Cultivar diversity in the mineral content of wheat grain under different nitrogen regimes.

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Abstract

Wheat is an important source of mineral nutrients in human diet. However, modern high-yield varieties have lost nutritional value relative to older ones. To correct this situation, we need to identify varieties with higher mineral concentrations in the grain and develop agronomic practices that favour nutrient accumulation in the grain. Here we compared nitrogen fertilization versus genetic variability as drivers of mineral content in the grain of wheat in Mediterranean semi-arid conditions, and alkaline soils. To do so, 12 winter wheat modern cultivars from different European provenance were grown on two consecutive years at three levels of top dressing N fertilization: optimal (105 Kg N ha^{-1}); -50%; and +30%. The variety was the most important factor affecting the mineral concentration in the grain, followed by the climatology of the year. The N-regime had very little effect. The cultivar CH-Nara had both the highest Ca, Fe, Mg, Mn, N, P, S, and Zn and the lowest GY.

Keywords

Wheat; mineral content; plant nutrition; heavy metals; nitrogen; grains

1. Introduction

Wheat is one of the main staple foods of the world, providing one fifth of the total available calories in human diet[1]. The global wheat production in 2019 was of 766 million tonnes (MT) and a total area of 216 million ha was dedicated to growing wheat worldwide[2]. Most of wheat grain is produced in temperate climate areas in the European Union (139 MT), China (134 MT), India (103 MT), and Russia (74 MT). Wheat is also a key global trade commodity, in ever-rising demand, and has extensive uses in the food industry[3,4]. The nutritional contribution of wheat to human diet is very important, especially in countries where most of the daily calorie intake comes from wheat. Wheat grains are rich in protein (15%) and dietary fibre (14% in wholemeal flour), and are a good source of polyphenols, vitamins, and essential mineral nutrients including iron (Fe) and zinc (Zn)[6,7]. Essential nutrients are necessary for human health, and their deficiency causes serious illness and malfunctions in our body. Iron and Zn are currently the main human nutritional deficiencies in the world, probably due to the green revolution strategy to base human diet in high-yielding crops with low micronutrient content[7]. Iron deficiency in humans is very widespread globally and causes anaemia, low blood cell count and immunity, heart issues, cognitive malfunction and retarded development, fatigue, increased maternal and perinatal mortality and morbidity[8]. Lesser known, Zn deficiency in humans can delay growth, impair cognitive function, compromise the immune system, increase the death rate for acute diarrhoea and other infections, and cause blindness and hypogonadism[9]. Besides the essential nutrients, toxic metals such as cadmium (Cd) and lead (Pb) can also accumulate in wheat grains and enter the food chain. These minerals are toxic to humans at trace concentrations and accumulate in our tissues. Lead is the oldest known toxic metal. It accumulates mainly in the nervous tissue, causing a plethora of neurological and cognitive dysfunctions, especially severe in children[10]. Lead can also cause blood and kidney disorders, reproductive issues, and miscarriage[10]. Cadmium exposure in humans can trigger cancer and cause toxic effects throughout the body: skeleton demineralization, neurological toxicity, impairment of the renal and reproductive functions, etc[11]. In summary, the mineral composition of wheat grains is a critical quality trait to guarantee the nutritional value and safety of our food.

There are several factors that explain mineral content in grains, including environmental factors, agronomic practices, plant traits, and physiological responses[12]. Among the environmental factors, the properties of the soil are key. The mineral composition, pH, clay content, cation exchange capacity, and redox activity of the soil have all been reported to affect nutrient bioavailability[13], and hence the nutritional content of the grain. The climate is another important environmental factor in determining mineral accumulation in the grain. Water scarcity during the growing season, especially during the grain-filling period, increases the concentration of micronutrients in the grain[14]. Pollution of irrigation water, soils, and air can cause the accumulation of toxic metals in wheat[15,16]. Cadmium is increasingly released into the environment from industrial sources, mining, atmospheric deposition, and the use of Cd-containing pesticides and fertilizers[17]. Due to the pollution of agricultural soils, Cd is a frequent contaminant in grain[18]. Agronomic practices such as liming, that increases the soil pH, and fertilizing can also impact the nutrient content of the grain[19–21]. Nitrogen fertilization increases nutrient mobility in the soil and promotes both root growth and fine roots, improving plant uptake of other nutrients[22]. Nitrogen fertilization may also lead to increased allocation of N to N-rich compounds such as ribulose bisphosphate carboxylase and chlorophylls, thus increasing the photosynthetic capacity of the plant and the demand for other nutrients[23]. Moreover, nitrogen is thought to play an important role in the mechanisms of mineral uptake, transport through the plant and loading in the grain[24]. Besides the environmental factors, genetic variability is a major factor in determining the nutritional content of wheat grains. Long-stemmed varieties and landraces typically have higher Fe, Mn, Zn content relative to the short-stemmed modern varieties[25,26]. Even among varieties of the same stature and antiquity, there is a great variability from one cultivar to another[27]. Genetic variability in mineral content cannot be traced back to a few QTL and many minerals seem to locate together, which makes genetical modification for this trait difficult[28]. The mineral content of the grain is controlled by a complex network of interconnected processes, from the uptake at the rhizosphere until the loading of nutrients and toxic metals in the grain[29]. For this reason, it has been stated that the best strategy to increase the mineral contents of wheat grains (so called biofortification) is breeding[30]. Hence, it is essential to identify which modern cultivars are interesting due to the accumulation of nutrients (especially Fe and Zn) in their grains.

Considering all the above, selecting the right wheat variety and providing enough N fertilization appears to be the most effective strategy to influence the nutritional quality of the grain at the farm level. However, there is very little information in the literature about the nutritional content of high-yielding wheat varieties of regular use in semi-arid environments with alkaline soils. These conditions are common in the Mediterranean region, home to 480 million people, and many other areas in the world. The impact of N fertilization in mineral accumulation in the grain in our conditions is also poorly known. Considering the yield gap that afflicts areas with similar water scarcity issues[31,32], this matter deserves further attention for its potential to improve agricultural productivity and climate change resilience for many communities. Hence, we focussed our research in those two factors.

Objectives: This study aims to assess the relative importance of N fertilization versus genetic variability as drivers of mineral content in the grain of high-yielding winter wheat varieties in Mediterranean climate. The results of this research will allow us to identify which varieties produce the most nutritious grains, and to investigate potential nutritional deficiencies and toxicities that may affect winter wheat productivity in the region. To do so, we set five objectives:

i) To evaluate the changes in the mineral content of the grain in twelve widely used high-yielding European bread wheat varieties in response to three different top dressing N- fertilization regimes,
ii) To identify which high-yielding varieties have the highest levels of nutrients and the lowest levels of toxic metals in the grain,
iii) To diagnose nutritional deficiencies and toxicities that might affect grain yield and quality locally and in similar settings,
iv) To evaluate the effect of water availability on the mineral content of the grain, and
v) To examine the associations between the mineral content and the yield, yield components, and carbon (C) and nitrogen (N) stable isotopes in the grain.

The study tested four working hypotheses: 1) The genotypic variability and water availability determine the mineral content of winter wheat grains grown under Mediterranean conditions; 2) specifically water deficit increases the mineral concentration in the grain; 3) increased N fertilization increases the concentration of some elements in the grain, especially micronutrients like Fe, Mn, or Zn; and 4) grain yield has a negative correlation with the concentration of micronutrients in the grain. The knowledge gained from this study will contribute to guide future breeding and agronomic practices in the region and guarantee food safety in the advent of climate change.

This research is associated with the European Consortium for Open Field Experimentation (ECOFE), which covers all major climatological regions of Europe. ECOFE aims to develop systematic investigations of the interactions between a plant genotype, environment, and agricultural management, i.e., studies of high-yielding wheat varieties across a range of farming practices and locations under highly standardized conditions. In that sense, the present study was performed in one of the experimental stations of the project.

2. Material and Methods

2.1. Experimental design

Field trials were conducted in the Castile-Leon Agriculture Technology Institute (ITACyL), in the Experimental Station of Zamadueñas (41° 39´ 8´´ N and 4° 43`24`` W, 690 m.a.s.l.), sited in Valladolid (Spain). A set of twelve high-yielding winter bread wheat cultivars (Benchmark, Bennington, Bologna, Chambo, CH-Nara, Henrik, Hondia, JB Diego, Julius, KWS-Siskin, RGT-Reform, and Soberbio)(Table S.1.) cultivated in European countries were evaluated under three contrasting overcasting nitrogen (N) fertilization regimes. The N regimes used were called: N2, optimal fertilization rate (105 Kg N ha^{-1}); N1, optimal -50%; and N3, optimal +30%. The main properties of the soil are listed on Table 1.

Table S. 1. List of winter wheat varieties used in this study with their qualities of interest. Adapted from de Lima et al, 2021[31].

