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Stable carbon and nitrogen isotopes and quality traits of fossil cereal grains provide clues on sustainability at the beginnings of Mediterranean agriculture[‡]

Mònica Aguilera[1], José Luis Araus[1†], Jordi Voltas[2], Maria Oliva Rodríguez-Ariza[3], Fernando Molina[4], Núria Rovira[5], Ramon Buxó[5] and Juan Pedro Ferrio[2,6*]

[1]Unitat de Fisiologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Spain
[2]Department of Crop and Forest Sciences, ETSEA-University of Lleida, Spain
[3]Centro Andaluz de Arqueología Ibérica, Universidad de Jaén, Spain
[4]Departamento de Prehistoria y Arqueología, Universidad de Granada, Spain
[5]Museu d'Arqueologia de Catalunya, Barcelona, Spain
[6]Chair of Tree Physiology, University of Freiburg, Germany

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We present a novel approach to study the sustainability of ancient Mediterranean agriculture that combines the measurement of carbon isotope discrimination ($\Delta^{13}C$) and nitrogen isotope composition ($\delta^{15}N$) along with the assessment of quality traits in fossil cereal grains. Charred grains of naked wheat and barley were recovered in *Los Castillejos*, an archaeological site in SE Spain, with a continuous occupation of ca. 1500 years starting soon after the origin of agriculture (ca. 4000 BCE) in the region. Crop water status and yield were estimated from $\Delta^{13}C$ and soil fertility and management practices were assessed from the $\delta^{15}N$ and N content of grains. The original grain weight was inferred from grain dimensions and grain N content was assessed after correcting N concentration for the effect of carbonisation. Estimated water conditions (i.e. rainfall) during crop growth remained constant for the entire period. However, the grain size and grain yield decreased progressively during the first millennium after the onset of agriculture, regardless of the species, with only a slight recovery afterwards. Minimum $\delta^{15}N$ values and grain N content were also recorded in the later periods of site occupation. Our results indicate a progressive loss of soil fertility, even when the amount of precipitation remained steady, thereby indicating the unsustainable nature of early agriculture at this site in the Western Mediterranean Basin. In addition, several findings suggest that barley and wheat were cultivated separately, the former being restricted to marginal areas, coinciding with an increased focus on wheat cultivation.

In recent years, information derived from the analysis of carbon isotope signatures in several kinds of fossil plant remains has been extensively used to reconstruct past climatic and environmental conditions.[1–3] An example involves the development of the methodological basis to infer the water status[4,5] and yield[6–8] of ancient agriculture in the Mediterranean. This approach involves carbon isotope discrimination ($\Delta^{13}C$) of fossil cereal grains recovered from archaeological sites. In contrast, the natural abundance of nitrogen stable isotopes has been widely used in palaeodietary studies;[9] however, its application remains more elusive in palaeoenvironmental reconstruction, although data on soil fertility and management practices in ancient agriculture might be obtained.[10,11]

Results from long-term experiments demonstrate that manuring (and, in general, the addition of any organic fertiliser) significantly raises the nitrogen isotope composition ($\delta^{15}N$) of winter cereals grains compared with unfertilised fields.[10,12,13] Indeed, modern plants growing near archaeological sites still reflect the impact of ancient management practices on their $\delta^{15}N$,[14–16] probably as a consequence of long-term animal/human nitrogen (N) inputs.[14,17] However, the miscellaneous factors that affect variation in ^{15}N in the soil-plant system complicate the interpretation of $\delta^{15}N$ in plants. Although the $\delta^{15}N$ of plant material reflects primarily the isotope signature of the N source, in natural systems the main N input is not easy to determine and discrimination processes may occur during N uptake, assimilation and redistribution within the plant.[18,19] Nevertheless, a number of common patterns have been described to trace the fate of ^{15}N in soil-plant systems.

**Correspondence to*: J. P. Ferrio, Chair of Tree Physiology, University of Freiburg, Georges-Köhler-Allee, Building 53/54, D-79110 Freiburg im Breisgau, Germany.
E-mail: pitter.ferrio@pvcf.udl.es
[†]Present address: International Maize and Wheat Center (CIMMYT), Mexico.
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Processes favouring N loss in soils translate into a more enriched $\delta^{15}N$ of the remaining available N, and this is reflected in plant matter $\delta^{15}N$.[18,20,21] Losses of N can be enhanced by certain environmental conditions (e.g. rainfall favours nitrate lixiviation), but these are driven mostly by N excess (i.e. inputs exceeding plant demand) and, thus, $\delta^{15}N$ values in both soil and plants are often positively related to the amount of N available to plants, particularly in the absence of mineral fertiliser.[12,22,23] In addition, $\delta^{15}N$ increases during decomposition and, consequently, N derived from organic matter rather than from mineral fertilisers or geologic substrate tends to be isotopically enriched.[12,21]

In summary, $\delta^{15}N$ in plants is, in addition to fractionation processes occurring within the plant, determined mostly by a combination of (1) $\delta^{15}N$ of N inputs, (2) the relationship between N inputs and plant demand, and (3) the proportion of N derived from organic matter decomposition. According to this simplified scheme, we would expect cereal crops cultivated without mineral fertilisers to show high $\delta^{15}N$ when grown in 'rich soils', that is, with large organic N pools and high water retention capacity. This situation would in turn increase both decomposition processes and N availability (points (2) and (3)). This effect would be enhanced if the soil received additional N inputs of animal origin (e.g. manure, sewage, food waste) by increasing the $\delta^{15}N$ of N inputs (point (1)). Consequently, $\delta^{15}N$ can be used as an indicator of the nutritional status of ancient crops. This measure has the potential to reflect not only the effect of manuring, but also the nutritional quality of agricultural soils. $\delta^{15}N$ determination can be particularly useful in Mediterranean and other arid environments, where N is, after water availability, the main limiting factor for crop growth.[24] However, a basic problem inherent to exploiting archaeobotanical remains in environmental reconstruction is related to their charred state. Although preliminary studies on experimentally charred grains and chaff show promising results on the preservation of the original ^{15}N signal, further work is still required to explore a broader range of crops and charring conditions.[10] This is also true for the assessment of possible shifts in grain N concentration, a parameter that may be more affected by carbonisation.[16]

