing the flow of CO₂, which decreases discrimination against ¹³C and leads to less negative $\delta^{13}\text{C}$ values. C₃ plant $\delta^{13}\text{C}$ values therefore tend to be negatively correlated with the amount of precipitation (Hartman and Danin 2010). C₄ plants follow a different photosynthetic pathway (Hatch-Slack) and tend to be more water-use efficient. C₄ plants represent a low percentage of the biomass in the Kastoria basin, limited to the dry season. These plants were not available to sample during the wet season and are not expected to contribute significantly to herbivore diets in this season.

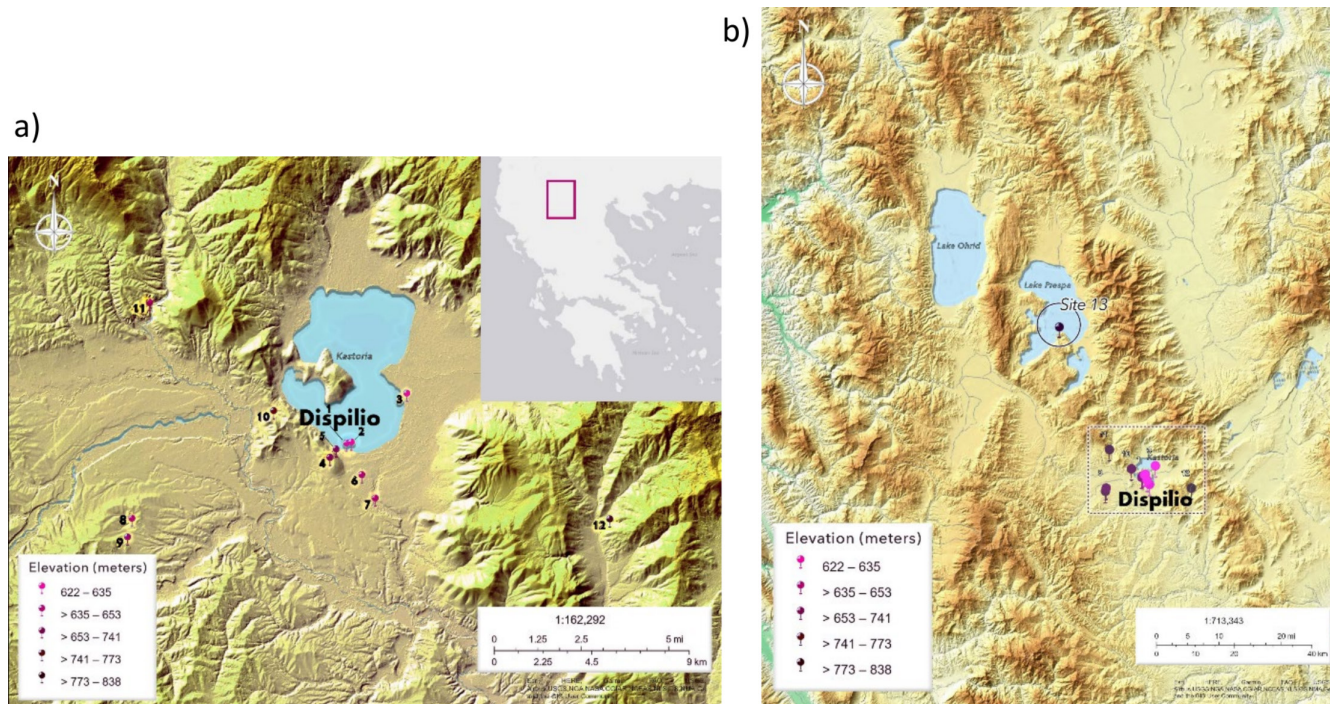


FIGURE 1 | (a) Map of Dispilio catchment area showing plant vegetation survey sampling locations. Sampling locations: 1. Dispilio archaeological site, 2. Dispilio archaeological site reconstruction, 3. Mavrochori, 4. Slope facing Argos Orestiko plain, 5. Dispilio ridge, 6. Between Dispilio/Ambelokipi, 7. Ambelokipi, 8. Avgi archaeological site, 9. Kouri near Avgi, 10. Maniakoi, 11. Koromilia, and 12. Germas. (b) Map of Lake Kastoria with sampling points and Lake Prespa with sampling location 13. Psarades. Maps created by Elizabeth Rosenbloom/Rosen Design LLC based on data from Garmin GPS.

Plant carbon isotopic composition has been shown to vary amongst life form types. Several studies have observed differences in $\delta^{13}\text{C}$ values between woody (tree, shrub) plants and herbaceous (herb, grass, vine) plants, with woody plants showing consistently higher $\delta^{13}\text{C}$ values (Benner et al. 1987). The higher $\delta^{13}\text{C}$ of woody plants may be explained by differences in chemical composition and the proportionally greater requirement of carbon to synthesize lignin. In contrast, the relatively low carbon content may be due to their low lignin content and relatively high growth rate (Lamlom and Savidge 2003; Johnson et al. 2007; Ma et al. 2018). The lignin content also tends to correlate with rooting depth and water source, with high-ligneous (deep-rooting) plants using groundwater, and low-ligneous (shallow-rooting) plants utilizing rainwater. There is some indication that shrubs may also utilize rainwater but more systematically depend on groundwater (Dodd et al. 1998). This carbon conservation of woody plants may also be related to the stomatal limitation of long-lived leaves aimed at avoiding excess water loss and potential leaf mortality later in the year (Escudero et al. 2008). Differences have also been observed in $\delta^{13}\text{C}$ values of different plant parts such as leaves, stems, roots, and other tissues, which may be due to the variable proportions of lipids, lignin, cellulose, sugars, and starches stored in different plant tissues (Badeck et al. 2005). This study chose to focus on leaves for comparability and because they represent the largest biomass contribution to domestic herbivore diets, but studies have found that leaves tend to be depleted in ^{13}C compared to other tissues by as much as 2–4‰ (Cernusak et al. 2009; Szpak et al. 2013). Thus, the consumption of nonphotosynthetic tissues may slightly elevate average $\delta^{13}\text{C}$ values.