Genotype	Country	Provider	Characteristics
Benchmark	Germany	Hannover University	High yield
Bennington	UK	Rothamsted Research?	High yield and septoria resistance
Bologna	Italy	University of Bologna	High yield and hard kernel
Chambo	Spain	Institute of Agrifood Research and Technology	High yield and rust resistance
CH-Nara	Switzerland	Agroscope	High yield and grain quality, baking quality, widely used
Henrik	Belgium	Ghent University	High and stable yield, widely used
Hondia	Poland	Institute of Soil Science and Plant Cultivation	High yield and grain quality, new variety
JB Diego	Ireland	National University of Ireland	High yield
Julius	Sweden	Swedish University of Agricultural Sciences	High yielding and first milling variety
KWS Siskin	UK	Rothamsted Research	High yielding and first milling variety, new variety
RGT Reform	Germany	Hohenheim University	High yield and grain quality, widely used
Soberbio	Spain	Castile-Leon Agriculture Technology Institute	High yield and grain quality traits

Table 1. Main soil properties in the experimental plots for each growth season, 2018/2019 and 2019/2020. Topsoil samples (0-10 cm) were collected before applying fertilizers and before sowing.

	2018	**2019**
pH in water (1:5)	8.52	8.62
Clay (%)	17.5	12.0
Sand (%)	35.0	33.0
Silt (%)	48.0	55.0
Texture	loam	silty loam
Phosphorus (mg P_2O_5 Kg^{-1})	64.2	45.4
Total carbon (%)		1.00
Total nitrogen (%)	0.0130	0.0670
Total nitrogen (mg Kg^{-1})	130	670
Potassium (mg K_2O g^{-1})	0.373	0.250
Potassium (mg Kg^{-1})	373	250
Active lime (%)	4.97	1.66
Electrical conductivity (µS cm^{-1})	117	136
Humidity (%)	1.43	1.62
Organic carbon (%)	0.16	
Organic matter (%)	0.28	1.17
Carbonates (%)		<3

The experimental design consisted of three replicates (individual plots) per genotype (twelve cultivars) and nitrogen regime (three regimes), totalling 108 plots per trial. The experiment was replicated in two consecutive years, harvested in 2019 and 2020. For the first trial, plots were 12 m long and 1.5 m wide, with 7 rows sown 21 cm apart (totalling 18 m^2 per plot). For the second trial, plots were 8 m long and 1.5 m wide, with 7 rows sown 20 cm apart (totalling 12 m^2 per plot).

The trials were sown on the 29/11/2018 (first trial) and the 28/10/2019 (second trial), at a rate of 450 seeds per m^2. In both trials, a basal dressing of 300 Kg ha^{-1} was applied as 8-15-15 (N-P-K) before seeding. Then, calcium ammonium nitrate (CAN, 27% N) was added during the tillering stage (date not recorded on 2019, and 14/1/2020) and Nitrosylsulphuric acid (NSA, 26% N) was applied as top-dressing during stem elongation (date not recorded on 2019, and 12/03/2020). Both NAC and NSA were applied at a rate of 200, 150, and 75 Kg ha^{-1} for the N1, N2, and N3 regimes, respectively. Phytosanitary treatment included the spraying of fungicides (Amistar, Syngenta; Prosaro, Bayer), insecticide (Karate Zeon, Syngenta), and herbicide (Amadeus top, Syngenta) at the recommended rates as needed during elongation, booting, and heading stages. Plants reached maturity in the second half of June. Plots were harvested mechanically on 17/07/2019 for the first trial and 21/07/2020 for the second trial.
During the first trial, the temperatures ranged -7.7 to 40.7 °C and the daily mean was on average 10.3 °C (Fig.1). Rainfall was very scarce with only 158 mm accumulated, therefore the conditions of this trial were those of intense drought. During the second trial, temperatures ranged from -4.8 to 36.2 °C, the daily mean averaged 11.2 °C, and the accumulated rainfall was 469.8 mm.

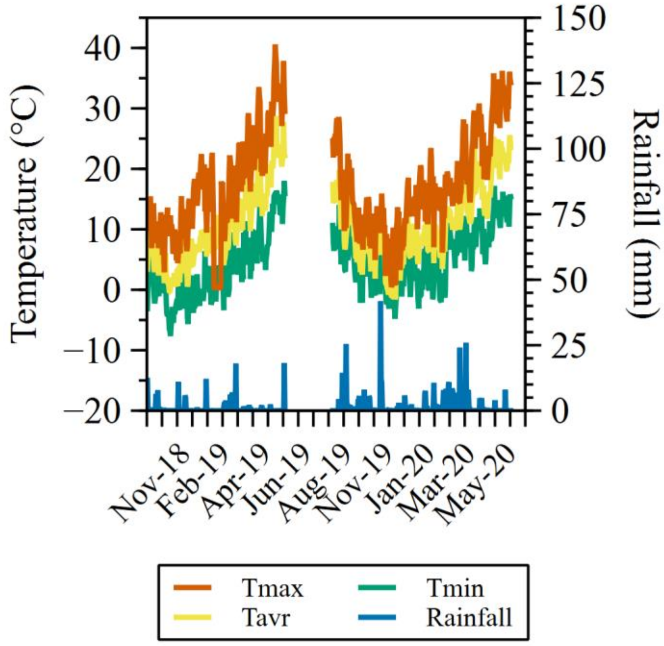

Figure 1. Climatic conditions recorded during the two field trials. Data represent daily temperatures and rainfall. Tmax = daily maximum temperature; Tavr = daily mean temperature; Tmin = daily minimum temperature; Rainfall = rainfall accumulated in 24h. Climatic data were collected from a meteorological station located in the experimental site, managed by the SIAR (Service of Agroclimatic Information for Irrigation, www.siar.es).

2.2. Yield and yield components

At the end of the experiment, the plots were harvested mechanically, and grain yield (GY) was determined for each plot and adjusted to a 10% moisture level. A subsample of around 20% was taken to determine thousand grain weight (TGW).

2.3. C and N content and stable isotopes

Grain samples were dried at 60 °C until constant weight and finely ground using a ball mill (MM 400, Retsch GmbH, Haan, Germany). Approximately 1 mg of the powdered samples were weighed in tin capsules (3.3 mm, Cromlab, Barcelona, Spain). The samples were taken to the facilities of the Scientific and Technical Centres of the University of Barcelona (CCiTUB, Barcelona, Spain) for C and N analyses. The total C and N content and $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were determined using an Elemental Analyser interfaced (EA) with an Isotope Ratio Mass Spectrometer (IRMS). For the first trial this was a Flash EA-1112-DeltaC IRMS, and for the second trial a Flash EA-IRMS-Delta V Advantatge (both ThermoFisher Scientific, Waltham, MA, USA). Total C and N contents in grains were expressed as the percentage (%) on dry matter basis. Stable isotope values were expressed in delta notation (δ), following equation (1):

$$\delta X_{sample}\ (‰) = \left[\frac{R_{sample}}{R_{standard}} - 1\right] \times 1000$$

Where δX_{sample} is the isotope composition of the sample expressed in ‰ ($\delta^{13}C$ or $\delta^{15}N$); R_{sample} is the isotope ratio of the sample ($^{13}C/^{12}C$ or $^{15}N/^{14}N$); and $R_{standard}$ is the isotope ratio of the secondary standard calibrated against the primary standard. The primary standards were Pee Dee Belemnite for C and N_2 from air for N. The secondary standards for C were IAEA-CH7 (International Atomic Energy Agency, Austria), UCGEMA K, UCGEMA CH, and fructose (in-house standards); and for N, IAEA N1, IAEA 600, UCGEMA K, UCGEMA CH, and UCGEMA. The analytical precision of the $\delta^{13}C$ and $\delta^{15}N$ analyses was 0.2‰.

2.4. Mineral content analyses

To determine the mineral content in the grain, 0.1 g of wheat flour were weighed in Teflon® beakers and digested in 2 ml HNO_3 and 1 ml H_2O_2 at 90°C overnight at the CCiTUB. Digests were diluted in 30 ml of MilliQ water (18.2 Ω) and refrigerated until analysis. The mineral content in the digests was determined by Induced Coupled Plasma Optical Emission Spectrometry for Ca, Fe, K, Mg, Na, P, S (ICP-OES, Optima 8300, Perkin Elmer, Waltham, MA, USA) and for Si (Optima 3200rl, Perkin Elmer); and by Induced Coupled Plasma Mass Spectrometry for B, Cu, Mn, Na, and Zn (Elan6000, Perkin Elmer). Per each 11 samples, a procedural blank and an aliquot of a certified reference materials were analysed in the same manner. The reference materials were BCR-596 (aquatic plant, Joint Research Centre, Brussels, Belgium) and BCR-279 (sea lettuce, JRC). We obtained 288 ± 3 (n = 12) $\mu g\ g^{-1}$ Zn, a 92% recovery relative to the certified values (313.0 ± 8.0). The digestion beakers were acid-washed and rinsed in Milli-Q water before use. The quality of the acids was 69-70 % Baker Instra – Analyzed Reagent 9598.34 for HNO_3, and 30% Suprapur Merck 1.07298.1000 for H_2O_2. Plastic tubes and caps were rinsed thrice in the digest before filling them for analysis.

2.5. Statistical analyses

Statistical analyses were conducted using R software version 3.4.0 for Windows[1] with the FSA package version 0.8.2660. For each dependent variable, the best mixed model was selected using the Akaike Information Criterion (AIC) and a three-way ANOVA was applied to the model with the lowest AIC score. The mixed models tested are shown in table Table S.2. We screened the data for outliers using Rosner's test and any outliers were removed from the dataset prior to analyses. The conditions of normal distribution and equal variances were not met completely in some cases. However, following[33] we trusted the robustness of the three-way ANOVA test and accepted its conclusions. The non-parametric Kruskal-Wallis (KW) ranks' test and Dunn's test with Benjamini-Hochberg adjustment (Dunn) were also applied to each factor separately, to confirm the results of the ANOVA. The scientific plotting package Veusz 1.23.2 (https://veusz.github.io/) was used to create the graphs.