The progressive aridification in the Mediterranean during the Holocene was triggered by changes in atmospheric global circulation and, in particular, in the North Atlantic Oscillation.[25] Palaeoenvironmental evidence indicates that the early- and mid-Holocene 'warm' phase, between 9000 and 6000 BP, was associated with moister than present conditions over much of the northern hemisphere.[3,26] Since then, drought has developed progressively in the Mediterranean. This trend is consistent with decreasing lake levels in Spain and Portugal between 6000 and 5000 BP,[27] and with changes during the last four millennia in the annual precipitation, estimated from fossil charcoal $\Delta^{13}C$.[28] In fact, typical Mediterranean vegetation, supposedly adapted to summer-arid climate, has been present for the last 5000 years, while the early- and mid-Holocene had a less arid climate.[29] However, several sources of evidence clearly indicate that the progression of aridification differed around the Mediterranean. In the case of Spain, the $\Delta^{13}C$ of fossil cereal grains suggests a gradual increase in drought conditions between 4000 and 1000 BCE, particularly in the south east of the country.[4] This trend towards aridity is also supported by pollen records[30] and olive cultivation in SE Spain.[31] Indeed this region of Spain is now one of the most eroded and arid areas of Mediterranean Europe. Regional factors such as those related to anthropogenic activities (i.e. agriculture and other land-use practices) may have contributed to accelerating aridification in this region.

Agriculture was already well established in SE Spain during the Copper Age (3500/3000 BCE). Agricultural remains show that the main crops were naked wheat (bread or durum) (*Triticum aestivum/durum*) and barley (*Hordeum vulgare* ssp. *nudum*).[32–34] An outstanding example of ancient agricultural settlements in SE Spain is the site of *Los Castillejos*, which shows a record of continuous cultivation of ca. 1500 years, starting soon after the origins of agriculture in this region, with naked wheat and barley as the main crops.[34–36] Taking *Los Castillejos* as a case study, here we present a novel methodological approach based on the analysis of stable C and N isotopic signatures and N concentration and size estimation of charred grains to study the sustainability of early Mediterranean agricultural systems. Changes in grain size, water status and yield of wheat and barley crops and fluctuations in soil fertility through time were inferred from charred grains recovered in several stratigraphic layers. The original grain size was estimated from dimensions of grain remains, crop water status and grain yield were determined from grain $\Delta^{13}C$, and information on soil fertility and management practices was obtained from $\delta^{15}N$ and total N content of fossil grains and the assemblage of weed seeds accompanying cereal remnants.

EXPERIMENTAL

Archaeobotanical material

Ancient grains of naked wheat (*T. aestivum/durum*, after Van Zeist and Bakker-Heeres[37]), naked barley (*Hordeum vulgare* ssp. *nudum* L.) and faba bean (*Vicia faba* L., only three remain from the Copper Age), along with seeds of weeds that occurred as contaminants with crop seeds, were collected from the archaeological site of *Los Castillejos*. Part of the large archaeological park of *Las Peñas de los Gitanos* located in Montefrío, Granada Province, SE Spain, *Los Castillejos* has a record of more than 4000 years of continued human occupation, from the Neolithic period until the Roman conquest. The latitude, longitude and altitude above sea level of the settlement are 37°20′02″N, 4°00′05″W and 900 mm, respectively. *Los Castillejos* shows a continuous cereal cultivation of ca. 1500 years, and comprises six cultural periods: Early, Middle and Late Neolithic, and Early, Middle and Late Copper Age. The site has been excavated by the University of Granada for more than 30 years,[35,36,38] and has an annual rainfall of about 600 mm and an average monthly maximum and minimum temperature of 21 and 7°C, respectively. The present-day natural vegetation in the region is Mediterranean, with evergreen oak (*Quercus ilex* L.) as the main tree species plus bushy species like the kermes oak (*Quercus coccifera* L.) and herbaceous plants like rosemary, halt and juniper. The land around the archaeological complex is currently extensively cultivated with olive trees and rain-fed cereal (wheat and barley). Fruit

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orchards and horticultural crops are also present where supplementary irrigation is available.

Crop grains and weed seeds were found in a carbonised state and were gathered in disparate fashion from domestic fires, cooking ovens, and floors. Soil samples were treated using a standard flotation tank in the field with 0.3 mm (flotation) and 2.5 mm (wet) sieves. Plant remains were then dried slowly before their transport and sorting. The total number of cereal fossil grains analysed, and their dates and cultural periods, are shown in Table 1. In addition, weed seeds were used as potential indicators of agricultural practices.[39] To this end, weeds were divided into two groups: ruderal and cereal weeds[40,41] (Table 2). The chronology of archaeobotanical samples was based on stratigraphic dating and radiocarbon ages. All radiocarbon determinations were performed on charcoal samples at Beta Analytic Inc. (Miami, FL, USA). Calibrated ages were determined using the computer programme CALIBTH3.[42] After calibration, the approximate range of dates for the material studied was 4000–2500 BCE (Table 1).

Stable isotope analyses

Carbonate crusts in fossil grains were removed by soaking each grain separately in 6 M HCl for 24 h at room temperature and then rinsing the grain repeatedly with distilled water.[16] All samples (modern and archaeological) were oven-dried at 60°C for 24 h before milling to a fine powder for isotope analyses. The stable isotope composition of carbon ($\delta^{13}C$, referred to the VPDB standard) and nitrogen ($\delta^{15}N$, referred to N_2 in air) as well as carbon and nitrogen concentration (%C, %N) were determined by elemental analysis and isotope ratio mass spectrometry (EA/IRMS) at the Isotope Services of the University of Barcelona (Barcelona, Spain). The overall analytical precision was about 0.1‰ for $\delta^{13}C$, 0.2‰ for $\delta^{15}N$, 0.6% for %C and 0.1% for %N. Carbon isotope discrimination of archaeological grains ($\Delta^{13}C$) was calculated from grain $\delta^{13}C$ and from $\delta^{13}C$ of atmospheric CO_2, as follows:

$$\Delta^{13}C(‰) = (\delta^{13}C_{air} - \delta^{13}C_{plant})/[1 + (\delta^{13}C_{plant}/1000)] \quad (1)$$

where $\delta^{13}C_{air}$ and $\delta^{13}C_{plant}$ denote air and plant $\delta^{13}C$, respectively.[43] $\delta^{13}C_{air}$ was inferred by interpolating a range of data from Antarctic ice-core records together with modern data from two Antarctic stations (Halley Bay and Palmer Station) of the CU-INSTAAR/NOAA-CMDL network for atmospheric CO_2,[44] as described elsewhere.[45] The whole $\delta^{13}C_{air}$ data set thus obtained covered the period from 16100 BCE to 2003 CE (data available from the internet[46]).