In addition to water availability and physiological properties, other factors that may influence $\delta^{13}\text{C}$ values include temperature, altitude (Jiang et al. 2024), soil moisture, and salinity, which relate to water availability (Van Groenigen and Van Kessel 2002), and light intensity (the “canopy effect”), plants growing in dense forests exhibiting lower $\delta^{13}\text{C}$ values than those in open areas due to lower rates of photosynthesis and the recycling of ^{13}C -depleted CO_2 (Vogel 1978).

Plant stable nitrogen isotope ($\delta^{15}\text{N}$) values express the ratio of ^{15}N relative to ^{14}N and reflect the nitrogen isotopic composition of the plants' nitrogen source (Virginia and Delwiche 1982; Högberg 1997). Like carbon, this process is heavily dependent on plant physiology and environmental conditions. Atmospheric nitrogen (N_2) enters the biosphere through the nitrogen cycle whereby N_2 is taken up by bacteria living in soil, which then fix it into compounds plants can use. Most plants are non- N_2 -fixing, meaning that they rely on additional microbial activity in the soil to transform the original N_2 into other compounds such as nitrate (NO_3^-) and ammonium (NH_4^+), which are bioavailable for them to take up. Because non- N_2 -fixers derive their nitrogen from soil that is subject to a variety of isotope fractionation processes, they are more susceptible to effects that can influence soil nitrogen composition. This in turn means that they tend to have $\delta^{15}\text{N}$ values that are more variable in relation to the atmospheric $\delta^{15}\text{N}$ (Virginia and Delwiche 1982; Högberg 1997; Evans 2001; Evans 2007). In contrast, N_2 -fixers derive their nitrogen through direct symbiotic relationships with bacteria residing in their root nodules and tend to have $\delta^{15}\text{N}$ values closer to that of atmospheric nitrogen ($\delta^{15}\text{N} = 0\text{‰}$). Several environmental factors can influence $\delta^{15}\text{N}$ values of soil and thus of plants.

The effect of bacterial denitrification is associated with waterlogging in anaerobic aquatic ecosystems and is thus particularly relevant to the wetland setting of Dispilio. In low-oxygen environments, denitrifying microbes in soil convert NO_3^- to N_2 gas during which the lighter isotope ^{14}N is lost from the ecosystem, leading waterlogged soils to become enriched in ^{15}N (Tiedje et al. 1982; Granger et al. 2008; Inglett and Reddy 2006; Sebilo et al. 2019). Another source of soil and plant ^{15}N -enrichment is the deposition of animal manure on fields during livestock grazing due to increased ammonia volatilization causing the loss of the lighter ^{14}N isotope in the form of gaseous NH_3 (Bogaard et al. 2007; Szpak 2014). Other environmental factors, which tend to increase plant $\delta^{15}\text{N}$ values are salinity (Van Groenigen and Van Kessel 2002), aridity, related to water availability (Handley et al. 1999; Aranibar et al. 2004), high rates of N mineralization (Kahmen et al. 2008), and processes related to local nitrogen cycling such as forest clearance by burning (Ehrmann et al. 2014), and high levels of organic N relative to plant demand (Aguilera et al. 2008).

4 | Materials and Methods

4.1 | Field Sampling Methods

The vegetation survey was conducted in the Dispilio site catchment area from 30th May, 2023 to 4th June, 2023 at the end of the wet season. Samples were taken from 13 sampling locations in total, spanning different altitudes, terrains, underlying geologies, and vegetational environments. Twelve of the locations sampled were within a 10-km catchment area of Dispilio (Figure 1a), which reflects the approximate distance to other contemporaneous Neolithic sites Avgi and Koromilia. One location, Psarades, is located approximately 50 km away (Figure 1b), situated on Lake Prespa and was chosen as a proxy for Dispilio in the past because it is currently less affected by human activity (Efthimiou et al. 2022). We used regional ecosystem classifications to define environmental zones as woodland, grassland, and wetland and sampled at least three locations corresponding to each type (Supplementary A, Figure S1, shows images of habitat types). High forests were not sampled as they were inaccessible at the time of sampling. Sampling locations were classified as open and semiopen based on the amount of shaded canopy. In open areas, sampling was conducted along a transect of 50–100 m (300 m in the case of the Psarades transect). In semiopen locations, samples were taken in an area of a radius of c. 10 m around a mature deciduous tree (usually oak) in a location where all the life forms were growing close to each other and without cultivation. Plants from different life forms were sampled, namely, trees, shrubs, grasses, and forbs (Supplementary A, Figure S2, shows images of plant forms). The plants were identified to taxon and, when possible, species level in the field. Where possible, the same taxa were collected across sampling locations for comparability. The species targeted are those that would be consumed by domestic herbivores, especially sheep, which are the predominant species at Neolithic Dispilio, followed by goats and cattle. All of the plants gathered are native and ubiquitous in the region. Sampling locations were chosen to avoid areas with significant disturbance or anthropogenic input, particularly agricultural activity. Coordinates were taken

with a Garmin GPS at each sampling location. The temperature range for Kastoria from 1st October 2022 to 31st May 2023 was -7.8°C to 28.6°C and the average precipitation was 37 mm per month, while the temperature range for Lake Prespa was -7.4°C to 25.7°C and the average precipitation was 41 mm per month (Meteo.gr, accessed 2024).