Table S.2. List of mixed models tested for each dependent variable. The best model for each dependent variable was selected using the Akaike Information Criterion (AIC).

Short name	Model
lme1	Y =parameter ~ year*nitrogen*genotype + (1|repeat)
lme2	Y =parameter ~ year*nitrogen*genotype + (year|repeat)
lme3	Y =parameter ~ year*nitrogen*genotype + (nitrogen|repeat)
lme4	Y =parameter ~ year*nitrogen*genotype + (1|repeat) + (year|repeat)
lme5	Y =parameter ~ year*nitrogen*genotype + (1|repeat) + (nitrogen|repeat)
lme6	Y =parameter ~ year*nitrogen*genotype + (1|repeat) + (year|repeat) + (nitrogen|repeat)

3. Results

3.1. Yield and TGW

Grain yield (GY, t ha^{-1}) was strongly affected by the year, variety, and their interaction (all three P <0.001) (Table 2). GY was the highest in 2020 (8.3±0.1 t ha^{-1}, mean of all varieties and nitrogen treatments), which was a rainier year, compared to the mean GY in 2019, which was 70% lower (2.5±0.1 t ha^{-1}). The variety Soberbio performed the best considering the two years, with a mean GY of 6.1±0.8 t ha^{-1}. By contrast, CH-Nara had the lowest GY (4.3±0.5 t ha^{-1}), 30% less on average than Soberbio. The interaction year x variety showed that CH-Nara and Bologna did not increase yield as much as other varieties in 2020.

Table 2. Grain yield (GY) and thousand grain weight (TGW) averaged per nitrogen fertilization regime, cultivar, and year of sowing. Values are reported as means ± standard errors. The number of replicates was n = 60, 71, and 69 for nitrogen regimes N1, N2, and N3 respectively; n = 17 for all cultivars, except Hondia (16) and Bologna (14); and n = 107 and 93 for growth seasons 2018/2019 and 2019/2020, respectively. Different letters indicate statistically significant groups.

	GY (Kg ha^{-1})	TGW (g)
nitrogen		
N1	4603±372a	30.9±0.81a
N2	5404±371a	32.5±0.69a
N3	5529±375a	32.8±0.59a
variety		
Benchmark	5125±831bcde	30.7±1.45abc
Bennington	5665±883efg	33.8±1.24cd
Bologna	4352±581a	24.8±1.48a
Chambo	5565±745defg	32.7±1.46bcd
CH-Nara	4326±496a	29.5±1.04a
Henrik	5959±824fg	35.1±0.88d

Hondia	4878±768abc	34.4±1.15d
JB_Diego	5254±818cdef	33.2±1.01bcd
Julius	4460±672ab	33.3±1.14bcd
KWS_Siskin	5655±803defg	30.4±1.30ab
RGT_Reform	4998±730abcd	32.4±1.29abcd
Soberbio	6074±781g	34.2±1.70d
year		
2019	2528±76a	28.2±0.42a
2020	8288±131b	36.6±0.32b
year	<0.001***	<0.001***
nitrogen	ns	ns
variety	<0.001***	<0.001***
year:nitrogen	ns	0.04
year:variety	<0.001***	<0.01**
nitrogen:variety	ns	ns
year:nitrogen:variety	ns	ns

The weight of one thousand grains (TGW, g) was strongly affected by the year (*P* <0.001), the variety (*P* <0.001), and their interaction (*P* = 0.005). The year x nitrogen interaction had some effect on TGW too but of less significance (*P* = 0.04). Following the same tendency as GY, the TGW was higher in 2020 (36.6±0.3g) than in 2019 (28.2±0.4g). The varieties Hondia, Soberbio, and Henrik reached the highest TGW, at an average of 34.4±1.2, 34.1±1.7, and 35.1±0.9 g, respectively. By contrast, CH-Nara had the lowest mean TGW (29.5±1.0g), 16% lower than Henrik. The interaction year x variety shows that Soberbio increased its TGW in 2020 much more than the other varieties. Further, the interaction year x nitrogen shows that N3 led to the highest TGW in 2019, but the lowest in 2020.

3.2. Element content – effect of the variety, year, and the N regime.

The most relevant factor to explain element content in the grain was the variety, which had a significant effect on all the elements quantified except C, Na, and Pb when the data from 2019 and 2020 were analysed together (Table 3). The variety CH-Nara showed the highest mean in [Ca], [Fe], [Mg], [Mn], N%, [P], [S], and [Zn]. Besides, KWS-Siskin had the highest [K], Bologna the highest [Cu], and both Bennington and JB Diego the highest [B]. The [Cd] in the grains was maximum in the variety Hondia and minimum in Benchmark. The second most important factor to explain the element content in the grain was the year of the harvest. Grains collected in 2019, under intense drought conditions, showed significantly higher levels of B, C, Ca, Cd, Cu, Fe, K, N, S, and Zn than grains collected in 2020. The same was probably true for Na, although the results were only significant either using a 0.1 confidence level (*P* = 0.09) or the KW test (*P* <0.001). By contrast, [Mg] was higher in the 2020 samples. The [Mn], [P], and [Pb] were also higher in 2020 according to the KW test (*P* <0.001), although the ANOVA was not significant (*P* = 0.12, 0.09, and 0.25, respectively). Nitrogen fertilization treatment had irrelevant effect in the mineral content of the grain when the two years were analysed together. However, some interactions with the year or the variety were noted. The interaction year x variety was significant for K, Mg, Ca, Fe, Mn, Cu, and Cd. This interaction is indicative of which varieties were most affected by the changes in the climatic conditions between the two years. JB Diego was the variety that most reduced its [Cu], [Fe], [Mg], and [Mn] in 2020 relative to 2019. Compared to the other varieties, Bologna did best at maintaining [Fe] and [Cu] in 2020. However, this variety also showed the highest reduction of [K] and increase of [Cd] in 2020. Benchmark increased [Ca] in 2020, while the other varieties decreased it or maintained it. Finally, the [K] of Julius decreased the least in 2020 compared to the other varieties. Further, the interaction year: nitrogen serves to assess if any of the regimes contributed to preserve the nutritional value of the grain in 2020. This interaction was

Table 3. Grain mineral composition per nitrogen fertilization regime, cultivar, and year of sowing. Values are reported as means ± standard errors. The number of replicates was n = 60, 71, and 69 for nitrogen regimes N1, N2, and N3 respectively; n = 17 for all cultivars, except Hondia (16) and Bologna (14); and n = 107 and 93 for growth seasons 2018/2019 and 2019/2020, respectively. Different letters indicate statistically significant groups.