Experimental charring

The effect of carbonisation on $\delta^{13}C$ and $\delta^{15}N$ has been studied on miscellaneous plant seeds and in some cases has shown erratic variations of up to 1.5‰.[16,47] However, in previous studies we reported that $\delta^{13}C$ in cereal grains is not significantly affected by carbonisation within the expected range for carbonisation and sample preservation (ca. 200–400°C), and that the original environmental signal of $\delta^{13}C$ is retained in charred grains.[33,48] Furthermore, Bogaard *et al*.[10] described no significant changes in $\delta^{15}N$ for grains carbonised at 230°C for up to 24 h. In the present study, we further assessed the impact of carbonisation on grain $\delta^{15}N$ and %N by comparing a range of experimental charring conditions. First, we used samples from a previous study in which grains of hulled barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) from the same field trial (UdL-IRTA experimental fields, Lleida, NE Spain) were carbonised at three temperatures (200, 250, and 300°C) and two atmospheric conditions (aerobic, anaerobic) to assess the effect of carbonisation on grain dimension.[49] We took a subset of five grains from each combination of temperature and atmospheric conditions to be analysed individually. As a reference value, a pool of intact grains of the same origin was also analysed.

Table 1. Number of archaeological seeds of naked wheat and naked barley recovered from *Los Castillejos* in which carbon concentration (%C), carbon isotope composition ($\delta^{13}C$), grain weight (GW), nitrogen concentration (%N) and nitrogen isotope composition ($\delta^{15}N$) were determined. Radiocarbon calibrated calendar age (BCE), corresponding cultural period, and inferred carbon isotope composition of atmospheric CO_2 ($\delta^{13}C_{air}$) are also included

Cultural period	Phase	Age (BCE)	$\delta^{13}C_{air}$ (‰)	N (%C, $\delta^{13}C$)	N (GW)	N (%N, $\delta^{15}N$)
Early Neolithic	1	4000-3967	−6.38	10	10	10
Early Neolithic	2	3967-3933	−6.38	10	5	5
Early Neolithic	3	3933-3900	−6.38	10	10	10
Early Neolithic	5	3867-3833	−6.38	5	5	5
Middle Neolithic	7	3800-3740	−6.37	14	5	5
Middle Neolithic	8	3740-3680	−6.37	4	4	4
Middle Neolithic	9	3680-3620	−6.37	5	5	5
Middle Neolithic	10	3620-3560	−6.37	6	5	6
Middle Neolithic	11	3560-3500	−6.37	1	1	1
Late Neolithic	12	3500-3433	−6.37	1	1	1
Late Neolithic	13	3433-3366	−6.37	1	1	1
Late Neolithic	14	3366-3300	−6.36	8	6	6
Late Neolithic	15	3300-3250	−6.36	9	8	9
Late Neolithic	16	3250-3200	−6.36	3	3	3
Early Copper Age	18	3050-2900	−6.35	15	7	10
Middle Copper Age	19	2900-2800	−6.34	17	10	10
Late Copper Age	22	2600-2500	−6.33	6	4	17
Total				125	90	108

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Table 2. Classification and number of weeds found in the archaeological site. Weeds associated with winter cereals crops (C); ruderal weeds (R)

Weed species	Type	Number	Percentage (%)
Bromus erectus	C	22	2.8
Bromus sp.	C	10	1.3
Linum sp.	C	26	3.3
Lolium sp.	C	100	12.5
Papaver dubium/rhoeas	C	62	7.8
Papaver somniferum ssp. *setigerum*	C	88	11.0
Phalaris sp.	C	123	15.4
Plantago lagopus/ovata	C	32	4.0
Rumex acetosella	C	14	1.8
Rumex crispus	C	13	1.6
Silene sp.	C	22	2.8
Total C		512	64.1
Apium graveolens	R	72	9.0
Chenopodium album	R	25	3.1
Chenopodium sp.	R	29	3.6
Malva sp.	R	84	10.5
Melilotus alba	R	25	3.1
Melilotus sp.	R	17	2.1
Urtica sp.	R	35	4.4
Total R		287	35.9

To strengthen the results from this experiment, we selected barley and wheat samples from five contrasting geographic origins across Spain (Table 3(a)), representative of a broad range of environmental conditions (water and nutritional status) and thus exhibiting large original variability in stable isotopes and %N. A subset of grains from each sample was analysed without treatment, whereas two additional subsets were carbonised at 250°C in two atmospheric conditions (aerobic, anaerobic) prior to analysis.

Estimation of grain weight in archaeological grains

We estimated grain weight from the products length × breadth (L × B) and length × thickness (L × T) of archaeological grains, following the models developed in a previous study.[49]

For wheat:

$$\mathrm{GrainWeight} = -15.4 + 2.47 \cdot (L \times B) \quad N = 95,\ r^2 = 0.80,\ P < 0.001 \tag{2}$$

$$\mathrm{GrainWeight} = -15.1 + 2.98 \cdot (L \times T) \quad N = 95,\ r^2 = 0.82,\ P < 0.001 \tag{3}$$

For barley:

$$\mathrm{GrainWeight} = 0.21 \times (L \times B)^{1.60} \quad N = 92,\ r^2 = 0.86,\ P < 0.001 \tag{4}$$

$$\mathrm{GrainWeight} = 0.78 \times (L \times B)^{1.28} \quad N = 92,\ r^2 = 0.85,\ P < 0.001 \tag{5}$$

Estimation of water input during grain filling and grain yield

The total water input (mm) during grain filling was estimated from the Δ^{13}C of charred grains, following the models developed by our team.[4,5,45]

For wheat:

$$\mathrm{WaterInput} = 0.225 \times e^{(0.364 \times \Delta^{13}\mathrm{C})} \quad N = 25,\ r^2 = 0.73,\ P < 0.001 \tag{6}$$

For barley:

$$\mathrm{WaterInput} = 0.175 \times e^{(0.376 \times \Delta^{13}\mathrm{C})} \quad N = 22,\ r^2 = 0.73,\ P < 0.001 \tag{7}$$

The grain yield (Mg ha^{-1}) in ancient crops was estimated from the Δ^{13}C of charred grains, as described elsewhere.[6–8]