4.2 | Laboratory Analysis

Stable carbon and nitrogen isotope analysis was undertaken for 146 plants, representing 51 species from 13 sampling locations. The samples were dried and pressed in the field. For comparability only leaves were sampled in this study. In the lab, leaves were separated, freeze-dried for 12 h, and crushed using a TissueLyser to create a homogenized powder. The homogenized samples were subsequently weighed out to c. 2 mg into tin capsules. The samples underwent dual carbon and nitrogen isotopic analysis on a precisiON isotope ratio mass spectrometer coupled to a vario PYRO cube high temperature elemental analyser at the Research Laboratory for Archaeology and History of Art (RLAHA), University of Oxford, UK. Out of the 146 plants characterized, 126 yielded adequate %N to qualify for subsequent statistical analyses. Stable carbon and nitrogen values were calibrated to the VPDB and AIR scales using USGS61 ($\delta^{13}\text{C} = -35.05 \pm 0.04\text{‰}$; $\delta^{15}\text{N} = -2.87 \pm 0.04\text{‰}$; University of Indiana), USGS 40 ($\delta^{13}\text{C} = -26.39 \pm 0.04\text{‰}$; $\delta^{15}\text{N} = -4.52 \pm 0.06\text{‰}$; IAEA) and an internal seal bone collagen reference material ($\delta^{13}\text{C} = -12.54 \pm 0.13\text{‰}$; $\delta^{15}\text{N} = 16.14 \pm 0.09\text{‰}$). Measurement uncertainty was monitored using two reference materials, namely, wheat flour ($\delta^{13}\text{C} = -27.21 \pm 0.13\text{‰}$; $\delta^{15}\text{N} = 2.73 \pm 0.17\text{‰}$; elemental microanalysis) and DL leucine ($\delta^{13}\text{C} = -28.31 \pm 0.03\text{‰}$; $\delta^{15}\text{N} = 6.33 \pm 0.07\text{‰}$; internal reference material from Sigma-Aldrich), coupled with the calibration standards and duplicate samples. Precision (u [Rw]) across all the runs was determined to be $\pm 0.09\text{‰}$ for $\delta^{13}\text{C}$ and $\pm 0.37\text{‰}$ for $\delta^{15}\text{N}$, accuracy (u [bias]) was $\pm 0.15\text{‰}$ for $\delta^{13}\text{C}$ and $\pm 0.44\text{‰}$ for $\delta^{15}\text{N}$. The total analytical uncertainty (u_c) was estimated to be $\pm 0.17\text{‰}$ for $\delta^{13}\text{C}$ and $\pm 0.57\text{‰}$ for $\delta^{15}\text{N}$. Values of raw and normalized isotope data of samples and standards can be found in Supplementary C. The statistical analysis and modeling were performed in R v.4.4.1.

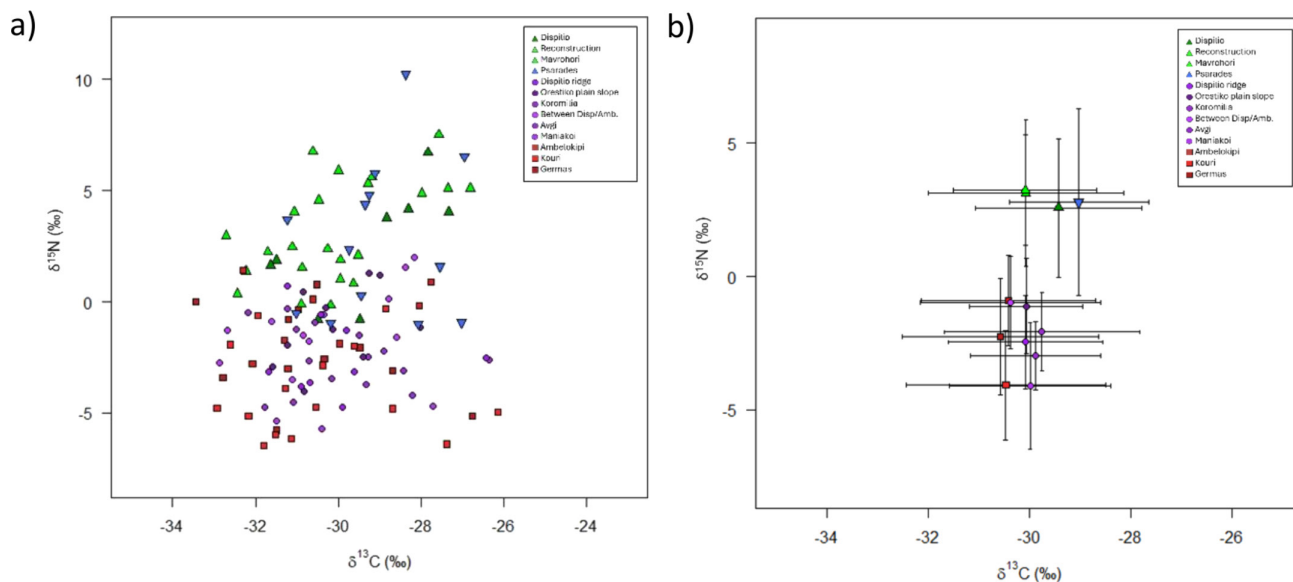
5 | Results

5.1 | Overall Variability in Plant $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Values

Table 1 presents a summary of the carbon and nitrogen isotope values by habitat type and life form groups, while the metadata containing all plant values are located in Supplementary B, and the raw isotope data can be found in Supplementary C. Figure 2a shows all the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values by sampling location, while Figure 2b shows the site mean values and their standard deviations. The foliar $\delta^{13}\text{C}$ values of all the plants range from -33.4‰ to -26.1‰ , with a mean value \pm standard deviation (SD) of $\delta^{13}\text{C} = -30.0 \pm 1.6\text{‰}$. When the sampling location of Psarades is excluded and only the Kastoria basin locations are considered, the mean $\delta^{13}\text{C}$ value is almost identical: $-30.1 \pm 1.6\text{‰}$. The foliar $\delta^{15}\text{N}$ values for all the plants used in

TABLE 1 | Carbon and nitrogen isotopic compositions of modern plants sampled in this study by habitat type and life form groups, with ligneous plants encompassing trees and shrubs and nonligneous plants encompassing grasses and forbs.