	K (g Kg^{-1})	P (g Kg^{-1})	S (g Kg^{-1})	Mg (g Kg^{-1})	Ca (g Kg^{-1})	Fe (mg Kg^{-1})	Mn (mg Kg^{-1})	Zn (mg Kg^{-1})	Na (mg Kg^{-1})	Cu (mg Kg^{-1})	B (mg Kg^{-1})	Cd (µg Kg^{-1})	Pb (µg Kg^{-1})
nitrogen													
N1	4.16±0.05^{a}	2.49±0.04^{a}	1.37±0.03^{a}	0.96±0.01^{a}	0.41±0.01^{a}	34.6±0.7^{a}	35.0±0.6^{a}	15.4±0.4^{a}	9.11±0.69^{a}	3.78±0.06^{a}	1.39±0.08^{a}	6.76±0.46^{a}	9.91±1.54^{a}
N2	4.15±0.05^{a}	2.51±0.05^{a}	1.35±0.03^{a}	0.97±0.01^{a}	0.41±0.01^{a}	33.4±0.6^{a}	35.0±0.6^{a}	15.4±0.5^{a}	8.91±0.49^{a}	3.83±0.06^{a}	1.34±0.07^{a}	6.65±0.46^{a}	8.4±1.81^{a}
N3	4.25±0.07^{a}	2.57±0.05^{a}	1.41±0.04^{a}	0.97±0.01^{a}	0.41±0.01^{a}	33.9±0.8^{a}	33.2±0.6^{a}	16.5±0.6^{a}	8.16±0.31^{a}	3.94±0.07^{a}	1.41±0.07^{a}	5.44±0.46^{a}	7.86±1.13^{a}
variety													
Benchmark	4.41±0.08^{de}	2.61±0.10^{ab}	1.29±0.06^{a}	0.91±0.02^{ab}	0.42±0.01^{cd}	35.3±1.6^{ab}	32.7±1.4^{abcd}	16.3±1.2^{ab}	7.88±0.80^{a}	3.69±0.11^{abc}	1.42±0.17^{ab}	4.93±0.63^{a}	9.49±2.70^{a}
Bennington	4.34±0.12^{de}	2.37±0.10^{a}	1.30±0.06^{a}	0.92±0.02^{abc}	0.39±0.01^{abc}	31.4±1.0^{a}	32.2±1.0^{a}	14.1±1.1^{a}	9.08±0.68^{a}	3.66±0.12^{ab}	1.51±0.14^{b}	5.26±0.67^{a}	9.1±3.12^{a}
Bologna	3.79±0.16^{a}	2.66±0.08^{ab}	1.52±0.06^{cd}	1.02±0.03^{de}	0.42±0.01^{abc}	33.5±1.6^{ab}	34.8±1.7^{bcde}	15.3±0.9^{ab}	8.25±0.75^{a}	4.39±0.08^{e}	1.43±0.17^{ab}	6.34±1.30^{abc}	9.46±2.19^{a}
CH-Nara	4.08±0.08^{bcd}	2.86±0.09^{b}	1.59±0.05^{d}	1.10±0.02^{f}	0.49±0.02^{e}	36.8±0.8^{b}	38.7±1.0^{e}	17.6±0.9^{b}	7.40±0.54^{a}	4.00±0.12^{cd}	1.34±0.14^{ab}	5.54±0.76^{ab}	6.81±1.57^{a}
Chambo	4.21±0.12^{bcde}	2.46±0.06^{a}	1.32±0.05^{a}	0.94±0.03^{bc}	0.38±0.01^{ab}	33.0±1.3^{ab}	35.7±1.1^{de}	15.2±0.7^{ab}	8.51±0.68^{a}	3.93±0.12^{bcd}	1.30±0.12^{ab}	5.51±0.81^{ab}	8.78±2.78^{a}
Henrik	4.25±0.09^{cde}	2.55±0.10^{ab}	1.30±0.05^{a}	0.98±0.02^{cde}	0.40±0.01^{abc}	35.6±1.4^{ab}	35.9±0.9^{de}	16.0±1.1^{ab}	8.37±0.63^{a}	3.89±0.13^{bcd}	1.44±0.15^{ab}	6.43±0.84^{abc}	10.1±2.6^{a}
Hondia	3.85±0.06^{ab}	2.68±0.08^{ab}	1.44±0.06^{c}	1.06±0.02^{ef}	0.38±0.01^{ab}	33.4±1.3^{ab}	35.3±1.3^{abcd}	17.1±1.4^{ab}	8.03±0.36^{a}	3.81±0.15^{bcd}	1.34±0.19^{ab}	8.22±1.22^{c}	5.60±1.25^{a}
JB Diego	4.32±0.11^{cde}	2.46±0.10^{a}	1.29±0.07^{a}	0.87±0.02^{a}	0.40±0.01^{abc}	33.7±1.7^{ab}	32.6±0.8^{ab}	16.0±1.3^{ab}	11.81±2.49^{a}	3.73±0.27^{abcd}	1.58±0.18^{b}	6.91±0.84^{abc}	10.6±4.4^{a}
Julius	4.18±0.08^{bcd}	2.54±0.08^{ab}	1.34±0.06^{ab}	0.97±0.02^{bcd}	0.38±0.01^{a}	34.5±1.4^{ab}	32.8±0.7^{abc}	16.6±1.2^{ab}	9.86±0.70^{a}	4.06±0.14^{de}	1.36±0.15^{ab}	6.49±0.89^{abc}	7.97±2.02^{a}
KWS-Siskin	4.52±0.06^{e}	2.34±0.09^{a}	1.34±0.05^{ab}	0.90±0.02^{ab}	0.46±0.02^{de}	31.0±1.0^{a}	33.1±1.2^{abcd}	14.3±0.9^{a}	8.02±0.70^{a}	3.72±0.10^{abc}	1.39±0.15^{ab}	6.82±0.90^{abc}	9.18±3.52^{a}
RGT-Reform	4.22±0.08c^{de}	2.39±0.05^{a}	1.44±0.06^{bc}	0.92±0.01^{abc}	0.42±0.02^{abc}	36.0±1.6^{ab}	34.5±0.7^{abcd}	15.2±0.9^{ab}	8.26±0.81^{a}	3.83±0.11^{bcd}	1.13±0.10^{a}	6.04±0.71^{abc}	6.29±1.76^{a}
Soberbio	3.96±0.12^{abc}	2.36±0.07^{a}	1.32±0.05^{ab}	1.02±0.03^{de}	0.42±0.01^{bcd}	33.7±1.5^{ab}	35.3±1.5^{cd}	14.9±0.9^{ab}	9.5±1.54^{a}	3.49±0.11^{a}	1.30±0.13^{ab}	7.55±1.34^{bc}	11.7±6.3^{a}
year													
2019	4.38±0.04^{b}	2.43±0.04^{a}	1.55±0.02^{b}	0.93±0.01^{a}	0.44±0.01^{b}	36.8±0.52^{b}	31.8±0.3^{a}	18.3±0.42^{b}	9.49±0.20^{a}	4.19±0.04^{b}	1.86±0.04^{b}	3.85±0.21^{b}	5.10±0.69^{a}
2020	3.96±0.03^{a}	2.62±0.03^{a}	1.17±0.01^{a}	1.00±0.01^{b}	0.38±0.00^{a}	30.7±0.41^{a}	37.5±0.5^{a}	12.8±0.17^{a}	7.92±0.61^{a}	3.45±0.04^{a}	0.83±0.01^{a}	9.15±0.34^{a}	12.9±1.7^{a}
year	<0.001***	ns	<0.001***	0.02*	0.02*	0.011*	ns	<0.001***	ns	<0.001***	<0.001***	0.012*	ns
nitrogen	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
variety	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	0.002**	<0.001***	0.005**	ns	<0.001***	0.03*	<0.001***	ns
year:nitrogen	0.03*	0.04*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
year:variety	<0.001***	ns	ns	<0.001***	<0.001***	0.018*	<0.001***	ns	ns	<0.001***	ns	<0.001***	ns
nitrogen:variety	ns	ns	0.04*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
year:nitrogen:variety	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

only significant for K and P, which were given together with N as the NPK fertilizer. Plants grown in the regime N3 did not reduce [K] so much in 2020. Besides, plants grown with the regimes N2 and N3 showed increased [P] in 2020. Finally, the interaction nitrogen:variety was only significant for S. Most of varieties had a higher [S] under the N1 treatment except these four: RGT-Reform and KWS-Siskin (N3), and Julius and Chambo (N2).

Next, we analysed the data for each year separately to identify any year-specific trends that did not appear in the joint analysis. In 2020, the variety had a significant effect on all the elements analysed except C%, [Cd], and [Na] (Tables S.3. and S.4.). Also in 2020, the variety CH-Nara showed the highest mean [Ca] (together with Benchmarck), [Fe], [Mg] (together with Soberbio), [Mn], N%, [P], [S], and [Zn]. Moreover, KWS-Siskin had the highest [K], Bologna the highest [Cu], and Bennington the highest [B]. Interestingly, in 2020 the variety had a significant effect on [Pb] (P = 0.04) which did not show when the two years were analysed together. The [Pb] was highest in Bologna grains and lowest in Hondia, Bennington and Soberbio. Besides, the interaction nitrogen:variety was significant for [Pb] (P<0.001), due to the variety Bologna showing an increased [Pb] in response to treatment N3. Finally, in 2020 the [Cd] increased with increasing N fertilization (P= 0.002), even if the posthoc contrasts did not separate the fertilization regimes into statistically significant groups. In the previous year, the variety had a significant effect on all the elements analysed except [B], C%, [Cd], N%, [P], [Pb], and [Zn] (Tables S.3. and S.4.). The variety CH-Nara showed the highest mean [Ca], [Mg], [Mn], and [S] in 2019. Furthermore, KWS-Siskin had the highest [K] and Bologna and Julius the highest [Cu]. For [Na], the ANOVA showed a significant effect of the variety (P = 0.04) that was not shown in 2020. However, the posthoc contrasts could not separate any statistically different groups. Finally, the [P] in the grain in 2019 decreased with increasing nitrogen (P =0.04), again an effect that was only observed this year.

3.3. Stable isotopes

The $\delta^{13}C$ was isotopically lighter in 2020 than in 2019 (P <0.001) (Table 4). Besides, there were clear differences between varieties (P <0.001), with CH-Nara showing the highest $\delta^{13}C$. By contrast, the $\delta^{15}N$ was isotopically lighter in 2019 (P <0.001) (Table 4) and did not differ across varieties. The nitrogen treatment had a significant effect on $\delta^{15}N$ according to the ANOVA (P = 0.013). However, the posthoc contrasts could not separate any significant groups. The interaction year x variety appeared significant for $\delta^{13}C$ (P = 0.049) and the interaction year x nitrogen for $\delta^{15}N$ (P = 0.0104). However, a closer inspection of the interaction plots did not reveal any biologically relevant effect (results not shown). When the two years were analysed separately, we still observed significant differences in the $\delta^{13}C$ between varieties (P <0.001) and CH-Nara was the variety with the highest $\delta^{13}C$, both in 2020 and in 2019. By contrast, $\delta^{15}N$ only showed significant differences between varieties in 2020 (P = 0.013), when CH-Nara had the highest $\delta^{15}N$. Besides, $\delta^{15}N$ decreased with increasing nitrogen fertilization only in 2019 (P = 0.0011).