For wheat:

$$\mathrm{Yield} = 10^{(0.1745 \times \Delta^{13}\mathrm{C} - 2.269)} \quad N = 508,\ r^2 = 0.72,\ P < 0.001 \tag{8}$$

For barley:

$$\mathrm{Yield} = 10^{(0.1156 \times \Delta^{13}\mathrm{C} - 1.415)} \quad N = 318,\ r^2 = 0.50,\ P < 0.001 \tag{9}$$

Equations (6) to (9) were originally fitted to present-day data collected from a wide range of genotypes and growing conditions. Grain yield estimates were subsequently corrected for the two main differences between old and modern crops not accounted for by the Δ^{13}C of ancient samples: atmospheric CO_2 levels and harvest index.[6–8] In addition, we applied a correction for changes in grain size over the period studied. For this purpose, we assumed the archaeological level with maximum average grain weight as being representative of the genetic potential for this trait in ancient genotypes. We then multiplied each level value by the ratio of average grain weight of the period to this maximum (potential) value. In this way, we attempted to consider the effect of reduced grain weight on crop yield.

Relationship between δ^{15}N and nutritional status in present crops

An additional sample collection of 13 geographic origins from Spain and Syria was used to assess the relationship between δ^{15}N, %N and total N content per grain (mg) (Table 3(b)). The locations were selected in order to maximise environmental variability for the aforementioned traits while having similar agronomic management to that expected in ancient crops. Therefore, we included unfertilised rain-fed fields or fields where only organic matter or low amounts of mineral fertilisers (up to 50 kg N ha^{-1}) were applied.

Statistical analyses

Data were subjected to analysis of variance (ANOVA) to ascertain the effect of charring treatments (temperature and atmospheric conditions) on δ^{15}N, %C and %N. A covariance analysis was used to quantify the association between %N and %C changes during carbonisation. A linear regression model was applied to predict changes in %N (carbonised minus control values) as a result of %C variation

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Table 3. Description of experimental sites where wheat and barley were cultivated to evaluate the impact of charring on grain nitrogen isotope composition (δ^{15}N) and nitrogen concentration (%N) (a) and to assess the relationships between δ^{15}N, %N and N content per grain (N_G) in intact grains (b). Average values of these traits in intact grains are also included. The sites cover a wide range of Mediterranean environments, selected to maximise the variation in δ^{15}N and %N. In addition, in (b) we included only sites where the crops were cultivated under rain-fed conditions and with null to low mineral fertilisation. $T_{a\text{-}m}$ and $P_{a\text{-}m}$, mean temperature and total precipitation during April and May (grain filling period); Irrig., additional water inputs through irrigation; N inputs, nitrogen inputs in the form of mineral fertiliser

Site, Country	Latitude	Longitude	Altitude (m.a.s.l)	$T_{a\text{-}m}$ (°C)	$P_{a\text{-}m}$ (mm)	Irrig. (mm)	N inputs (kg N ha^{-1})	δ^{15}N (‰)	%N	N_G (mg)
a) Sites used to assess the effect of charring										
Barley										
Arkaute, Spain[a]	42°51′N	2°41′W	525	11.1	121	—	50	4.46	2.13	0.81
Calaf, Spain[a]	41°45′N	1°30′E	660	13.3	98	—	134	5.73	2.33	0.83
Guadahortuna, Spain[a]	37°33′N	3°24′W	978	14.0	43	—	82	3.70	2.95	0.93
Barcelona, Spain[b]	41°23′N	2°07′W	63	17.0	86	100	140	8.14	1.97	—
Gimenells, Spain[a]	41°40′N	0°25′E	250	15.7	88	220	206	3.90	2.27	0.69
Wheat										
Arkaute, Spain[a]	42°51′N	2°41′W	525	11.1	121	—	50	5.94	2.31	0.88
Calaf, Spain[a]	41°45′N	1°30′E	660	13.3	98	—	134	4.15	2.25	0.80
Guadahortuna, Spain[a]	37°33′N	3°24′W	978	14.0	43	—	82	2.21	2.65	0.84
Barcelona, Spain[b]	41°23′N	2°07′W	63	17.0	86	100	140	5.78	2.38	—
Gimenells, Spain[a]	41°40′N	0°25′E	250	15.7	88	220	206	3.87	2.65	0.69
b) Sites used to assess relationships between N traits in intact grains										
Barley										
Cinco Casas, Spain[a]	39°17′N	3°22′W	654	12.8	60	—	50	3.91	2.23	0.75
Cobeja, Spain[a]	40°10′N	3°51′W	499	14.7	87	—	—	1.44	2.91	0.74
Ocaña, Spain[a]	39°58′N	3°29′W	731	13.5	97	—	—	0.68	2.16	0.64
La Tallada, Spain[a]	42°03′N	3°04′E	18	15.8	77	—	—	3.03	2.55	0.65
Vic, Spain[ac]	41°96′N	2°22′E	520	14.8	71	—	18	6.42	2.95	0.94
Arkaute, Spain[a]	42°51′N	2°41′W	525	11.1	121	—	50	4.46	2.13	0.81
Abu Galgal, Syria[d]	36°26′N	38°05′E	418	18.9	44	—	—	0.72	2.12	0.76
Tell Halula, Syria[d]	36°25′N	38°12′E	335	18.9	43	—	—	1.65	1.43	0.63
Wheat										
Arkaute, Spain[a]	42°51′N	2°41′W	525	11.1	121	—	50	5.94	2.31	0.72
La Tallada, Spain[a]	42°03′N	3°04′E	18	15.8	77	—	—	4.15	2.25	0.62
Breda, Syria[e]	36°01′N	36°56′E	284	18.4	57	—	—	1.80	3.19	0.71
Abu Galgal, Syria[d]	36°26′N	38°05′E	418	18.9	44	—	—	0.79	2.03	0.71
Membij, Syria[d]	37°56′N	36°31′E	460	18.8	47	—	—	−0.44	1.57	0.53