Habitat	Life form	<i>n</i>	C:N	$\delta^{13}\text{C}_{\text{mean}}$	$\delta^{13}\text{C}_{\text{SD}}$	$\delta^{15}\text{N}_{\text{mean}}$	$\delta^{15}\text{N}_{\text{SD}}$	$\Delta^{13}\text{C}_{\text{mean}}$
Woodland	Ligneous	8	26.4	-28.0	1.2	-3.3	2.4	22.2
	Nonligneous	25	21.4	-31.3	1.1	-2.6	2.3	25.7
Grassland	Ligneous	14	23.4	-28.6	1.2	-2.2	1.5	22.9
	Nonligneous	35	22.3	-30.6	1.1	-2.0	2.0	25.0
Wetland	Ligneous	7	22.9	-28.3	1.0	1.1	2.0	22.6
	Nonligneous	37	18.6	-29.9	1.5	3.3	2.6	24.2

**FIGURE 2** | (a) All $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values by sampling locations. Each color represents a different sampling location, while each symbol corresponds to a different habitat (triangle = wetland, circle = grassland, square = woodland). (b) All $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ sampling locations means with corresponding standard deviations.

this analysis range from -6.5‰ to 10.2‰ , with a mean value of $\delta^{15}\text{N} = -0.5 \pm 3.4\text{‰}$. When the wetland sites are excluded, the mean $\delta^{15}\text{N}$ value = $-2.3 \pm 2.1\text{‰}$. There were no statistically significant outlier values.

5.2 | The Variability in Plant $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Values by Life Form

Figure 3a shows boxplots of how plant $\delta^{13}\text{C}$ values vary by life form. A $\delta^{13}\text{C}$ Shapiro–Wilk normality test for $\delta^{13}\text{C}$ revealed a normal distribution for trees ($W=0.92$, $p=0.32$), shrubs ($W=0.96$, $p=0.57$), grasses ($W=0.96$, $p=0.47$) and forbs ($W=0.98$, $p=0.55$). According to Levene's test for equality of variance there was not a significant difference in variance ($F_{(3,122)}=0.56$, $p=0.64$). Nested analysis of variance (with $\delta^{13}\text{C}$ values nested by sampling location) found a significant difference in $\delta^{13}\text{C}$ values amongst the life forms ($F=24.77$, $p<0.0001$). The least squares mean of $\delta^{13}\text{C}$ values of trees is -28.8‰ (95% CI = -29.6 to -27.9‰); of shrubs is -28.1‰ (95% CI = -28.8 to -27.4‰); of grasses is -29.8‰ (95% CI = -30.4 to -29.3‰); and of forbs is -30.8‰ (95% CI = -31.2 to -30.4‰).

Figure 3b shows boxplots of how plant $\delta^{15}\text{N}$ values vary by life form. The $\delta^{15}\text{N}$ Shapiro–Wilk normality test revealed a normal distribution for trees, shrubs, and forbs with p values <0.05 but not for grasses ($W=0.92$, $p=0.04$). Nested analysis of variance did find a significant difference in $\delta^{15}\text{N}$ values amongst the life forms ($F=2.77$, $p<0.04$) meaning sampling location may have had an influence on the relationship between life form type and $\delta^{15}\text{N}$ values. The least squares mean of $\delta^{15}\text{N}$ values of trees is -0.88‰ (95% CI = -3.01 to 1.25‰); of shrubs is -1.79‰ (95% CI = -3.73 to 0.15‰); of grasses is 0.13‰ (95% CI = -1.69 to 1.95‰); and of forbs is -0.67‰ (95% CI = -2.35 to 1.00‰).

5.3 | The Variability in Plant $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Values Among Habitats

Figure 4a shows the $\delta^{13}\text{C}$ values plotted by habitat. The habitat categories are grassland, woodland, and wetland. The $\delta^{13}\text{C}$ Shapiro–Wilk normality test revealed a normal distribution for woodlands ($W=0.95$, $p=0.15$), grasslands ($W=0.97$, $p=0.29$), and wetlands ($W=0.97$, $p=0.41$) and Levene's test for equality of variance demonstrated that the variance between categories

