The Evolutionary Adaptation of the C282Y Mutation to Culture and Climate During the European Neolithic

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ABSTRACT

Objectives: The C282Y allele is the major cause of hemochromatosis as a result of excessive iron absorption. The mutation arose in continental Europe no earlier than 6,000 years ago, coinciding with the arrival of the Neolithic agricultural revolution. Here we hypothesize that this new Neolithic diet, which originated in the sunny warm and dry climates of the Middle East, was carried by migrating farmers into the chilly and damp environments of Europe where iron is a critical micronutrient for effective thermoregulation. We argue that the C282Y allele was an adaptation to this novel environment.

Materials and Methods: To address our hypothesis, we compiled C282Y allele frequencies, known Neolithic sites in Europe and climatic data on temperature and rainfall for statistical analysis.

Results: Our findings indicate that the geographic cline for C282Y frequency in Europe increases as average temperatures decrease below 16°C, a critical threshold for thermoregulation, with rainy days intensifying the trend.

Discussion: The results indicate that the deleterious C282Y allele, responsible for most cases of hemochromatosis, may have evolved as a selective advantage to culture and climate during the European Neolithic. Am J Phys Anthropol 160:86–101, 2016.

Humans not only adapt to their environment but also, alter their immediate environment by extracting food and water, and developing shelter and technologies. This process of niche construction may affect the direction of evolution (Odling-Smee et al., 1996; Laland and Brown, 2006). An example of culturally induced niche construction is the Neolithic diet, with a shift to domestication, beginning in the Middle East about 11,000 years ago (Cordain et al., 2002; Laland and O'Brien, 2010). The best known example of adaptation to the Neolithic diet is the spread of the lactase persistence allele allowing the consumption of milk postweaning (Cochran and Harpending, 2009; Laland and O'Brien, 2010; Gerbault et al., 2011; Rogers, 2011). Unlike other organisms, humans have the ability to carry or adopt a niche that was constructed in one particular environment to new and novel environments. Such is the case with domestication that was developed in the sunny warm climates of the Middle East and spread throughout Europe from east-to-west and south-to-north by 6,000 years ago (4000 BCE) (Pinhasi et al., 2005). This migrating Neolithic niche presumably put additional pressures on Europeans to adapt to a new diet in a novel environment. Here we argue that the C282Y mutation is an example of genetic adaptation to a culturally induced stressor of the Neolithic diet crossing into the locally novel chilly and damp environments of Europe.

Hereditary hemochromatosis (HH) is a recessive genetic disorder characterized by increased iron absorption (up to two to three times normal uptake), leading to excess iron retention (Andrews, 2000). Common effects of hereditary hemochromatosis include weakness, enlarged liver and, more life threatening, cirrhosis (Crownover and Covey, 2013). The disease also results in deposition of iron in the anterior pituitary gland, leading to impaired hormone release and a cascaded failure of sexual function in the advanced stage (Rosenbaum and Morgan, 2013). However, frequencies of overt clinical hemochromatosis are rare before age 40 for both males and females (Edwards et al., 2000).

The human hemochromatosis protein (HFE) is responsible for down regulation of iron absorption (Salter-Cid et al., 1999). Mutations in the gene for the HFE protein are the cause of most cases of HH (Whittington and Kowdley, 2002; Beutler et al., 2003). Although HH can manifest through multiple mutations of the HFE gene, the two most clinically relevant mutations are C282Y and H63D. The point mutation in the C282Y allele (HFE; OMIM 235200. 6p22.2) deactivates HFE's ability

Additional Supporting Information may be found in the online version of this article.

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to regulate, and thus results in increased iron absorption and frequently, hemochromatosis (Sheftel et al., 2012; Muckenthaler, 2014). By contrast, the H63D allele (HFE; OMIM 613609. 6p22.2), has a much weaker effect on iron absorption, and does not prevent cell surface expression as in C282Y, but rather simply reduces HFE's efficacy in downregulating iron uptake (Feder et al., 1998). C282Y homozygosity is the predominant form associated with clinical symptoms in 80 to 92% of cases (Feder et al., 1996; Hanson et al., 2001; Aguilar-Martinez et al., 2011). H63D is unlikely to produce hemochromatosis, even in homozygotes (Beutler, 2006; Adams, 2014; Kelley et al., 2014). Compound heterozygotes, C282Y/H63D, and simple heterozygotes of either mutation carry a very low risk of HH disease (Waalen et al., 2002; Gurrin et al., 2009). However, there is evidence for elevated serum ferritin levels and improved iron status in C282Y carriers and compound heterozygotes with H63D (Bulaj et al., 1996; Datz et al., 1997; Waalen et al., 2002; Beutler et al., 2003; Gurrin et al., 2009; Aguilar-Martinez et al., 2011). The C282Y and H63D mutations also differ in their timing and allele frequency distributions. H63D appears to be thousands of years older than C282Y, having arisen at least twice in different locations and is distributed worldwide (Cullen et al., 1998; Rochette et al., 1999; Merryweather-Clarke et al., 2000; Candore et al., 2002). Since H63D has negligible impact on iron absorption compared to C282Y, here we focus our investigation on the C282Y allele.