[a]Check-genotypes from the GENVCE network of recommendation trials (Spain).
[b]Wheat and barley genotypes grown in small plots at the experimental fields of the University of Barcelona.[74,75]
[c]Also fertilised with 60 Mg ha^{-1} of cattle dung.
[d]Durum wheat and barley grains sampled in near-subsistence crops, where mineral N additions are not expected.
[e]Mean across 24 genotypes of bread wheat.[76]

in charred grains, and simple correlations were used to assess the relationship between different variables (δ^{15}N and %N in charred and intact grains, as well as δ^{15}N, %N, total N content and climatic variables in intact grains). Unless otherwise stated, differences were considered statistically significant when $P < 0.05$. We fitted locally weighted least-squares regression curves (LOESS[50]) to the archaeological data sets to summarise overall trends in the time series of variables studied (grain weight, Δ^{13}C, δ^{15}N, %N, yield). Each variable was LOESS-fitted after choosing the smoothing parameter (*span*) that minimised a bias-corrected Akaike statistic. All analyses were carried out using standard SAS-STAT procedures.[51]

RESULTS AND DISCUSSION

Use of δ^{15}N to infer ancient agronomic conditions

Impact of charring on %N, %C and δ^{15}N

In the initial carbonisation experiment we detected strongly significant temperature effects on both %N and %C ($P < 0.001$), whereas the atmospheric condition affected only %C ($P < 0.05$). No significant interactions were found among cereal type, temperature and atmospheric condition on these traits. Given that %N shifts caused by charring resembled those for %C, we applied a covariance analysis to determine the extent by which changes in %N could be explained by %C variability. After including %C as a covariate in the ANOVA, the effect of temperature on %N became non-significant ($P = 0.561$, see also Fig. 1). Considering that %C in extant cereal grains is nearly constant regardless of the species and environmental conditions,[52,53] we fitted a linear regression model relating the original grain %N (%$N_{corr.}$) to the %N and %C values of charred grains, as follows ($N = 60$, $r^2 = 0.62$, $P < 0.001$):

$$\%N_{corr.} = \%N - 0.094 \times \%C + 3.98 \qquad (10)$$

This model was tested on an independent collection of samples (Fig. 2(a)). A significant association ($N = 20$, $r = 0.59$, $P < 0.01$) between the %N of intact grains and the estimated values for carbonised grains was observed after applying Eqn. (10). The intercept and slope of the relationship did not

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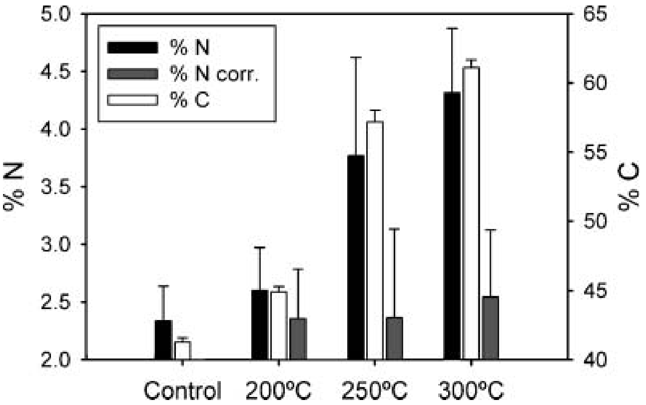

Figure 1. Changes in nitrogen and carbon concentration (%N, %C) in cereal grains charred at different temperatures. %N corr., estimated values of the original grain %N after correction for the effect of charring (Eqn. (10)). Values are means ± standard deviation of 4–5 grains. As a reference, two replicate analyses of a pool of intact grains are included (Control).

differ significantly from zero and unity, respectively. Thus, despite the high variability of carbonised values, modelled values were comparable with those for intact samples, thereby allowing for a semi-quantitative estimation of %N that could complement the potential information included in δ^{15}N.

Apart from significant changes in element concentration during carbonisation, we did not find any significant difference among charring environments (i.e. temperature and atmospheric conditions) for δ^{15}N in the initial experiment. This observation was further confirmed after comparing intact (control) and charred grains for δ^{15}N in an independent collection of samples (Fig. 2(b)). This result indicates that this trait is not altered by carbonisation and that the original environmental signal is well preserved in charred grains. This finding agrees with a previous study showing no significant charring effects on the δ^{15}N of cereal grains.[10]

What can δ^{15}N reveal about the nutritional status of crops?

To gain information about a possible association between grain δ^{15}N and crop nutritional status, we compared δ^{15}N values with both %N and total N content per grain in cereal samples collected from a representative range of Mediterranean conditions (Table 3(b)). The δ^{15}N and %N values were not significantly correlated, but there was a significant relationship between δ^{15}N and N content ($r = 0.61$, $P < 0.05$). Although positive associations between %N and δ^{15}N have been reported for cereal grains,[13,22] the total N content integrates the effect of N availability on grain size and %N simultaneously, and thus it is potentially more sensitive to nutritional deficits.[54]

Grain δ^{15}N correlated negatively with temperature ($r = -0.67$, $P < 0.01$) and positively with precipitation ($r = 0.56$, $P < 0.05$), but these climate variables were related neither to %N nor to total N content in grains. Thus, the relationship observed between δ^{15}N and N content could not be explained through the indirect effect of climate on these two traits. This finding indicates that plant nutritional status, rather than environmental variation, is the main determinant of the abovementioned common patterns involving N-related traits. Therefore, our results indicate that grain δ^{15}N can be used as an integrative indicator of plant nutritional status in spite of showing significant fractionations with respect to the original N source.[19,55] Grain δ^{15}N is thus of potential use in archaeological contexts, where other plant parts (e.g. leaves or roots) are rare, and provides a reasonable background to assess, at least qualitatively, the nutritional status of ancient crops from the Mediterranean area.