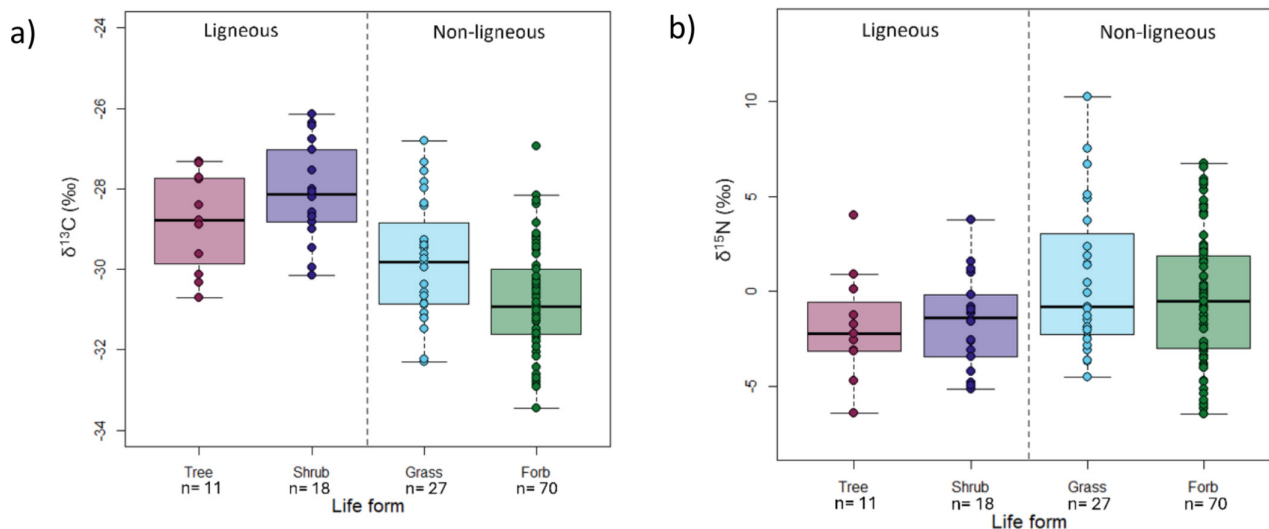


FIGURE 3 | Comparison of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of plants by life form. (a) Box plot of plant $\delta^{13}\text{C}$ values categorized as tree, shrub, grass, or forb. (b) Box plot of plant $\delta^{15}\text{N}$ values categorized as tree, shrub, grass, or forb. Boxes represent the quartiles, the bold lines represent the medium, and the whiskers represent $1.5 \times$ the interquartile range. The points signify individual plants.

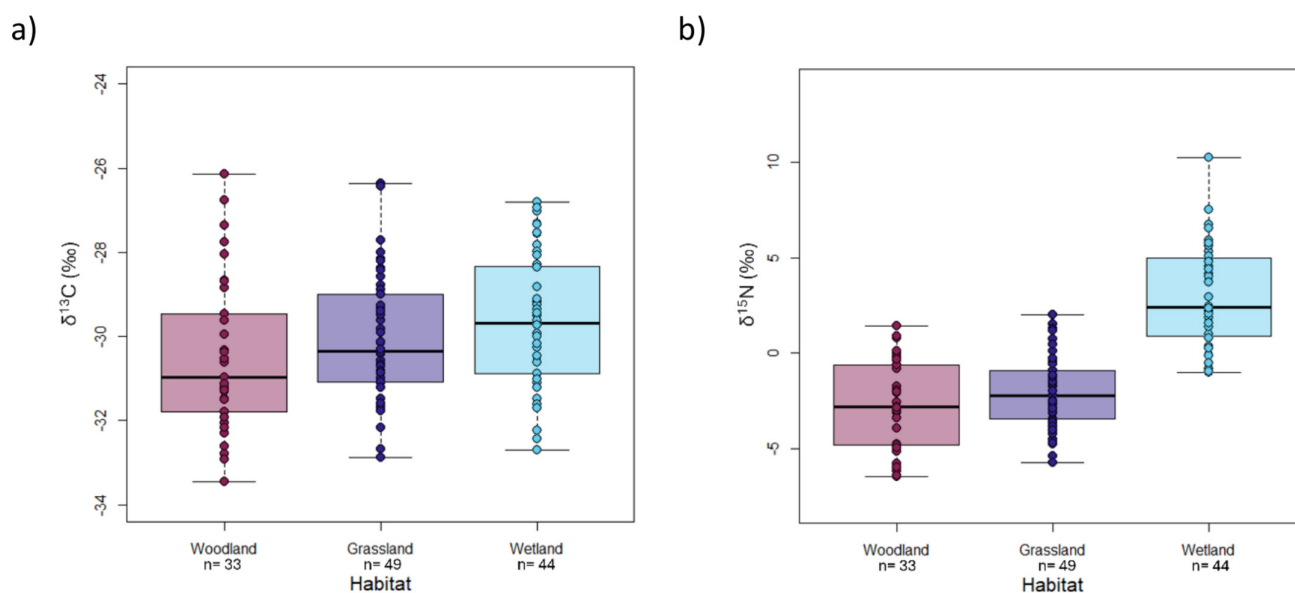


FIGURE 4 | Comparison of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of plants by habitat. (a) Box plot of plant $\delta^{13}\text{C}$ values by habitat categories of woodland, grassland and wetland. (b) Box plot of plant $\delta^{15}\text{N}$ values by habitat categories of woodland, grassland and wetland. Boxes represent the quartiles, the bold lines represent the medium, and the whiskers represent $1.5 \times$ the interquartile range. The points signify individual plants.

was not statistically significant ($F_{(2,123)} = 0.68, p = 0.51$). Nested analysis of variance did not show a significant difference in $\delta^{13}\text{C}$ values amongst the habitat types ($F = 2.56, p < 0.13$). The least squares mean of $\delta^{13}\text{C}$ values of woodlands is -30.5‰ (95% CI = -31.1 to -29.9‰), grasslands -30.0‰ (95% CI = -30.5 to -29.5‰), and wetlands -29.6‰ (95% CI = -30.2 to -29.1‰). The effect of canopy cover on $\delta^{13}\text{C}$ values was also tested for the woodland sites. The plant $\delta^{13}\text{C}$ values from semiopen areas were normally distributed ($W = 0.95, p = 0.38$), while the plant $\delta^{13}\text{C}$ values from open areas were not ($W = 0.83, p = 0.02$). The canopy values did however display homogeneity of variance ($F_{(1,31)} = 1.80, p = 0.19$) and no significant difference was found with nested analysis ($F = 0.03, p < 0.87$). The least squares mean of $\delta^{13}\text{C}$ values of semiopen areas is -30.4‰ (95% CI = -32.2 to -28.6‰) and of open areas is -30.5‰ (95% CI = -32.8 to -28.3‰).