The C282Y allele is found predominantly among European populations reaching polymorphic levels in the northwest (Merryweather-Clarke et al., 1997, 2000). Highest frequencies are found in Ireland (mean 10.1%) and prevalence declines to near 0% in southeast Europe (Fairbanks, 2000; Olsson et al., 2011). Current analyses suggest that the temporal origin of the C282Y mutation, occurred 200 to 250 generations ago or at approximately 6,000 BP (4000 BCE) (Raha-Chowdhury and Gruen, 2000) in central Europe (Symonette and Adams, 2011). Distante et al. (2004) concur and argue that the timing of the C282Y mutation coincides with the Neolithic farmers expanding into Central Europe and the spread of the allele may be an adaptation to a dietary shift from hunting and gathering wild foods rich in iron to domesticated cereal and dairy food poor in iron. Other researchers have also suggested that the C282Y mutation may have spread as a genetic adaptation to dietary iron deficiency but did not offer an explanatory mechanism for this occurrence (Motulsky, 1979; Rotter and Diamond, 1987; Salter-Cid et al., 1999; Rossi et al., 2000; Fairbanks, 2000; Toomajian et al., 2003).

Current research in ancient DNA studies show that the initial expansion of the Neolithic into Europe was a demic diffusion of peoples genetically similar to present-day Near Easterners and distinct from the indigenous hunter-gatherer populations in the area (Lazaridis et al., 2014). The Neolithic first appears in Europe on the Island of Cyprus about 10,000 years ago (Pinhasi et al., 2005). Once the Neolithic reached the Hungarian Plains, by about 7,000 years ago, it rapidly moved throughout central Europe following the Danube River and its tributaries and settled on the rich loess soils ideal for farming (Jochim, 2000; Price, 2000, 2003; Price et al., 2001; Shennan, 2009, 2012; Bentley, 2013). The first farmers in Mainland Europe are associated with material remains known as the Linearbandkeramik Culture (LBK) whose temporal fingerprint is at its height between 7500 and 6900 BP (Whittle, 1996). The Neolithic stalled in Mainland Europe not reaching the British Isles until about 6200 BP where, within less than 400 years, the Neolithicization of Britain and Ireland was rapid and complete (Richards et al., 2003; Shennan, 2009; Cunliffe, 2013; Whitehouse et al., 2013; Cramp et al., 2014; Woodbridge et al., 2014).

Before the Neolithic, the subsistence strategies of European Mesolithic foragers were highly variable depending on local environments but were generally iron rich, based on wild flora and fauna including but not limited to game, fish, shellfish, insects, nuts, roots, and vegetables (Stiner and Munro, 2002; Eaton, 2006; Kuipers et al., 2010) as evidenced by the bioarchaeological skeletons from Europe (Price, 2000). The Neolithic ushered in the era of domesticated plants and animals, principally grains and dairy (Cordain et al., 2002; Carrera-Bastos et al., 2011). Where grains added calories, dairy products provided a protein substitute for meat and fish, thus shifting human subsistence toward a high carbohydrate but low-iron diet (Cordain et al., 2002). The grain-dairy combination of the Neolithic subsistence strategy created a dual nutritional problem not present before domestication. Phytates, found on the surface of cereal grains have a chelating effect resulting in dietary iron being physiologically unavailable (Hurrell, 2003; Naugler, 2008), and unlike human breast milk that has iron bearing lactoferrin, calcium rich bovine milk has very poor iron bioavailability (Pennington et al., 1987; Howcroft et al., 2012). In addition, bovine milk may cause iron deficiency through occult blood loss and impairs absorption of non-heme iron (Ziegler, 2011).