Pattern of cereal cultivation over time in *Los Castillejos*

Changes in grain weight and grain yield

Grain weight decreased progressively in both wheat and barley during the first millennium of the site occupation,

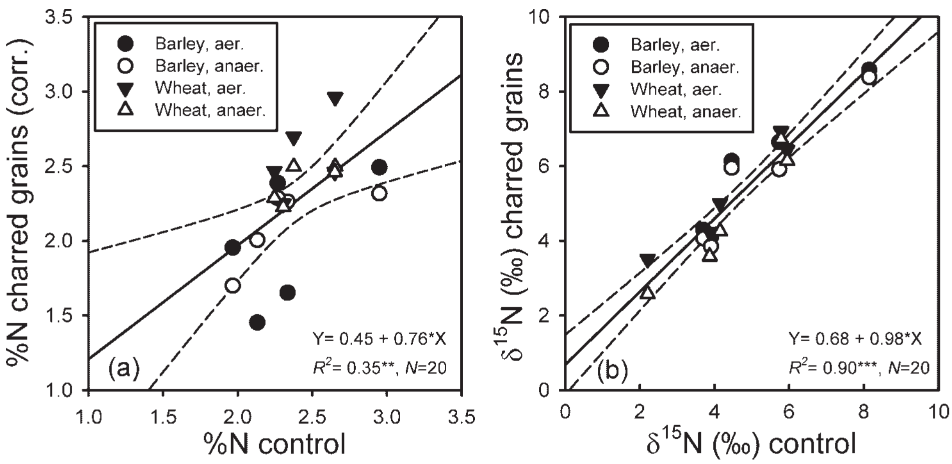

Figure 2. Relationship between nitrogen concentration (%N) (a) and nitrogen isotope composition (δ^{15}N) (b) in intact and carbonised grains of wheat and barley. %N values of carbonised grains were corrected according to Eqn. (10). Wheat and barley grains from locations described in Table 3(a) were charred at 250°C under two types of atmospheric conditions (aerobic and anaerobic). Dashed lines indicate 95% confidence intervals of the regression line. $^{**}P < 0.01$; $^{***}P < 0.001$.

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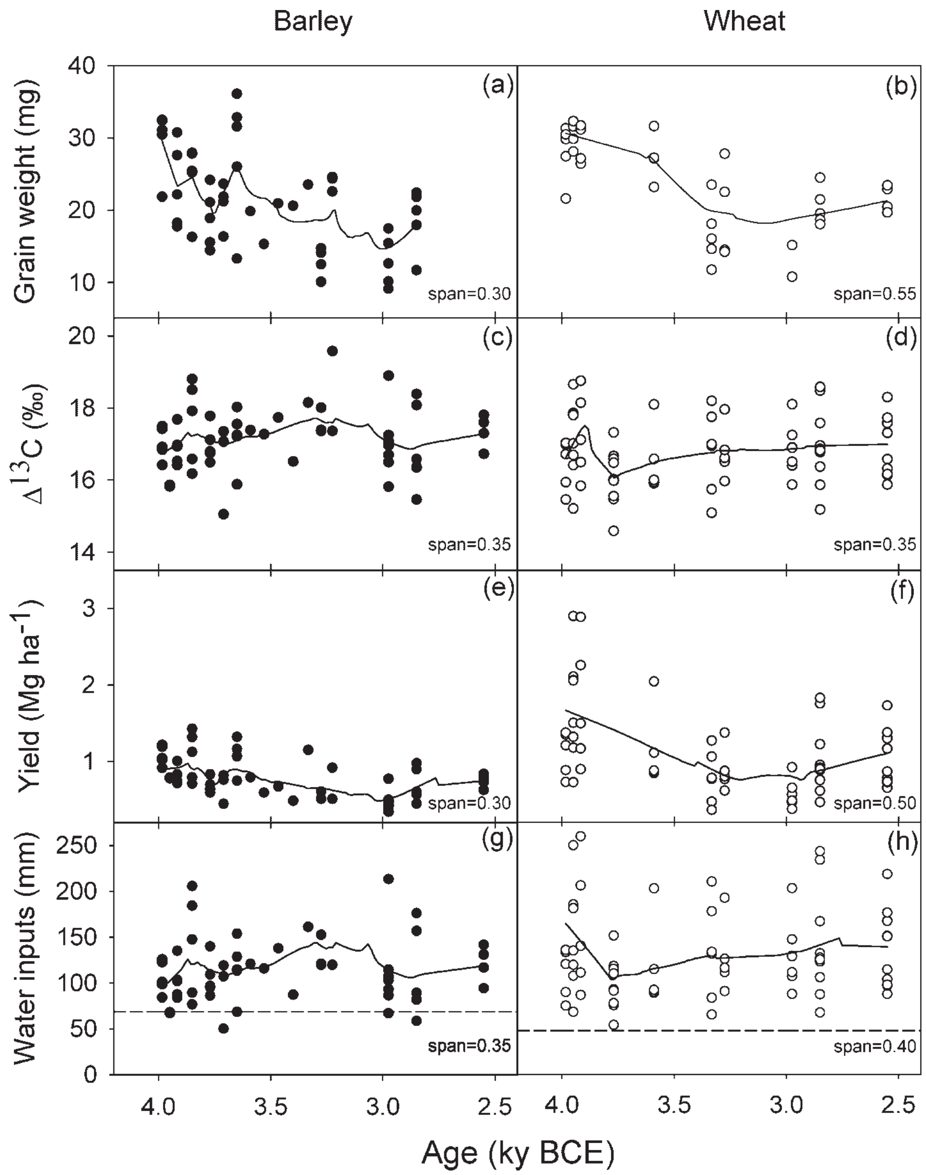

Figure 3. Pattern of changes through time in grain size (a, b), carbon isotope discrimination ($\Delta^{13}C$) (c, d), grain yield (e, f) and total water input during grain filling (g, h) in barley and wheat. Trend lines depict locally weighted least-squares regression curves (LOESS) fitted to the data. Water input and grain yield were estimated from $\Delta^{13}C$ values (see text for details). Broken lines refer to the present-day water input for barley and wheat in the area. All the analyses and calculations were derived from charred grains recovered at *Los Castillejos*. Barley, dotted circles; wheat, open circles.

starting from roughly 30 mg (ca. 4000 BCE) and reaching nearly half their original values by 3000 BCE (Figs. 3(a) and 3(b)). Weights of about 30 mg are considered in the lower range for current barley and wheat cultivated under adequate agronomic conditions.[56,57] Although the comparison of landraces and cultivars suggests that modern cereals have not increased grain size substantially,[8,58,59] a significant contribution of selection practices to favouring a higher grain weight from archaeological material to current traditional landraces cannot be completely ruled out.[60,61] Nevertheless, for both crops, a weight of ca. 15–20 mg is likely to be the outcome of inadequate growing conditions,[54] either because of water scarcity[62,63] and related stresses such as high temperature or because of low soil fertility, basically lack of available N.[64,65] These two alternatives were examined from the C and N composition of archaeological grains (see below).