Table 4. Grain carbon and nitrogen concentration and stable isotope composition per nitrogen fertilization regime, cultivar, and year of sowing. Values are reported as means ± standard errors. The number of replicates was n = 60, 71, and 69 for nitrogen regimes N1, N2, and N3 respectively; n = 17 for all cultivars, except Hondia (16) and Bologna (14); and n = 107 and 93 for growth seasons 2018/2019 and 2019/2020, respectively. Different letters indicate statistically significant groups

	C(%)	N(%)	$\delta^{13}C$(‰)	$\delta^{15}N$(‰)
nitrogen				
N1	41.9±0.3a	2.35±0.08a	-23.8±0.31a	2.07±0.08a
N2	41.8±0.23a	2.23±0.06a	-24.3±0.28a	2.04±0.09a
N3	40.9±0.41a	2.25±0.06a	-24.1±0.28a	1.88±0.10a
variety				
Benchmark	40.25±0.987a	2.19±0.136a	-24.11±0.562abc	2.09±0.194a
Bennington	41.64±0.749a	2.14±0.144a	-24.37±0.589ab	1.97±0.160a
Bologna	42.37±0.529a	2.43±0.165a	-23.89±0.630a	1.72±0.206a
CH-Nara	41.50±0.640a	2.54±0.112b	-23.51±0.593d	2.11±0.168a
Chambo	41.41±0.689a	2.19±0.126a	-24.19±0.64abc	1.69±0.203a
Henrik	41.66±0.538a	2.20±0.115a	-24.06±0.542abc	1.99±0.212a
Hondia	41.62±0.539a	2.14±0.130a	-24.05±0.597cd	1.89±0.227a
JB Diego	41.48±0.578a	2.23±0.138ab	-24.09±0.634abc	2.13±0.165a
Julius	42.04±0.542a	2.39±0.147ab	-23.85±0.573cd	2.15±0.180a
KWS_Siskin	41.04±0.600a	2.20±0.120a	-24.33±0.530abc	2.03±0.181a
RGT_Reform	41.80±0.592a	2.39±0.118ab	-23.98±0.566bcd	2.06±0.188a
Soberbio	41.42±0.635a	2.24±0.140ab	-24.49±0.602a	2.03±0.172a
year				
2019	43.29±0.145b	2.67±0.036b	-21.95±0.057b	1.46±0.057a
2020	39.45±0.218a	1.82±0.030a	-26.53±0.049a	2.61±0.037b
year	<0.01**	<0.001***	<0.001***	<0.001***
nitrogen	ns	ns	<0.01**	<0.05*
variety	ns	<0.001***	<0.001***	ns
year:nitrogen	ns	ns	ns	<0.05*
year:variety	ns	ns	ns	ns
nitrogen:variety	ns	ns	ns	ns
year:nitrogen:variety	ns	ns	ns	ns

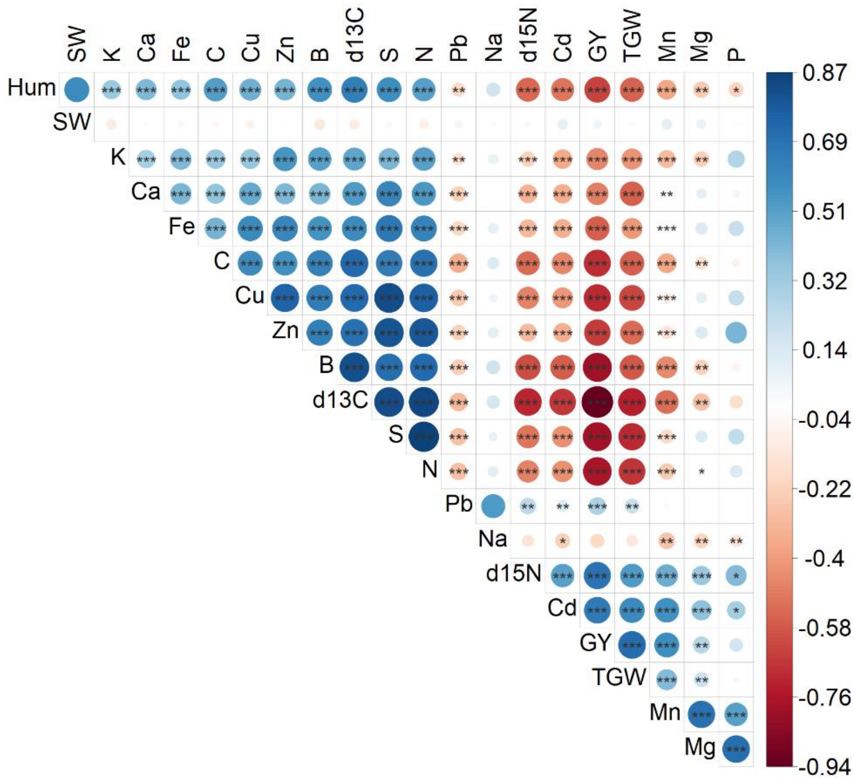

Figure 2. Pearson's correlation correlogram for the grain concentration of several minerals, grain yield (GY), thousand grain weight (TGW), grain humidity (Hum), $\delta^{13}C$, $\delta^{15}N$, and shoot weight (SW). Both growth seasons are considered together. Significance levels are reported as P<0.001 (***), P<0.01 (**), and P<0.05 (*).

3.4. Correlations

The variables used in this study displayed several significant correlations with the mineral content of the grain (Fig. 2). The complete correlation matrix with coefficients and significance values is presented in Table S.5. The GY, TGW, and $\delta^{15}N$ correlated negatively with B, C, Ca, Cu, Fe, K, N, S, and Zn (Fig. 2, Table S.5.). The same three parameters showed positive correlations with Cd and Mn, and even with Mg and Pb if we include the weak correlations. By contrast, the $\delta^{13}C$ and C% showed exactly the opposite trends as GY, TGW, and $\delta^{15}N$. Besides, we observed several correlations between the nutrients, which were generally positive except when either Cd, Pb, or Mn were involved. Sulphur and N correlated positively with Cu, Zn, and B. Zinc and Cu were also strongly correlated, as were P and Mg. All the correlations described in this paragraph were highly significant (*P*<0.001).

When the correlations were calculated separately for 2020 and 2019, the results obtained were different. In 2020, the GY only showed a moderate negative correlation with P (-0.304, *P* <0.01) and a moderate positive correlation with TGW (0.389, *P* <0.001) (Table S.5.). The TGW correlated negatively with the concentration of Ca, P, S, Cu, Zn, Fe, and N in grains (-0.3 to -0.6, *P* <0.001). As for the isotopic signatures, the $\delta^{13}C$ correlated negatively with nutrients N, S, P, and Zn (-0.3 to -0.6, *P* <0.001), while the $\delta^{15}N$ did not show any relevant associations. Finally, several positive correlations between nutrients were observed in 2020. In essence, all minerals correlated positively with one another except B, Cd, K, Pb, and Na. In 2019, even fewer correlations were observed between the productivity and physiological parameters and the mineral content in the grain (Table S.5.). The GY only showed a moderate negative correlation with [Zn] (-0.341, P <0.001) and no relevant associations were observed for TGW. As for the stable isotope composition, the $\delta^{13}C$ correlated positively with Cu, Mn, Mg, N, P, S, and Zn (0.3-0.6, P<0.001); and the $\delta^{15}N$ with N, P, S, and Zn (0.3-0.4, P<0.01). Besides, we observed several positive correlations between nutrients. Namely, P correlated with B, C, Cu, Fe, K, Mg, Mn, N, S, and Zn (0.4-0.8, P<0.001); S with C, Ca, Cu, Fe, Mg, Mn, N, P, and Zn (0.4-0.7, P<0.001); and N with C, Cu, K, Mg, Mn, P, S, and Zn (0.3-0.7, P<0.001). Besides, both Fe and Mg correlated positively with Cu, Zn, and Mn (0.3-0.6, P<0.01). Finally, Zn and Cu had a strong positive correlation (0.70, P<0.001).

4. Discussion

4.1 Nutritional value of the grain. Potential deficiencies and toxicities.

The mineral levels in the grain samples analysed are in line with the literature for B, Cd, Cu, Fe, K, Mg, Mn, Na, Pb, and Zn[34–37]. The mean concentration of Cu, Fe, K, Mg, Mn, and P in the grain was enough to make nutritional food claims following Regulation (EU) No 1169/2011[38]. According to this regulation, nutrient levels at least 15% of the daily recommended intake constitute a significant amount, making a foodstuff valuable for human nutrition. The threshold levels are 1.5 mg Kg^{-1} Cu, 21 mg Kg^{-1} Fe, 3000 mg Kg^{-1} K, 563 mg Kg^{-1} Mg, 3 mg Kg^{-1} Mn, and 1050 mg Kg^{-1} P for solid food[7]. This is generally in agreement with Ciudad-Mulero *et al.*[37], who found significant amounts of Cu, Fe, K, Mg, Mn, and Zn in wholemeal flour from wheat grown in similar conditions to ours. In our study, however, many samples did not reach the required levels for Zn (15 mg Kg^{-1}). Samples in the lower end of the range for Fe, P, and Zn concentration were below the threshold of nutritional deficiency for wheat (21 mg Kg^{-1} Fe[39], 2000 mg Kg^{-1} P[28], and 10 µg Kg^{-1} Zn[29]). The soil in our experiment had a high pH, which is typical of Mediterranean soils and the main cause for low Fe, P, and Zn bioavailability[42]. This factor alone can fully explain the low Fe, P, and Zn concentrations in some of our grain samples. Besides, Zn and Fe in the grain are mostly stored in the aleurone layer, bound to phytate (a phosphate-rich compound) and sequestered in the protein storage vacuoles[43]. Therefore, P deficiency in wheat can reduce both Fe and Zn accumulation in the grain. Finally, the high GY of modern wheat varieties causes a dilution effect in the grain that reduces the concentration of nutrients, a trend that started several decades ago[44]. Fertilization with P, Zn, and Fe could probe a useful strategy to improve the nutritional status of the crops in our region, and in general to increase GY and quality in wheat grown in alkaline soils and semi-arid conditions[45–47].