The Neolithic subsistence strategy of Europeans 6000 BP was highly variable ranging from a strong mix of wild resources and fish, to a substantial reliance on dairying and the inclusion of grains. Reconstructing diet from archaeological context relies on identification of archaeobotanical and archaeozoological remains, stable isotope analysis on human remains, and lipid and protein residue analysis from potshards (Kovačiková et al., 2012; Layman et al., 2012; Brown and Brown, 2013; Colledge and Conolly, 2014). Based on one or more of these analyses, a picture of the European Neolithic diet has begun to emerge. The Pitted Ware Culture and the Comb-Ware Culture of coastal Scandinavia and the Baltic Sea is primarily identified by the Neolithic material culture, principally pottery, but the diet is similar to the strategies of the Mesolithic peoples with a reliance on wild resources and fish, (Malmström et al., 2009; Schulting, 2011; Isaksson and Hallgren, 2012; Lahtinen and Rowley-Conwy, 2013). Therefore, these material cultures do not represent the diet of Neolithic farmers. However, mainland Europe, associated with the LBK complex as well as some parts of Scandinavia known as the Funnel Beaker Culture, reflect a subsistence strategy based on grains and dairying but with locally variable reliance on wild resources and meat consumption (; Robinson, 2003; Malmström et al., 2009; Oelze et al., 2011; Isaksson and Hallgren, 2012; Gerbault et al., 2013). At the other end of the spectrum, the British Isles show a sharp shift in subsistence from wild meat, birds and fish to a heavy reliance on dairying as well as the inclusion of grains (Richards et al., 2003; Brown, 2007; Tipping et al., 2009; Collard et al., 2010; Schulting, 2013; Cramp et al., 2014; McClatchie et al., 2014; Whitehouse and Kirleis, 2014;

Serjeantson, 2014; Smyth and Evershed, 2015; Rowley-Conwy and Legge, 2015).

Unfortunately some of these techniques used to reconstruct subsistence strategies have limited utility. Recovery and identification of archaeobotanical remains may artificially inflate the amount of wild plant materials since those remains may just be an artifact of the natural flora of the area (Colledge and Conolly, 2014). Additionally, high levels of nitrogen^{15} (δ^{15}N) from stable isotope analysis has been interpreted as representing a high proportion of animal proteins in the diet; however, crop manuring of grain fields can produce equally high δ^{15}N levels (Schulting et al., 2010; Bogaard et al., 2013). Therefore, levels of δ^{15}N may be a questionable diagnostic measurement with regard to the proportion of animal protein but rather, indicative of a high grain diet. Regardless of the analyses used, the Neolithic farmers of Europe unquestionably had grains and dairy as part of their diet.

Beyond the diet, European Neolithic farmers are inherently more sedentary than European Mesolithic foragers (Bellwood and Oxenham, 2008; Gerbault et al., 2011). Increased sedentism resulted in high fertility and rapid population growth, a benchmark feature of the European Neolithic farmer (Bocquet-Appel and Bar-Yosef, 2008; Bocquet-Appel, 2011; Shennan, 2012). The high caloric intake and reduction in parental investment in offspring resulted in shorter interbirth intervals (IBIs) and increased fecundity (Shennan, 2008; Helle et al., 2014). Bocquet-Appel and Bar-Yosef (2008) estimate that fertility ratios increased 1:3 from foraging to sedentism. Pregnancy requires iron at a rate that exceeds the amount that can be absorbed from even an optimal intake (Bothwell, 2000). With shorter IBIs a woman would have very little opportunity to replenish her iron stores before the next pregnancy. A combination of the above factors seen among European Neolithic farmers potentially placed them at risk for iron deficiency.

The bioarcheaological record regarding iron deficiency anemia is also unclear due to the difficulty in establishing the etiology of skeletal pathologies associated with the disease. Traditionally, cribra orbitalia and porotic hyperostosis were held as markers for iron deficiency anemia and/or scurvy (Cohen, 2008). However, Walker et al. (2009), argue that these lesions are probably caused by Vitamin B_{12} deficiency, and cannot be produced by iron deficiency. Recently, the validity of this assessment has been questioned (Oxenham and Cavill, 2010; McIlvaine, 2013). As of the writing of this article, no techniques for detecting the presence of anemia in skeletal remains have gained wide acceptance. Despite the controversy, it is difficult to imagine a scenario in which iron deficiency did not exist to some significant degree during the Neolithic transition to domestication. Even in contemporary times, iron deficiency remains the single most common micronutrient disorder in the world (Stoltzfus, 2001).