For barley, average (i.e. LOESS-fitted) grain $\Delta^{13}C$ values ranged mostly between 17.0 and 17.5‰ over the period studied, while for wheat they were usually below 17.0‰ (Figs. 3(c) and 3(d)). Grain yield was inferred from the grain $\Delta^{13}C$ and further corrected by taking into account changes in grain weight through time. Crop productivity decreased with time from about 1.0 to 0.6 tons per hectare for barley, and from 1.5 to 1.0 tons per hectare for wheat (Figs. 3(e) and 3(f)). In fact, the total number of cereal seeds recovered in the archaeological site peaked in layers corresponding to the first third of the occupation period, and further decreased thereafter.[34] Although yield values attained in this site are comparable with those inferred from ancient written sources

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(see references in Araus *et al.*[8]), the clear trend towards a decrease with time further supports the increasing occurrence of stress conditions, either of climatic nature or due to changes in soil fertility.

Water status

The water input during grain filling, as determined from grain $\Delta^{13}C$, remained fairly stable for barley and wheat over the period studied, with average values between 100 and 140 mm (Figs. 3(f) and 3(g)). These values correspond to fairly humid environments,[4,33,45,66] i.e. about twofold wetter than present-day conditions in the region (Figs. 3(f) and 3(g)). However, crop cultivation probably did not use irrigation, as concluded from several lines of evidence. First, grain $\Delta^{13}C$ was slightly higher in barley than in wheat throughout the period, which may be related to differences in crop phenology. Barley usually reaches maturity earlier than wheat, therefore having less evapotranspirative demand and thus avoiding water stress during grain formation.[4,33] Secondly, average grain $\Delta^{13}C$ values were clearly below 18‰, a threshold indicating full irrigation for cereals grown in South Iberian Peninsula.[33] It must be noted, however, that a number of grains reached $\Delta^{13}C$ values of 18‰ or higher, from which it might be concluded that crops were occasionally irrigated. In contrast, the range of grain $\Delta^{13}C$ values within each stratigraphic layer was 3‰ or higher, which can be regarded as a sizeable yearly variability in moisture conditions during the grain-filling period.[59] A large standard deviation in grain $\Delta^{13}C$ occurs preferably in sites where cultivation relies on rainfall as the only water source, thereby reflecting seasonal rainfall unpredictability rather than irregular external water inputs to the crop.

In addition to high-frequency interannual fluctuations in water inputs, inherent to Mediterranean climates, the crop water status remained fairly constant through 1500 years of continued cultivation in this site. This finding diverges from trends observed for the region during the same period. Thus, the $\Delta^{13}C$ of charred cereal grains recovered from a set of archaeological sites in NE and SE Spain indicate a progressive increase in aridity between 4000 and 1000 BCE, particularly in SE Spain.[4] However, this study did not cover a continuous temporal sequence for any of the sites involved. We believe that the lack of agreement between local and regional trends is not inconsistent from a climatic standpoint, and may be the consequence of a particular microclimate (i.e. wet and cool conditions) in *Los Castillejos* during this period.

Nitrogen status and soil fertility

The average grain %N in barley decreased from about 2.0% to 1.4% during the initial half millennium and then remained steady (data not shown). In wheat, however, the average grain %N remained steady (about 2.4%) throughout the whole period. Overall, these values fit the range for present-day crops well,[54,63,67] and are also consistent with a smaller %N in barley than in wheat.[52] In contrast, the N content per grain decreased by half for both crops during the first millennium of cultivation (Figs. 4(a) and 4(b)), which points to a progressive decrease in soil N fertility. A reduction in total N per organ rather than in %N is the typical response to a lack of N availability in plant sinks

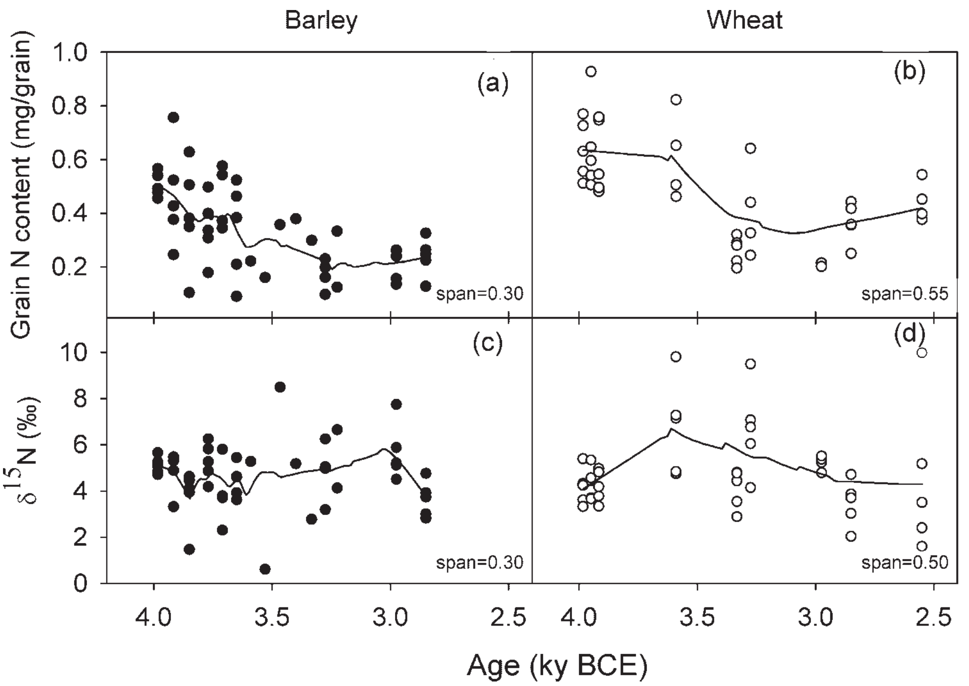

Figure 4. Pattern of changes through time in total N content per grain (a, b) and $\delta^{15}N$ (c, d) in charred barley and wheat grains recovered at *Los Castillejos*. Trend lines depict locally weighted least-squares regression curves (LOESS) fitted to the data. Total N content was calculated for each grain as the product between grain weight and N concentration of charred grains, corrected using Eqn. (10). Barley, dotted circles; wheat, open circles.