Thus, the canopy effect was not observed (Supplementary A, Figure S3). The effect of altitude on $\delta^{13}\text{C}$ values was also tested along an altitudinal gradient of 622 to 838 masl. The $\delta^{13}\text{C}$ values are normally distributed ($W = 0.98, p = 0.08$). A generalized least squares model did not find a significant relationship between plant $\delta^{13}\text{C}$ values and sampling location elevation (masl) ($\beta = 0.001, \text{SE} = 0.002, t = 0.70, p = 0.49$). The model produced a marginal R^2_{LR} of 0.004 (Supplementary A, Figure S4).

Figure 4b shows the $\delta^{15}\text{N}$ values plotted by habitat. The $\delta^{15}\text{N}$ values are normally distributed within habitat categories: woodlands ($W = 0.95, p = 0.15$), grasslands ($W = 0.98, p = 0.77$) and wetlands ($W = 0.96, p = 0.17$). However, Levene's test for equality of variance was violated ($F_{(2,123)} = 3.29, p = 0.04$). The least squares mean of $\delta^{15}\text{N}$ values of woodlands is -2.6‰

(95% CI = -3.9 to 1.2‰), grasslands -2.14‰ (95% CI = -3.1 to -1.2‰), and wetlands 2.95‰ (95% CI = 1.8 to 4.1‰). The 95% CIs for the woodlands and grasslands overlap, while the 95% CI for the wetland does not overlap with the other two. These two groups broadly correspond to terrestrial and wetland environments. Because of the unequal variance, a nested ANOVA test was not possible. Instead, a Welch *t* test was used to compare the $\delta^{15}\text{N}$ values of terrestrial and wetland environments and a significant difference was found ($t(124) = 12.22$, $p < 0.0001$, 95% CI = -6.16 to -4.44‰) with wetland sites showing consistently higher $\delta^{15}\text{N}$ values.

5.4 | Psarades Wetland Transect $\delta^{15}\text{N}$ and Proximity to Water Trend

The Psarades wetland is plotted separately here because of its geographical location and because it is grazed by feral cattle, which may affect nitrogen values. Figure 5 shows the $\delta^{15}\text{N}$ values of plants sampled along a transect from closest to farthest from water. Because sampling points were not equidistant (50–130 m), an ordinal number was assigned to each sampling area based on its relative distance from the water. To evaluate the relationship between $\delta^{15}\text{N}$ values and relative distance from the water, a nonparametric Spearman's rank correlation coefficient test was conducted, and there was a significant negative relationship between $\delta^{15}\text{N}$ values and distance from water, ($r_{s(11)} = -0.92$, $p < 0.0001$). A similar trend is observed at the nonpasture wetland site of Dispilio Reconstruction, where another transect was performed (Supplementary A, Figure S5).

6 | Discussion

6.1 | Plant Life Forms and Precipitation

The results show a significant correlation between $\delta^{13}\text{C}$ values and plant life forms (Figure 3). The statistical analyses distinguished between three categories, namely, trees and shrubs combined, grasses, and forbs. These categories correspond to functional groups identified by other studies and distinguished by their similar responses to environmental conditions and effect on ecosystem processes (Lavorel et al. 1997). For the purposes of this study, the distinction between ligneous (trees and shrubs) and nonligneous (grasses and forbs) plant groups is more informative for animal dietary studies. Ligneous plants showed consistently less negative $\delta^{13}\text{C}$ values ($\delta^{13}\text{C} = -28.4 \pm 1.2\text{‰}$) than nonligneous plants ($\delta^{13}\text{C} = -30.5 \pm 1.4\text{‰}$). Although ligneous plants formed a comparatively smaller sample in this study ($n = 29$), the results are consistent across sampling sites. This is surprising, given that woody plant leaves have been found to contain more lignin than herbaceous leaves (Kendall et al. 2019) and lignin is depleted in ^{13}C relative to whole-plant material (Benner et al. 1987). It will be interesting to see whether this difference holds between ligneous and nonligneous plant groups in the dry season, when shallower-rooting grasses and forbs are likely to be more sensitive to lower water availability and therefore may have higher $\delta^{13}\text{C}$ values. It is worth noting that the Kastoria basin $\delta^{13}\text{C}$ values across all life forms are, as expected, more negative than those measured by Hartman and Danin (2010) during the wet season in the Eastern Mediterranean (Israel) and also those of plants measured in