Iron deficiency regularly co-presents with other forms of malnutrition, and in fact, other forms of malnutrition can produce anemia that is functionally similar to that of iron deficiency by affecting iron metabolism or absorption (Jackson, 2007). For example, scurvy produces iron deficiency anemia through two means, direct blood loss due to connective tissue microhemorrhaging and impairment, as well as inhibiting the ability to absorb dietary iron (Fain, 2005; Delanghe et al., 2013). Celiac disease is an adverse reaction to gluten that when consumed results in malabsorption of nutrients and may contribute to iron deficiency (Butterworth et al., 2002). Moreover, LBK skeletal remains, especially for neonates and children, have a high prevalence of Harris lines, transversal enamel hypoplasia, and/or porotic hyperostosis, all signs of malnutrition (Wittwer-Backofen and Tomo, 2008). Given a diet regime that includes grains and dairy along with a preponderance of evidence indicating suboptimal health (Harper and Armelagos, 2013), it is likely that a significant proportion of Neolithic farmers in Europe were at best undernourished with insufficient iron stores.

Although we concur with the above assessment that the Neolithic diet created conditions favoring the C282Y allele for iron absorption, such an argument fails to address why the C282Y allele would have had a selective advantage in Northwest Europe, less so in Southern Europe and virtually non-existent in other geographic areas where the same Neolithic diet was adopted. Below, we argue that climate was a contributing factor.

Humans evolved in tropical Africa and their thermoneutral range is relatively narrow (25–27°C, 77–81°F) (Snodgrass, 2012). Outside of this range, the human body has mechanisms to return to thermal homeostasis, which require iron as a micronutrient (Rosenzweig and Volpe, 1999). In healthy people, exposure to cold temperatures 16°C (61°F) or below results in potentially fatal thermoregulation stress (Parsons, 2014). As seen among ancestral peoples of the north, one such adaptive response to cold stress is to increase metabolism, which is triggered by the release of hormones (Beal et al., 2012; Snodgrass, 2012). But iron deficiency inhibits the release of thyroid stimulating hormones (TSH) necessary for regulating metabolism (Beard et al., 1990; Beard and Durward, 2012). Therefore, below the lower critical temperature of 16°C (61°F) for humans, iron deficient individuals are less able to regulate body temperature in response to thermal stress (Beard et al., 1990, 1996; Brigham et al., 1996; Lukaski et al., 1990). Moreover, Beal et al., (2012) show that at 16°C (61°F) manual dexterity begins to fail and at 12°C (54°F) tactile sensation disappears. Adding moisture, even humidity, to the equation results in significantly greater body temperature loss due to conduction and evaporative cooling (Parsons, 2014). Exposure to wet and cold environments result in dramatically higher metabolic stress and thermal loss than in dry conditions (Thompson and Hayward, 1996; Weller et al., 1997; Yamane et al., 2010). Thompson and Hayward (1996) reported a 10°C reduction in rectal temperature in healthy men after just 2 h of exposure to wet and cold conditions. Small bodied neonates, adolescents, and even adult women are at a greater risk for hypothermia than are adult men because of their greater surface to mass ratio (Stocks et al., 2004; Parsons, 2014). Iron deficiency under chilly and damp conditions results in increased thermoregulatory stress and may lead to selective pressure to improve iron absorption.

From this discussion the authors generate the following hypothesis: The spread of the C282Y allele was a genetic adaptation to the chilly and damp environments of Neolithic Europe where adequate iron was required for physiological thermoregulation. Here we test three predictions. The C282Y allele frequency has 1) an inverse linear relationship with mean daily temperatures, 2) an inverse linear relationship with the mean maximum temperatures, and 3) a positive linear relationship with mean wet days per year.