The threshold levels for Cd in wheat flour are either <0.1 mg Kg^{-1} (EU) or <0.2 mg Kg^{-1} (China) according to international food regulations[48–50]. The Cd content of the grains used in this study was always very low and within the legal limits. In agreement with the literature[51], Cd concentration in the grain increased with GY and was higher in some varieties. However, Cd levels in the grain were not affected by the fertilization regime. Ata-Ul-Karim and co-workers[51] recently reported increasing Cd levels in wheat grains with increasing N fertilization, reaching up to 0.26 mg Kg^{-1}. The conditions of their experiment, however, were very different to ours: the soil was acidic (pH 6.0); the climate was humid (>1800 mm $year^{-1}$, 78% humidity); and the rates of N fertilization were higher (up to 300 Kg N ha^{-1}). The semi-arid climatology, the alkalinity of the soil, and the lower N levels in our experiment compared to At-Ul-Karim's work[51] might have contributed to a lower accumulation of Cd in the grain, by either reducing Cd availability in the soil or limiting bulk flow. Further, Pb is another non-essential trace element toxic to living organisms and commonly found in wheat[52,53]. The maximum Pb levels permitted in wheat [48–50] are <0.2 mg Kg^{-1}, akin to those of Cd. In most of the samples, Pb was either very low or absent, but we found a few with abnormally high Pb levels (≈ 0.1 mg Kg^{-1}). This can be attributed to the presence of random Pb sources in the soil, like old birdshot. Still, all the samples analysed were within the safe limit for Pb.

4.2 Effect of the genotype on the mineral content in the grain.

The choice of cultivar was the factor that most influenced the concentration of minerals in the grain in our study. Following previous studies by the group[31,54], we used an array of 12 high-yielding varieties popular with farmers from different European regions, not only from the Mediterranean but also from central and north Europe countries, which adapt well to our conditions. The variety CH-Nara showed the highest mineral content overall. A cultivar developed in Switzerland, CH-Nara produces top quality grain with a high protein content, while the yield is low compared to other high-yielding varieties. Our results show that the nutritional quality could be an interesting trait of this variety. There is little information in the literature about the concentration of minerals in the grain for many commonly used wheat varieties, especially those grown in Spain and other arid and semi-arid environments. We could not find any mineral concentration data for CH-Nara. Hence, to assess the nutritional potential of this variety, we compared our results for CH-Nara with other varieties grown in similar environments. Marcos-Barbero and co-authors[34] recently examined the nutritional composition of grain from 10 genotypes cultivated in growth chambers simulating Mediterranean conditions. As compared with the genotype with the highest concentration for each nutrient, CH-Nara had higher Ca, Fe, and S concentrations (26%, 62%, and 16x more, respectively); lower Cu, Mg, P, and Zn (25%, 16%, 48%, and 54% less, respectively); and similar levels of B, K, and Na. It must be noted that the [S] in[34] was exceptionally low. Similarly, as compared with the maximum values for the variety Cajeme reported by Ciudad-Mulero *et al.*[37], CH-Nara showed higher Ca, Fe, K, and Mn in grains (2.6x, 47% more, 3.3x, and 6.5x, respectively); lower Cu, Na, and Zn (60%, 98%, and 41% less, respectively); and similar Mg concentration. Vázquez *et al*.[55] reported the Fe and Zn grain concentration for several landraces and commercial wheat varieties in a wide range of soil types. CH-Nara mean Fe levels reported here are within the range given for both landraces and commercial varieties. However, the Zn concentration was much lower. Compared to the maximum values for landraces and commercial varieties in Vázquez *et al*.[28], CH-Nara had 91% and 72% less Zn, respectively. In summary, CH-Nara grains are high in Ca and Fe, but low in Zn, as compared to other wheat varieties grown in similar conditions.

In our study, the effect of the genotype was not equally strong for all the minerals analysed: Ca, K, Mg, and S differed greatly between varieties; B, Fe, P, and Zn showed a low variability across the genotypes; and Na and Pb did not show any statistical differences between varieties. Similarly, Marcos-Barbero *et al.*[16] found that the variation of B, Fe, Na, and Zn across 10 wheat genotypes did not reach statistical significance. Further, Guttieri and co-workers[56] quantified the heritability of the concentration of several minerals in the grain and found differences between the elements. In their experiment, heritability was high for Cd, Li, and Ca; moderate for Ni, Mn, Cu, and Fe; and low for Zn. They also observed very low heritability for the mineral concentration in the grain in one of the trial sites. This suggests that the environment can be more relevant than the genotype for some locations. The genetic basis underlying the diversity in grain mineral content across genotypes is currently poorly understood. However, some alleles have been linked to increased mineral allocation to the grain. In Swedish spring wheat, varieties carrying the wildtype allele of the gene *NAM-B1*, involved in leaf senescence and nutrient remobilization to the grain, accumulate more P and K in the grain[57]. Recently, a genome-wide association study on 205 winter wheat accessions identified 101 loci affecting the content of Ca, Fe, Zn, Se, Cu, Mn, Cd, As, and Pb in the grain[28].

4.2 Effect of the yield and yield components on the mineral content in the grain.

To understand which traits might lead to increased mineral accumulation in some varieties but not in others, we will now discuss the relationship between the mineral content in the grain and the grain yield (GY) and a key yield component (TGW). In the literature, an increase in GY is generally followed by a decrease in the nutritional value[44]. This seems to agree with our results, since the mineral content in the grain decreased with increasing GY and TGW when the two years were analysed together. Besides, the variety with the highest mineral content (CH-Nara) also had the lowest GY. However, the GY and the mineral content did not generally correlate when we separated the data from different years. Moreover, the TGW correlated negatively with several nutrients in 2020 but not in 2019. The most suitable explanation for these results lays in the location of the minerals in the grain. The mineral content is higher in the outer layers of the grain than in the starchy endosperm[58]. Therefore, the varieties with bigger and rounder grains due to higher starch accumulation might have higher TGW but a lower concentration of nutrients. A higher TGW does not always lead to higher GY, because the total number of grains, rather than the TGW, use to be more important determining GY. This could explain why, within each year, the GY generally did not correlate with the mineral content of the grain. Hence, we propose that the mineral content of wheat grain in our conditions might be influenced by the shape of the grain, which can be a trait associated to the variety. However, this effect disappeared under drought, as can be seen by the absence of correlations of TGW with the mineral content in the grain in 2019. The reason for that is probably that the grain filling could not be properly completed in these unfavourable conditions, reducing the roundness of the grains in all varieties. If our hypothesis is correct, a good strategy to tackle the dwindling nutritional value of wheat crops could be to breed for long grains that maintain a high surface to volume ratio even after grain filling.

4.4 Effect of the N fertilization regime on the mineral content in the grain.

Several researchers have informed that increasing the dose of N fertilizer promoted the accumulation of various nutrients in the grain, such as Ca, Cu, Fe, K, Mn, N, Ni, P, and Zn[59,60]. This can be attributed to the beneficial effect of N on root growth and synthesis of storage proteins, increasing the capacity of the plant to acquire nutrients and accumulate them in the grain[20,62]. However, important decreases in Ca, Cu, Fe, K, Mg, Mn, P, and Zn have also been reported in response to N application[63,64]. These reductions could be due to N fertilization increasing the yield and causing a dilution effect when the proportion of starch increases versus the nutrients accumulated[64]. The N fertilization regimes tested in our study barely had any effect in the mineral composition of the grain. Similarly, Jaskulska and co-authors[65] found very few changes on the mineral composition of the grain after applying N, only a small increase in the concentration of Ca. After examining the literature, we could not identify any single factor (pH, organic matter, etc.) that provided a consistent explanation for the variability of the effects of N fertilization on the concentration of minerals in the kernels. We conclude that the response of the mineral concentration in wheat grains to N fertilization is highly site-specific, and probably the result of complex interactions between several factors, such as the environmental conditions, management schemes, cultivars used, etc. In our case, the most likely explanation for the little response to N fertilization is that N was not a key limiting factor for nutrient acquisition in our conditions, the main issue being water scarcity. Studies dealing with the nutritional content of wheat grain mostly focus on temperate climates and acidic-neutral soils, and wheat cultivars that grow well in those conditions (*e.g.*, [47,52]). Seeing the strong influence of the environment in the accumulation of minerals in the grain, it is crucial to increase the number of studies like the present one, in arid and semi-arid environments and alkaline soils.