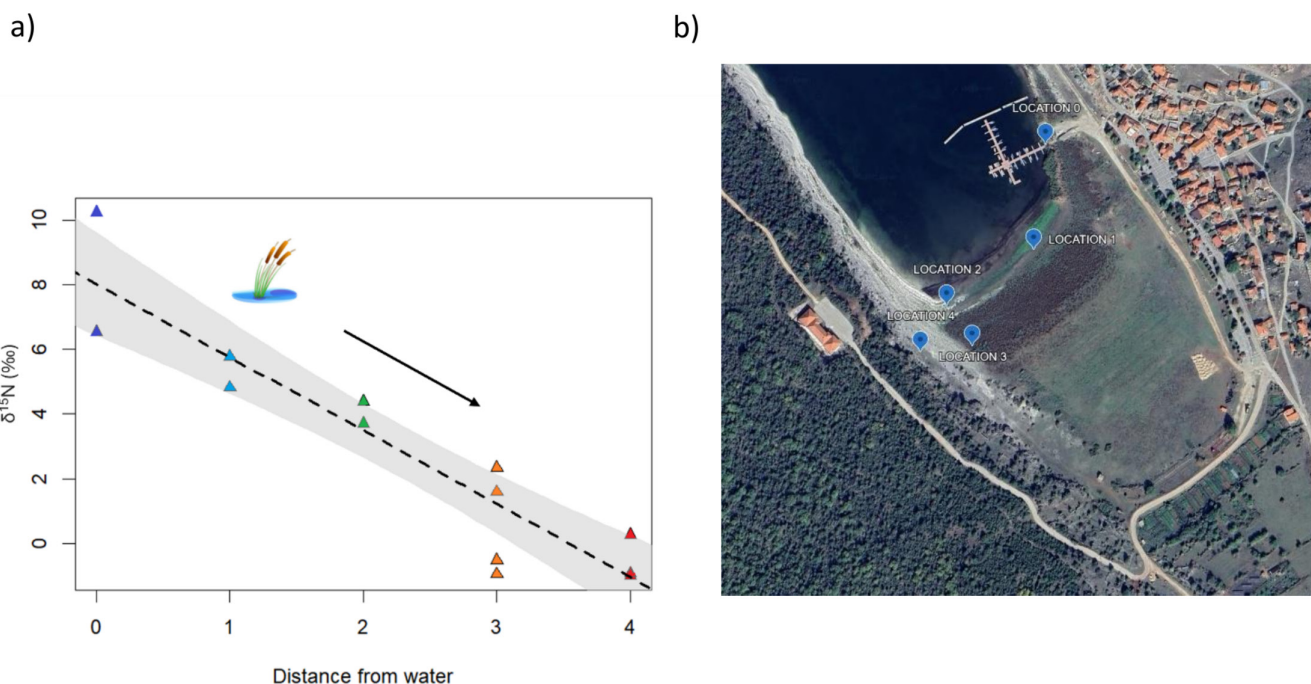


FIGURE 5 | The $\delta^{15}\text{N}$ values of plants sampled along a transect at Psarades plotted against arbitrarily assigned distance from water based on sampling area with 0 representing plants growing in the lake, 1 representing plants growing on the lake edge, and 2, 3, and 4 representing increasing distance from water. The dotted line represents a fitted linear model relating $\delta^{15}\text{N}$ values and sampling areas. The grey shading represents the 95% confidence interval of this relationship.

the southwestern Peloponnese, Greece, during the dry season (Norström et al. 2017). The wet-season mean $\delta^{13}\text{C}$ value (-30‰) of Kastoria more closely approximates the mean $\delta^{13}\text{C}$ value estimated for C_3 plants in temperate northern Europe (Wang et al. 2024).

The mean $\delta^{15}\text{N}$ values did not vary systematically across life forms. This is best observed in terrestrial sampling locations when N_2 -fixing plants are separated. The mean $\delta^{15}\text{N}$ value of ligneous plants is $-1.7 \pm 2.5\text{‰}$, while the nonligneous plants have a mean value of $-0.1 \pm 3.9\text{‰}$, and N_2 -fixing plants ($n = 18$) have a mean value of $-0.4 \pm 0.8\text{‰}$. The wet season $\delta^{15}\text{N}$ values of non- N_2 -fixing plants show an apparent depletion in relation to atmospheric nitrogen ($\delta^{15}\text{N} = 0\text{‰}$), probably because colder and wetter climatic systems are generally more effective in conserving and recycling mineral nitrogen (Handley et al. 1999). Other studies have observed a similar trend to more negative $\delta^{15}\text{N}$ values during periods of high precipitation (Austin and Vitousek 1998; Swap et al. 2004; Hartman and Danin 2010).

6.2 | Habitat Variability in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Values

No correlation was found between $\delta^{13}\text{C}$ values and habitat (Figure 4a). All the habitats sampled had a range from -33.4‰ to -26.1‰ , with $\delta^{13}\text{C}$ mean values of around -30‰ . This range falls within that expected for C_3 plants. This study also tested the relationship between canopy density and $\delta^{13}\text{C}$ values and no effect was found. The $\delta^{13}\text{C}$ values of plants growing under a semiopen and open canopy were not significantly different. This is likely because the semiopen canopy is not sufficiently dense to cause the ^{13}C -depletion associated with the canopy effect (Styring et al. 2024). The lack of a truly closed forest amongst the sampling sites precluded the measurement of canopy effect in a densely forested environment.

There was a significant correlation between $\delta^{15}\text{N}$ values and habitat, principally between wetland and terrestrial environments (Figures 4b and 5). The terrestrial habitats had a range from -6.5‰ to 2.0‰ with a mean value of $\delta^{15}\text{N} = -2.3 \pm 2.1\text{‰}$, while the wetlands exhibited a range from -1.0‰ to 10.2‰ with $\delta^{15}\text{N}$ mean values of $\delta^{15}\text{N} = 3.0 \pm 2.7\text{‰}$, representing a difference of $\pm 5.3\text{‰}$ in their mean $\delta^{15}\text{N}$ values. The $\delta^{15}\text{N}$ -enrichment effect in wetland settings has been observed in other studies and attributed to bacterial denitrification (Wang et al. 2015; Sebilo et al. 2019). Lake Kastoria is a naturally eutrophic lake and the wetland $\delta^{15}\text{N}$ -enrichment effect observed in this study would have been present in the past (Natura 2000 site GR1320001). To ensure that this effect was not exacerbated by urban and agricultural activities in Kastoria, a transect was measured at Psarades on Lake Megali Prespa and the effect was present to the same degree. Because the Psarades sampling transect is also frequented as pasture by feral indigenous dwarf cattle, the higher $\delta^{15}\text{N}$ values close to the lakeshore at Psarades compared to that at the Dispilio Reconstruction could be due to manure derived ^{15}N -enrichment on top of denitrification due to waterlogging. However, we infer that any manuring effect by grazing cattle is minor in comparison to the effect of bacterial denitrification, decreasing with distance from the seasonally flooded lakeshore.