TABLE 1. Weighted mean C282Y allele frequencies by European geographic location

Population	No. studies	Subjects examined (*n*)	Weighted mean C282Y %	References
Austria	2	758	4.56	Datz et al. (1997); Kazemi-Shirazi et al. (1999)
Bosnia-Herzegovina	1	200	2.25	Terzić et al. (2006)
Bulgaria	1	100	0	Ivanova et al. (1999)
Croatia	1	200	3.3	Ristić et al. (2003)
Czech Republic	2	239	4.75	Zdárský et al. (1999); Hrachovinova et al. (1999)
Denmark	6	18,534	5.67	Pedersen et al. (2008); Steffensen et al. (1998); Merryweather-Clarke et al. (1999); Simonsen et al. (1999); Ellervik et al. (2001); Milman et al. (2004)
France	9	10,104	5.8	Merryweather-Clarke et al. (2000); Mercier et al. (1998); Mura et al. (1999); Jouanolle et al. (1997); Jézéquel et al. (1998); Borot et al. (1997)
Germany	6	1,419	4.38	Nielsen et al. (1998); Gottschalk et al. (2000); Höhler et al. (2000); Hellerbrand et al. (2001); Braun et al. (1998); Raddatz et al. (2003)
Greece	2	297	0.43	Merryweather-Clarke et al. (1997); Papanikolaou et al. (2000)
Hungary	3	1,721	3.42	Tordai et al. (1998); Andrikovics et al. (2001); Szakony et al. (1999)
Ireland	2	259	9.45	Ryan et al. (1998); Merryweather-Clarke et al. (2000)
Italy	13	3,717	1.99	Borgna-Pignatti et al. (1998); Candore et al. (2002); Pozzato et al. (2001); Racchi et al. (1999); Piperno et al. (1998); Sampietro et al. (1998); Cassanelli et al. (2001); Merryweather-Clarke et al. (1997); Longo et al. (1999); Campo et al. (2001)
Italy, Sardinia	1	61	0	Candore et al. (2002)
Italy, Sicily	1	106	0	Candore et al. (2002)
Lithuania	1	1,011	2.6	Kucinskas et al. 2012
Norway	4	2,643	6.89	Merryweather-Clarke et al.(1997); Undlien et al. (1998); Distante et al. (2000); Distante et al. (1999)
Poland	1	871	3.11	Moczulski et al. (2001)
Portugal	2	640	3.41	Cardoso et al. (2001)
Republic of Macedonia	1	306	0	Davalieva et al. (2002)
Republic of Serbia	1	318	1.6	Šarić et al. (2006)
Romania	1	225	3.1	Trifa et al. (2012)
Slovenia	1	1,282	3.6	Cukjati et al. (2007)
Spain	5	1,132	3.11	Moreno et al. (1999); Alvarez et al. (2001); Fábrega et al. (1999); Gimferrer et al. (1999); Sánchez et al. (1998)
Spain, Balearic Islands	1	210	2.6	Guix et al. (2000)
Sweden	1	206	7.5	Beckman et al. (1997)
Switzerland	2	189	3.72	Claeys et al. (2002); Beris et al. (1999)
The Netherlands	4	1,630	6.15	Roest et al. (1999); Cobbaert et al. (2012)
UK—England	2	485	6.41	Merryweather-Clarke et al. (1997); Grove et al. (1998)
UK—Jersey Island	1	411	8.3	Merryweather-Clarke et al. (1998)
UK—Northern Ireland	1	409	9.9	Murphy et al. (1998)
UK—Orkney Islands	1	103	4.9	Merryweather-Clarke et al. (2000)
UK—Scotland	1	184	8.4	Miedzybrodzka et al. (1999)
UK—Wales	4	12,312	8.19	Roberts et al. (1997); Willis et al. (1997); Merryweather-Clarke et al. (2000); Jackson et al. (2001)