4.3 Effect of water availability and N fertilization on C and N stable isotopes.

As discussed before, the drought conditions of 2019 increased the mineral content of the grain. This we attributed to a change in the geometry of the grain caused by insufficient grain filling, which limited the dilution effect of starch accumulation. Besides, the $\delta^{13}C$ correlated positively with the mineral content, both when the two years were analysed together and separately. The $\delta^{13}C$ varies with the internal CO_2 concentration of the leaves, which greatly depends on the stomatal aperture[67]. Hence, lower $\delta^{13}C$ values indicate higher transpiration rates and GY values[35]. Higher transpiration rates could potentially increase nutrient acquisition through the bulk flow. However, that does not necessarily mean that the nutrient content of the grain should increase. The higher GY and the subsequent dilution effect will tend to lower the mineral concentration in the grain[44]. When crop growth is very fast, the mechanisms for mineral uptake, transport and allocation to the grain cannot increase in the same proportion as the synthesis of photoassimilates[56]. Our $\delta^{13}C$ data are consistent with the dilution effect being more relevant for the mineral content of the grain than the transpiration rate. The mineral content correlated positively with $\delta^{13}C$ and was lower in 2020, even if this was a relatively wet year that allowed plants to keep higher transpiration rates.

In 2019, $\delta^{15}N$ decreased with increasing N application and was lower than in 2020. The relationship between $\delta^{15}N$ and N fertilization was not significant in 2020. This points to plants acquiring N from different pools in 2019 and 2020. The activity of soil bacteria, including the biological fixation of nitrogen, is inhibited by intense drought[57]. Hence, a possible interpretation of the $\delta^{15}N$ data is that in 2019 most of the N came from the fertilizer. Chemical fertilizer, including calcium ammonium nitrate, exhibit lower $\delta^{15}N$ than available soil N coming from decomposition of organic matter. By contrast, the higher $\delta^{15}N$ in 2020 and lack of response to increasing fertilizer rates indicates that plants were using additional sources of N, such as N from organic matter decomposition. However, N metabolism in plants is very complex, and many steps during N uptake, assimilation, and remobilization could have altered the $\delta^{15}N$ of the original N source. Therefore, we explore alternative explanations for these results. Opposite to $\delta^{13}C$, $\delta^{15}N$ decreases in response to stress[58]. In C3 plants such as wheat, water deficit restricts stomatal aperture and reduces the intracellular CO_2/O_2 ratio, increasing photorespiration rates[58]. Photorespiration is strongly connected to N assimilation and acts as the intersection between N and C assimilation. In agreement, lower $\delta^{15}N$ were associated with higher mineral concentrations in the grain and lower GY and TGW.

5. Conclusions

Of the four working hypotheses tested, our findings support hypothesis 1, 2, and 4. The genotype and water availability were key to determine the mineral content of wheat grain. The mineral concentration in the grain increased with water scarcity but had a negative correlation with grain yield. This supports the idea of a dilution effect as the most likely mechanism for the dwindling nutritional value of the grain in modern wheat varieties. However, our data are not compatible with accepting hypothesis 3. We did not observe that higher N-fertilization rates had any beneficial effect on the concentration of micronutrients like in the grain. We attribute this to the fact that N was not a key limiting factor for nutrient acquisition in our conditions, the main issues being water scarcity or other nutritional deficiencies. We identified three potential nutritional deficiencies (P, Zn, and Fe), likely caused by the high soil pH. Correcting these deficiencies might improve wheat GY and nutritional value in our area and others with similar conditions (semi-arid, alkaline soils). We propose that specific fertilization regimes providing those three elements and soil amendments aiming to reduce soil pH could be the best strategies to close the yield gap and improve the grain nutrient concentration in this kind of setting. We identified CH-Nara as a cultivar of interest for the high nutritional value of the grain, while maintaining an adequate yield for modern standards. The knowledge gained from this study will contribute to guide future agronomic practices in the region and other areas with similar climate and soil properties, and guarantee food safety and quality for the local communities.

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7. Reference List

1. Food and Agriculture Organization of the United Nations. Wheat - the largest primary commodity. https://www.fao.org/resources/infographics/infographics-details/es/c/240943/.
2. FAOSTATS. Crops and livestock products. (2019).
3. Day, L., Augustin, M. A., Batey, I. L. & Wrigley, C. Wheat-gluten uses and industry needs. *Trends Food Sci. Technol.* **17**, 82–90 (2006).
4. Shewry, P. R. Wheat. *J. Exp. Bot.* **60**, 1537–1553 (2009).
5. Kumar, P. *et al.* Nutritional Contents and Medicinal Properties of Wheat: A Review. *Life Sci. Med. Res.* **LMSR-22**, (2011).
6. Frølich, W., Åman, P. & Tetens, I. Whole grain foods and health - a Scandinavian perspective. *Food Nutr. Res.* (2013) doi:10.3402/fnr.v57i0.18503.

7. Graham, R. D., Knez, M. & Welch, R. M. Chapter One - How Much Nutritional Iron Deficiency in Humans Globally Is due to an Underlying Zinc Deficiency? in *Advances in Agronomy* (ed. Sparks, D. L.) vol. 115 1–40 (Academic Press, 2012).

8. Abbaspour, N., Hurrell, R. & Kelishadi, R. Review on iron and its importance for human health. *J. Res. Med. Sci. Off. J. Isfahan Univ. Med. Sci.* **19**, 164–174 (2014).

9. Prasad, A. S. Discovery of Human Zinc Deficiency: Its Impact on Human Health and Disease123. *Adv. Nutr.* **4**, 176–190 (2013).

10. Wani, A. L., Ara, A. & Usmani, J. A. Lead toxicity: a review. *Interdiscip. Toxicol.* **8**, 55–64 (2015).

11. Rafati Rahimzadeh, M., Rafati Rahimzadeh, M., Kazemi, S. & Moghadamnia, A. Cadmium toxicity and treatment: An update. *Casp. J. Intern. Med.* **8**, 135–145 (2017).

12. Johansson, E. *et al.* Genotypic and Environmental Effects on Wheat Technological and Nutritional Quality. in *Wheat Quality For Improving Processing And Human Health* (eds. Igrejas, G., Ikeda, T. M. & Guzmán, C.) 171–204 (Springer International Publishing, 2020). doi:10.1007/978-3-030-34163-3_8.

13. Naidu, R. *et al.* Chapter 1 Chemical bioavailability in terrestrial environments. in *Developments in Soil Science* (eds. Hartemink, A. E., McBratney, A. B. & Naidu, R.) vol. 32 1–6 (Elsevier, 2008).

14. Zhao, C.-X., He, M.-R., Wang, Z.-L., Wang, Y.-F. & Lin, Q. Effects of different water availability at post-anthesis stage on grain nutrition and quality in strong-gluten winter wheat. *C. R. Biol.* **332**, 759–764 (2009).

15. Bermudez, G. M. A., Jasan, R., Plá, R. & Pignata, M. L. Heavy metals and trace elements in atmospheric fall-out: Their relationship with topsoil and wheat element composition. *J. Hazard. Mater.* **213–214**, 447–456 (2012).

16. Huang, M., Zhou, S., Sun, B. & Zhao, Q. Heavy metals in wheat grain: Assessment of potential health risk for inhabitants in Kunshan, China. *Sci. Total Environ.* **405**, 54–61 (2008).

17. Kubier, A., Wilkin, R. T. & Pichler, T. Cadmium in soils and groundwater: A review. *Appl. Geochem.* **108**, 104388 (2019).

18. Abedi, T. & Mojiri, A. Cadmium Uptake by Wheat (Triticum aestivum L.): An Overview. *Plants* **9**, 500 (2020).

19. Antoun, L. W., Zakaria, S. M. & Rafla, H. H. INFLUENCE OF COMPOST, N-MINERAL AND HUMIC ACID ON YIELD AND CHMICAL COMPOSITION OF WHEAT PLANTS. *J. Soil Sci. Agric. Eng.* **1**, 1131–1143 (2010).

20. Dolijanović, Ž. *et al.* Mineral profile of the winter wheat grain: effects of soil tillage systems and nitrogen fertilization. *Appl. Ecol. Environ. Res.* **17**, 11757–11771 (2019).

21. Chauhan, N., Sankhyan, N. K., Sharma, R. P., Singh, J., & Gourav. Effect of long-term application of inorganic fertilizers, farm yard manure and lime on wheat (Triticum aestivum L.) productivity, quality and nutrient content in an acid alfisol. *J. Plant Nutr.* **43**, 2569–2578 (2020).

22. Rekaby, S. A., Eissa, M. A., Hegab, S. A. & Ragheb, H. M. Wheat Response to Nitrogen and Irrigation under Semi-Arid Conditions. *World J. Agric. Soil Sci.* **1**, 1–6 (2019).

23. Hamnér, K., Weih, M., Eriksson, J. & Kirchmann, H. Influence of nitrogen supply on macro- and micronutrient accumulation during growth of winter wheat. *Field Crops Res.* **213**, 118–129 (2017).

24. Shi, R. *et al.* Influence of long-term nitrogen fertilization on micronutrient density in grain of winter wheat (Triticum aestivum L.). *J. Cereal Sci.* **51**, 165–170 (2010).

25. Ficco, D. B. M. *et al.* Phytate and mineral elements concentration in a collection of Italian durum wheat cultivars. *Field Crops Res.* **111**, 235–242 (2009).

26. Hussain, S., Maqsood, M. A., Rengel, Z. & Khan, M. K. Mineral bioavailability in grains of Pakistani bread wheat declines from old to current cultivars. *Euphytica* **186**, 153–163 (2012).

27. Murphy, K. M., Reeves, P. G. & Jones, S. S. Relationship between yield and mineral nutrient concentrations in historical and modern spring wheat cultivars. *Euphytica* **163**, 381–390 (2008).