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(e.g. grains).[54] Genetic changes in N assimilation capacity associated with crop domestication and further selection can be discarded. For example, an earlier study did not detect differences in plant N pools (%N in leaves and stems, N content per unit leaf area) among wild ancestors, landraces and modern cultivars of wheat and barley.[68]

To infer differences in soil N availability, the grain $\delta^{15}N$ was analysed over the period. In wheat, average $\delta^{15}N$ values increased by about 2‰ during the initial 400 years, decreasing progressively thereafter. In contrast, average $\delta^{15}N$ values for barley remained fairly stable, at between 4 and 6‰ (Figs. 4(c) and 4(d)), and decreased at the end of the period. These results suggest a decrease with time in soil N availability and, especially, for the case of wheat soon after the onset of agriculture in this site. Contrasting with these results, barley showed a steeper decrease in total N content in grains. Differences in agronomic conditions between these two cereals may account for these observations. Thus, the time course of changes in frequency of barley grains recovered in the site followed a similar pattern to that of ruderal weeds, whereas the wheat frequencies roughly followed the same pattern to those of weeds typically associated with winter cereal crops (Fig. 5). These findings indicate that barley was cultivated as a secondary crop in marginal areas, rather than in continuously cultivated cereal fields. In these marginal areas, the soil N availability might have been comparable with that of the soils used for wheat crops (thus showing similar $\delta^{15}N$). However, the intense competition with ruderal weeds would have depleted the N available for barley. Indeed, several studies have shown that barley plants grown together with weeds are less efficient in N uptake, particularly under low N levels.[69,70] Furthermore, changes in the frequency of weeds associated with winter cereals suggest an increase in the area devoted to cereal cultivation (probably wheat) towards the end of the period, probably at the expense of pastures and unploughed land (Fig. 5).

Spatial and agronomic differences in cultivation

Our results indicate that wheat and barley were cultivated under different cropping systems. Naked barley would have probably been found in marginal fields, where ruderal weeds predominated, whereas wheat would have been cultivated in selected areas as the main crop, hence leading to a weed assemblage typical of winter cereals. It should be noted that wheat and barley remains were always mixed, and thus it was not possible to establish a direct association between each crop and the proportion of weeds. However, we observed that in the seed assemblages where wheat was clearly dominant, ruderal weeds were rare, becoming more common when barley was more abundant. Compared with wheat, the lower yield (Fig. 3) and N content (Fig. 4) of barley across all cultivation periods further support this assumption. Cereal remnants found in storage pits at several Copper Age sites in SE Spain such as *Campos* (Cuevas de Almanzora, Almería), *Cerro de la Virgen* (Orce, Granada) or *Cueva de Nerja* (Malaga) also indicate that barley and wheat were cultivated separately.[32,34] In *Los Castillejos*, changes over time in the frequency of wheat and barley grains indicate that the former displaced the latter as the main cereal soon after the onset of agriculture (Fig. 5), naked barley becoming a secondary cereal thereafter.

The few grains of faba bean analysed showed a mean $\Delta^{13}C$ of 18.0‰ (±0.7 SD), which suggests cultivation under wetter conditions than cereals. These grains also exhibited a lower $\delta^{15}N$ (2.3 ± 0.8‰), probably due to symbiotic N_2 fixation metabolism.[17] These differences in $\delta^{15}N$ between cereals and legumes would negate joint cultivation in the same fields, either as intercropping or in a rotation scheme. In fact, a strong distinction between irrigated gardens and non-irrigated farmland is a typical feature of agriculture in Spain,[29] with the oldest lines of evidence originating from the same region in which *Los Castillejos* is located.[71,72] Our findings help to trace this phenomenon back to at least the Copper Age, in accordance with earlier studies reporting that

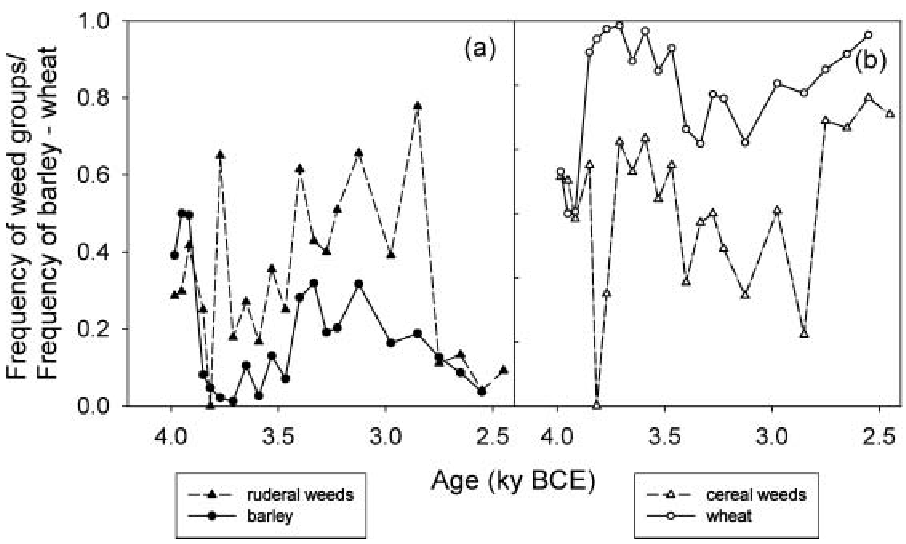

Figure 5. Evolution through time of frequencies in ruderal weed seeds, in relation to total weed seeds, and barley grains, in relation to total cereal grains (a); and frequencies in cereal weed seeds, in relation to total weeds seeds, and wheat grains, in relation to total cereal grains (b), recovered at *Los Castillejos*.

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beans were irrigated but cereals were not.[33] As Grove and Rackham[29] state, every Mediterranean community in Spain would have had its *regadío* (irrigated) gardens surrounded by a much larger area of *secano* (rainfed) arable land and *despoblado* (pasture and savanna) areas for many centuries. This kind of distribution was already common in the area in mediaeval times,[73] but could have started much earlier, as exemplified in *Los Castillejos*.

CONCLUSIONS

This is the first report on a combined analysis of carbon and nitrogen isotopic signatures together with quality traits from crop remains to provide an overall description of the environmental conditions prevailing in ancient agriculture. Although spatial and agronomic differences in cultivation were already established in this early agricultural site, we can conclude that early agricultural systems in the Mediterranean were probably not sustainable, but exposed to a progressive lost of efficiency. This conclusion is based on the observed decrease in grain size as well as in crop yield and total nitrogen content per grain through time. This pattern does not appears to be related to changes in water availability during cultivation, but rather to decreased soil fertility, particularly for wheat, the main crop in this agricultural system. Our results reinforce the negative role of early agricultural activities in shaping the environmental and ecological conditions in the Mediterranean.

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