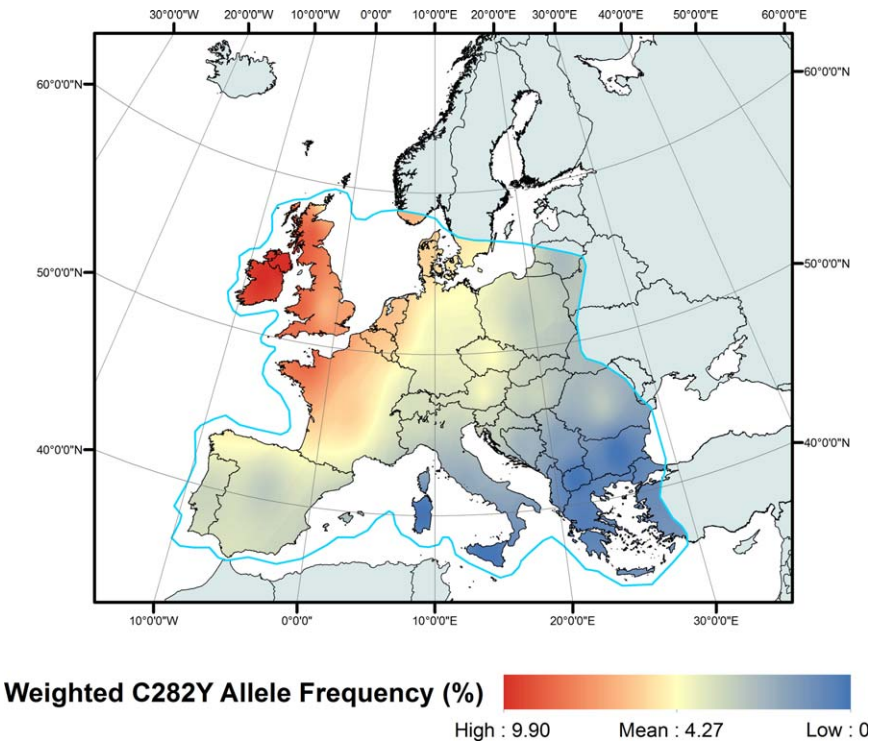

Fig. 1. Visual depiction of weighted C282Y allele frequency (%) cline from northwest to southeast within the study area in Europe.

MATERIALS AND METHODS

The study is based on information collected from three European sources: documented contemporary C282Y allele frequencies, identified Neolithic sites, and climatic data compiled from weather stations. Climate data were joined with European Neolithic site locations to explain the clinal distribution of the C282Y allele in Europe. Present-day C282Y allele frequency is used as a proxy for data from the Neolithic period, reflecting the residual adaptive significance for this allele. Therefore, our study area represents European locations where both C282Y allele frequencies have been identified and where Neolithic sites occur along with their corresponding weather station. The specific procedures used in sampling and analyses in this research are detailed below (see Supporting Information S1—Spreadsheet).

Samples

Stoneking (2006) argues for the usefulness of analyzing contemporary variation in genetics for understanding ancestral population as the selection forces in the past will leave a fingerprint in the present (Stoneking and Krause, 2011). Therefore, we compiled data on the frequency of the C282Y allele throughout contemporary Europe populations from Milman and Pedersen (2003) and expanded by more recent studies resulting in 99 published case studies with a minimum sample size of 50 subjects. Since our research concerns the selective advantage of the C282Y allele during the Neolithic, we eliminated 14 published studies from our analyses.

First, published studies on the frequency of the C282Y allele from The Faroe Islands (Merryweather-Clarke et al., 1998; Milman et al., 2005) and Iceland (Merryweather-Clarke et al., 1997, 1999) are not included in our study. Current archaeological evidence suggests that the Faroe Islands may have been sparsely occupied before the Vikings as early as the 4th century ACE (Church et al., 2013) but the evidence is not associated with Neolithic occupation. As for Iceland, the Icelandic Sagas place the first settlements at 870 ACE, which is supported by volcanic ash dates of 871 + 2 ACE (Sveinbjarnardóttir, 2012). Therefore, these locations represent Post-Neolithic occupation. Second, published studies on the frequency of the C282Y allele from Finland (Beckman et al., 1997; Tuomainen et al., 1999), Estonia (Mikelsaar et al., 1999), and Mordovia (Beckman et al., 1997) are also not included in our study. Although Late Neolithic sites have been documented in Finland, Estonia and Mordovia, they have been identified primarily from Neolithic artifacts but the presence of Neolithic farming has not been clearly identified (Vybornov, 2011; Lahtinen and Rowley-Conwy, 2013). Since our research question concerns the adaptive significance of the C282Y allele among European Neolithic farmers, these locations are also excluded from our analysis. Third, we are not including studies on the Basque (Merryweather-Clarke et al., 1997; Baiget et al., 1998; Mercier et al., 1998), Roma (Szakony et al., 1999; Gabriková et al., 2012) or Saami (Beckman et al., 2001) as they are considered separate ethnic groups and do not represent the national population.

The remaining 85 published case studies of the C282Y allele frequency vary from countrywide to regions within countries to cities. In several cases, more than one analysis was conducted at the same location. We averaged the C282Y allele frequency by country where necessary for those cases. England, Northern Ireland, Scotland and Wales were kept as discrete regions of the United Kingdom and the islands of Sicily, Sardinia, Balearic, Jersey, and Orkney were also

counted as discrete regions. The reconfiguration of the C282Y allele weighted mean frequency data resulted in 33 case studies presented in Table 1, and illustrated in Figure 1.

Locations of Neolithic sites throughout Europe were partially compiled from Pinhasi et al. (2005) and reduced or expanded to reflect regions where C282Y allele frequency data were documented (Whittle, 1996; Tomaž, 2010; Kilhavn, 2013). This resulted in 600 Neolithic sites located in 33 European countries corresponding to our study area as seen in Figure 2.