28. Wang, W. *et al.* Identification of novel genomic regions associated with nine mineral elements in Chinese winter wheat grain. *BMC Plant Biol.* **21**, 311 (2021).

29. Palmgren, M. *et al.* Zinc biofortification of cereals: problems and solutions. *Trends Plant Sci.* **13**, 464–73 (2008).

30. Velu, G., Ortiz-Monasterio, I., Cakmak, I., Hao, Y. & Singh, R. P. Biofortification strategies to increase grain zinc and iron concentrations in wheat. *J. Cereal Sci.* **59**, 365–372 (2014).

31. de Lima, V. J. *et al.* Comparative Performance of High-Yielding European Wheat Cultivars Under Contrasting Mediterranean Conditions. *Front. Plant Sci.* **12**, 1190 (2021).

32. Hatfield, J. L. & Beres, B. L. Yield Gaps in Wheat: Path to Enhancing Productivity. *Front. Plant Sci.* **10**, 1603 (2019).

33. Knief, U. & Forstmeier, W. Violating the normality assumption may be the lesser of two evils. *Behav. Res. Methods* (2021) doi:10.3758/s13428-021-01587-5.

34. Marcos-Barbero, E. L., Pérez, P., Martínez-Carrasco, R., Arellano, J. B. & Morcuende, R. Genotypic Variability on Grain Yield and Grain Nutritional Quality Characteristics of Wheat Grown under Elevated CO2 and High Temperature. *Plants* **10**, 1043 (2021).

35. Irmak, S. & Vapur, H. Correlation Between Boron Contents of Soils and Wheat Plants (Triticum spp.) in The Cukurova Plain in Turkey. *Asian J Chem* **21**, 10 (2009).

36. Nyachoti, S., Adebayo, S. & Godebo, T. R. Elemental composition of teff (a gluten-free grain), maize and wheat: Staple crops in the Main Ethiopian Rift Valley. *J. Food Compos. Anal.* **100**, 103660 (2021).

37. Ciudad-Mulero, M. *et al.* Potential Health Claims of Durum and Bread Wheat Flours as Functional Ingredients. *Nutrients* **12**, E504 (2020).

38. European Parliament. REGULATION (EU) No 1169/2011 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004 (Text with EEA relevance). https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32011R1169&from=ES#d1e2308-18-1.

39. Celletti, S., Pii, Y., Mimmo, T., Cesco, S. & Astolfi, S. The characterization of the adaptive responses of durum wheat to different Fe availability highlights an optimum Fe requirement threshold. *Plant Physiol. Biochem. PPB* **109**, 300–307 (2016).

40. D.J. Reuter & Robinson, J. B. *Plant Analysis: An Interpretation Manual*. (CSIRO publishing, 1997).

41. Alloway, B. J. Soil factors associated with zinc deficiency in crops and humans. *Environ. Geochem. Health* **31**, 537–48 (2009).

42. Rengel, Z. Availability of Mn, Zn and Fe in the rhizosphere. *J. Soil Sci. Plant Nutr.* **15**, 0–0 (2015).

43. Borrill, P. *et al.* Biofortification of wheat grain with iron and zinc: integrating novel genomic resources and knowledge from model crops. *Front. Plant Sci.* **5**, 53 (2014).

44. Fan, M.-S. *et al.* Evidence of decreasing mineral density in wheat grain over the last 160 years. *J. Trace Elem. Med. Biol.* **22**, 315–324 (2008).

45. Aciksoz, S. B., Yazici, A., Ozturk, L. & Cakmak, I. Biofortification of wheat with iron through soil and foliar application of nitrogen and iron fertilizers. *Plant Soil* **349**, 215–225 (2011).

46. Lakshmi, P. V. *et al.* Long-Term Zinc Fertilization in Calcareous Soils Improves Wheat (Triticum aestivum L.) Productivity and Soil Zinc Status in the Rice–Wheat Cropping System. *Agronomy* **11**, 1306 (2021).

47. Sánchez-Rodríguez, A. R. *et al.* Combining P and Zn fertilization to enhance yield and grain quality in maize grown on Mediterranean soils. *Sci. Rep.* **11**, 7427 (2021).

48. GB 2762-2017. Food safety national standard. Contaminant limits in food. (2017).

49. COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. (2006).

50. CF/12 INF/1 2018. WORKING DOCUMENT FOR INFORMATION AND USE IN DISCUSSIONS RELATED TO CONTAMINANTS AND TOXINS IN THE GSCTFF (Prepared by Japan and the Netherlands). (2018).

51. Ata-Ul-Karim, S. T., Cang, L., Wang, Y. & Zhou, D. Effects of soil properties, nitrogen application, plant phenology, and their interactions on plant uptake of cadmium in wheat. *J. Hazard. Mater.* **384**, 121452 (2020).

52. Wu, X., Cai, Q., Xu, Q., Zhou, Z. & Shi, J. Wheat (Triticum aestivum L.) grains uptake of lead (Pb), transfer factors and prediction models for various types of soils from China. *Ecotoxicol. Environ. Saf.* **206**, 111387 (2020).

53. Ma, C. *et al.* Mechanism of Pb absorption in wheat grains. *J. Hazard. Mater.* **415**, 125618 (2021).

54. Fernandez-Gallego, J. A. *et al.* Low-cost assessment of grain yield in durum wheat using RGB images. *Eur. J. Agron.* **105**, 146–156 (2019).

55. José Francisco Vázquez, Efraín Antonio Chacón, José María Carrillo, & Elena Benavente. Grain mineral density of bread and durum wheat landraces from geochemically diverse native soils. *Crop Pasture Sci.* **69**, 335–346 (2018).

56. Guttieri, M. J. *et al.* Variation for grain mineral concentration in a diversity panel of current and historical great plains hard winter wheat germplasm. *Crop Sci.* **55**, 1035–1052 (2015).

57. Asplund, L., Bergkvist, G., Leino, M. W., Westerbergh, A. & Weih, M. Swedish Spring Wheat Varieties with the Rare High Grain Protein Allele of NAM-B1 Differ in Leaf Senescence and Grain Mineral Content. *PLOS ONE* **8**, e59704 (2013).

58. Brouns, F., Hemery, Y., Price, R. & Anson, N. M. Wheat Aleurone: Separation, Composition, Health Aspects, and Potential Food Use. *Crit. Rev. Food Sci. Nutr.* **52**, 553–568 (2012).

59. Wozniak, A. & Makarski, B. Content of minerals in grain of spring wheat cv. Koksa depending on cultivation conditions. *J. Elem.* **17**, (2012).

60. Svecnjak, Z., Jenel, M., Bujan, M., Vitali, D. & Dragojević, I. V. Trace element concentrations in the grain of wheat cultivars as affected by nitrogen fertilization. *Agric. Food Sci.* **22**, 445–451 (2013).

61. Svoboda, P., Kurešová, G., Raimanová, I., Kunzová, E. & Haberle, J. The Effect of Different Fertilization Treatments on Wheat Root Depth and Length Density Distribution in a Long-Term Experiment. *Agronomy* **10**, 1355 (2020).

62. Zhen, S. *et al.* Effect of high-nitrogen fertilizer on gliadin and glutenin subproteomes during kernel development in wheat (Triticum aestivum L.). *Crop J.* **8**, 38–52 (2020).

63. Andruszczak, S. SPELT WHEAT GRAIN YIELD AND NUTRITIONAL VALUE RESPONSE TO SOWING RATE AND NITROGEN FERTILIZATION. *J. Anim. Plant Sci.* **28**, 1476–1484 (2018).

64. Smith, E. G., Janzen, H. H. & Ellert, B. H. Effect of fertilizer and cropping system on grain nutrient concentrations in spring wheat. *Can. J. Plant Sci.* **98**, 125–131 (2017).

65. Jaskulska, I. *et al.* Mineral Composition and Baking Value of the Winter Wheat Grain under Varied Environmental and Agronomic Conditions. *J. Chem.* **2018**, e5013825 (2018).

66. Klikocka, H. & Marks, M. Sulphur and Nitrogen Fertilization as a Potential Means of Agronomic Biofortification to Improve the Content and Uptake of Microelements in Spring Wheat Grain DM. *J. Chem.* **2018**, e9326820 (2018).

67. Farquhar, G. D., Ehleringer, J. R. & Hubick, K. T. Carbon isotope discrimination and photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 503–537 (1989).

68. Foulkes, M. J., DeSilva, J., Gaju, O. & Carvalho, P. Relationships between δ13C, δ18O and grain yield in bread wheat genotypes under favourable irrigated and rain-fed conditions. *Field Crops Res.* **196**, 237–250 (2016).

69. Jarrell, W. M. & Beverly, R. B. The Dilution Effect in Plant Nutrition Studies. in *Advances in Agronomy* (ed. Brady, N. C.) vol. 34 197–224 (Academic Press, 1981).

70. Manzoni, S. & Katul, G. Invariant soil water potential at zero microbial respiration explained by hydrological discontinuity in dry soils. *Geophys. Res. Lett.* **41**, 7151–7158 (2014).

71. Yousfi, S., Serret, M. D. & Araus, J. L. Comparative response of δ13C, δ18O and δ15N in durum wheat exposed to salinity at the vegetative and reproductive stages. *Plant Cell Environ.* **36**, 1214–1227 (2013).