6.3 | Implications for Livestock Diet/Management Studies

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic variability is closely linked to processes in the local environment, and understanding the driving forces of variation has value for studies of ancient livestock management. The observed difference in $\delta^{13}\text{C}$ values between the leaves of the trees and shrubs (ligneous) and the grasses and forbs (nonligneous) has implications for interpreting $\delta^{13}\text{C}$ values of browsers and grazers. Grazers such as cattle and especially sheep tend to prefer eating more grassy and herbaceous vegetation, while browsers like goats tend to feed on shrubby vegetation, for example in the hedges of fields. Thus, given the difference in the $\delta^{13}\text{C}$ values of these two vegetation types, there should be an observable difference between the $\delta^{13}\text{C}$ values of faunal remains of cattle and sheep versus goats. In contrast, no difference in the faunal values may imply human intervention in the animals' feeding habits that limit their natural preferences. Several archaeological studies have demonstrated that the use of fodder, differential pasture use, diverse herding practices, and human control of movement have a significant and observable influence on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in animal bone collagen (Makarewicz 2014; Makarewicz and Pederzani 2017; Isaakidou et al. 2022). Ethnographic sources attest to the traditional use of leaf hay as winter fodder and of freshly cut branches as supplementary fodder at other times of year in the mountains of northern Greece (Halstead 1998). This study did not observe any canopy effect in $\delta^{13}\text{C}$ values of the sampled open woodland environments, but paleoenvironmental records show greater woodland cover in the past in relatively close proximity to the site (Kouli and Dermitzakis 2008). Thus, the canopy effect on $\delta^{13}\text{C}$ values cannot be conclusively excluded from interpretation of faunal isotope values at Dispilio.

The $\delta^{15}\text{N}$ values observed in the wetland environment greatly exceed those of terrestrial environments and may afford insight into the nature of animal diets and pasture locations. Animals feeding near the lakeshore would be expected to exhibit elevated $\delta^{15}\text{N}$ values. Furthermore, local ethnographic sources suggest that wetland areas provided seasonal graze for animals and that cattle in the recent past limited growth of the reed beds (Halstead et al. *in press a.*). However, ^{15}N -enrichment is also caused by a cumulative manuring effect in long-term pasture fields (Elmore and Craine 2011; Makarewicz et al. 2016). Various studies have also attested to intensive agricultural systems in the past that benefited from the integration of cultivation and animal husbandry, with animals being utilized for manure to enrich crops and as labor to plough fields and, in turn, gaining access to ^{15}N -enriched crops through grazing on stubble fields and ingestion of manured seeds and straw (Halstead 2006; Bogaard et al. 2013). Identifying the source of ^{15}N -enrichment is complex, but further research may aid in disentangling some of these influences. For instance, further isotopic characterization of archaeobotanical remains may lend insight into the expected degree of nitrogen enrichment in cultivated crops. Regardless of whether any ^{15}N -enrichment effect is the result of grazing near the lake shore, the use of long-term pasture, or the feeding of livestock with manured crops, it would suggest animal management fairly close to the site rather than further afield or in the surrounding uplands.

Conversely, if no effect is observed, it may imply more extensive management of animals at some distance from the site.

7 | Conclusion

The stable carbon and nitrogen isotope values of modern plants in the Dispilio catchment area within the Kastoria basin provide vital information about the driving forces of isotopic variation in the local environment during the wet season. The $\delta^{13}\text{C}$ results show distinctions between life forms (trees/shrubs, grasses, and forbs), while no such effect was seen in $\delta^{15}\text{N}$ values. The $\delta^{15}\text{N}$ results reveal significant differences between terrestrial and wetland environments. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are expected to be negatively correlated with seasonal precipitation. No correlation is found between isotopic values and moderate altitudinal differences or canopy density in open woodland environments. The high degree of isotopic variability within sampling locations for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values suggests that differences in faunal stable isotope values should be interpreted cautiously.

These insights will inform studies of ancient animal management. The observed difference in $\delta^{13}\text{C}$ values, between ligneous and nonligneous plants, and in $\delta^{15}\text{N}$ values, between wetland and terrestrial settings, should enable discrimination of past grazers from browsers and of lake-edge from dryland feeders, respectively. These results will thus help interpret archaeological faunal isotopic measurements in terms of animal diet, hence animal husbandry practices and the utilization of local habitats by early farmers. Understanding the adaptability of past agricultural systems within the context of local ecosystems will inform contemporary discussions of sustainable farming practices and the conservation of Mediterranean wetlands, one of Europe's most vulnerable biodiversity hotspots.

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Data Availability Statement

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

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Example of different habitats sampled, (a) woodland, (b) grassland, and (c) wetland environment. **Figure S2:** Example of different life forms sampled, (a) tree, (b) shrub, (c) grass, and (d) forb. **Figure S3:** Box plot of plant $\delta^{13}\text{C}$ values of plants sampled in semiopen and open canopy areas at the three woodland localities. Boxes represent the quartiles, the bold lines represent the median, and the whiskers represent $1.5 \times$ the interquartile range. The points signify individual plants. **Figure S4:** Comparison of $\delta^{13}\text{C}$ values of plants sampled in this study plotted against sampling location elevation (masl). The dotted line represents a fitted linear model relating $\delta^{13}\text{C}$ values and elevation, while the grey shading represents the 95% confidence interval of this relationship. There is no correlation observed between $\delta^{13}\text{C}$ values and elevation. **Figure S5:** The $\delta^{15}\text{N}$ values of plants sampled along a transect at the Dispilio reconstruction plotted against arbitrarily assigned distance from water based on sampling area with 3 representing plants sampled on the lake edge and 2, 1, and 0 representing plants sampled in increasingly deeper water. The dotted line represents a fitted linear model relating $\delta^{15}\text{N}$ values and sampling areas. The grey shading represents the 95% confidence interval of this relationship. The arrow represents the direction of the trend. **Data S1:** Supporting information. **Data S2:** Supporting information.