Climate data were collected from the European Climate Assessment and Dataset (www.ecad.eu) (Klein Tank et al., 2002) for a 30-year period from 1961 to 1990 as a proxy for conditions in Europe 6000 BP. A 30-year series normalizes variations, providing a more representative sample of the true conditions. For the geographic area of our study, the overall climatic pattern was stable since 6000 BP (Davis et al., 2003; Wanner et al., 2008). Extensive climatic modeling for the mid-Holocene to the near present (1kya) also suggest little temporal variability in rainfall patterns in our study area (Braconnot et al., 2007). This stable climatic pattern coincides temporally with the prevalence of the Neolithic diet in Europe and the emergence of the C282Y mutation.

A total of 7,848 climate stations are in the European Climate Assessment & Database. Of these, 3,371 stations were within our study area. To confine the available climate data to places associated with Neolithic human habitation, only climate stations nearest to each identified Neolithic site were selected for inclusion in our database, resulting in 600 climate stations. To determine the climate station nearest to each Neolithic site, both the climate database and the Neolithic site database were projected as point data onto an equidistant conic projection of continental Europe in ArcGIS 10.1. Then, a near analysis was performed between the two datasets. Mountain top climate stations were eliminated from our data. Each climate station is identified by a station-number along with the latitude, longitude plus a myriad of climatic information; however, not all stations collect the same climatic data. For our chilly and damp environment hypothesis, we were interested in the average temperatures and number of days with rainfall for each select station. The most frequently recorded climatic information regarding our hypothesis was mean of mean daily temperature, mean maximum daily temperature, and mean wet days per year >1 mm (herein called mean wet days).

The climate associated with Neolithic habitation in Europe may be considerably different than the blanket climate data, which includes all elevations and

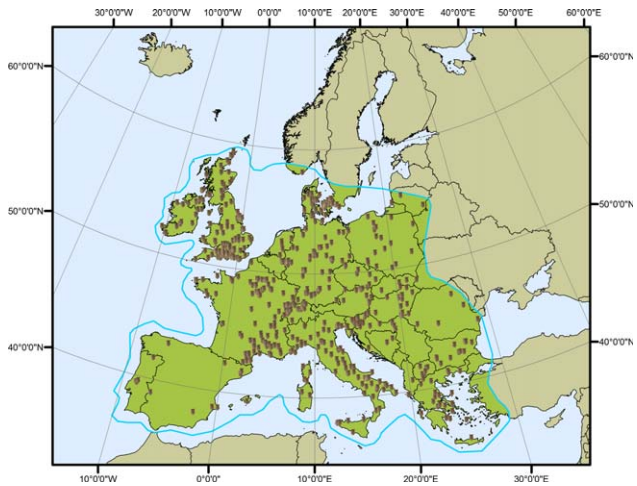

Fig. 2. Map of the geographic distribution of 600 European Neolithic sites partially compiled from Pinhasi et al. (2005).

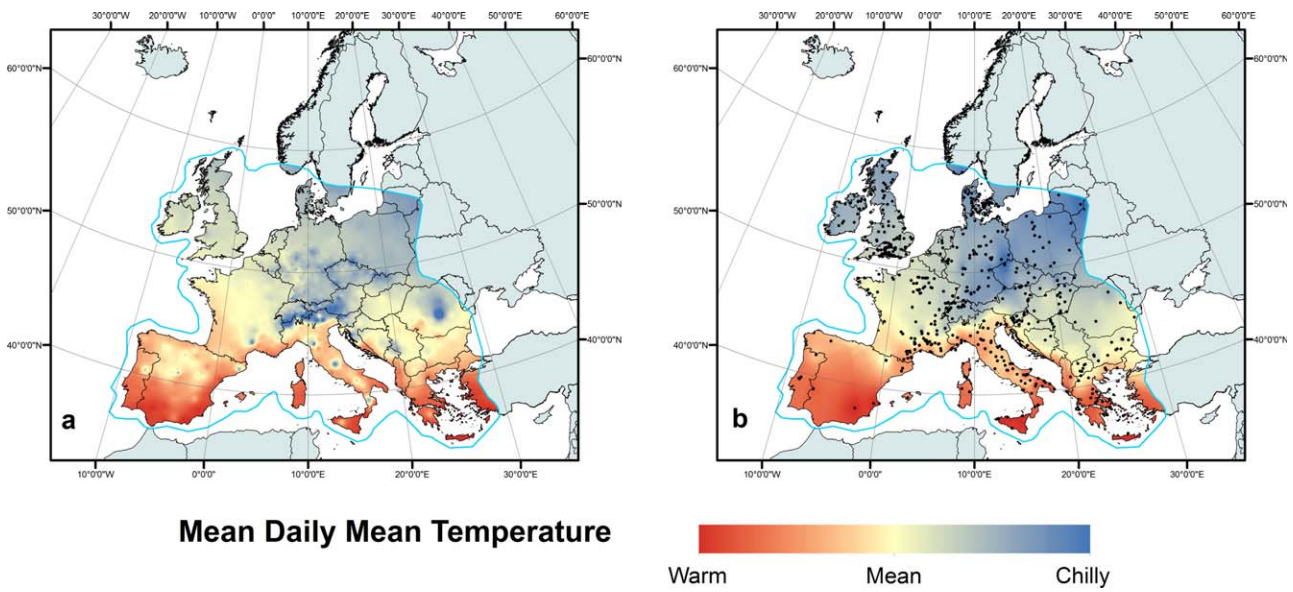

Fig. 3. Visual depiction of mean daily mean temperature cline from northwest to southeast within our study area. Hard-blue represents the lowest temperatures (chilly) while hard-red represents the highest temperatures (warm). **A**, Map represents mean daily mean temperature for 1,308 climate stations within our study area. **B**, Map represents mean daily mean temperature for 370 climate stations nearest Neolithic sites within our study area.

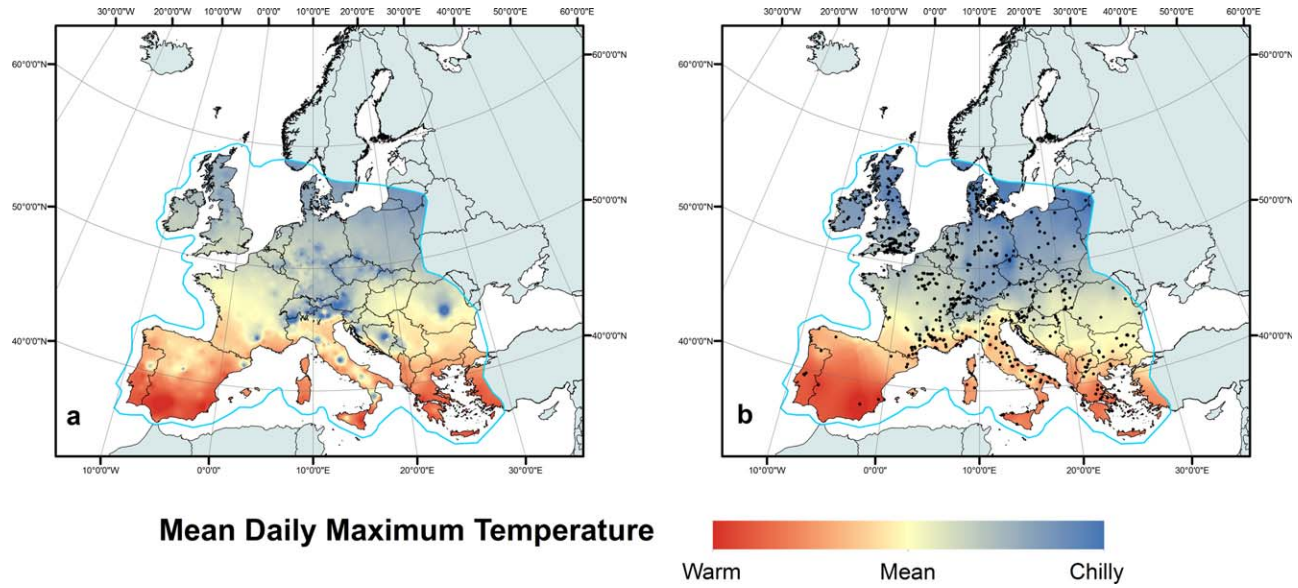

Fig. 4. Visual depiction of mean daily maximum temperature cline from northwest to southeast within our study area. Hard-blue represents the lowest temperatures (chilly) while hard-red represents the highest temperatures (warm). **A**, Map represents mean daily maximum temperature for 1,438 climate stations within our study area. **B**, Map represents mean daily maximum temperature for 457 climate stations nearest Neolithic sites within our study area.

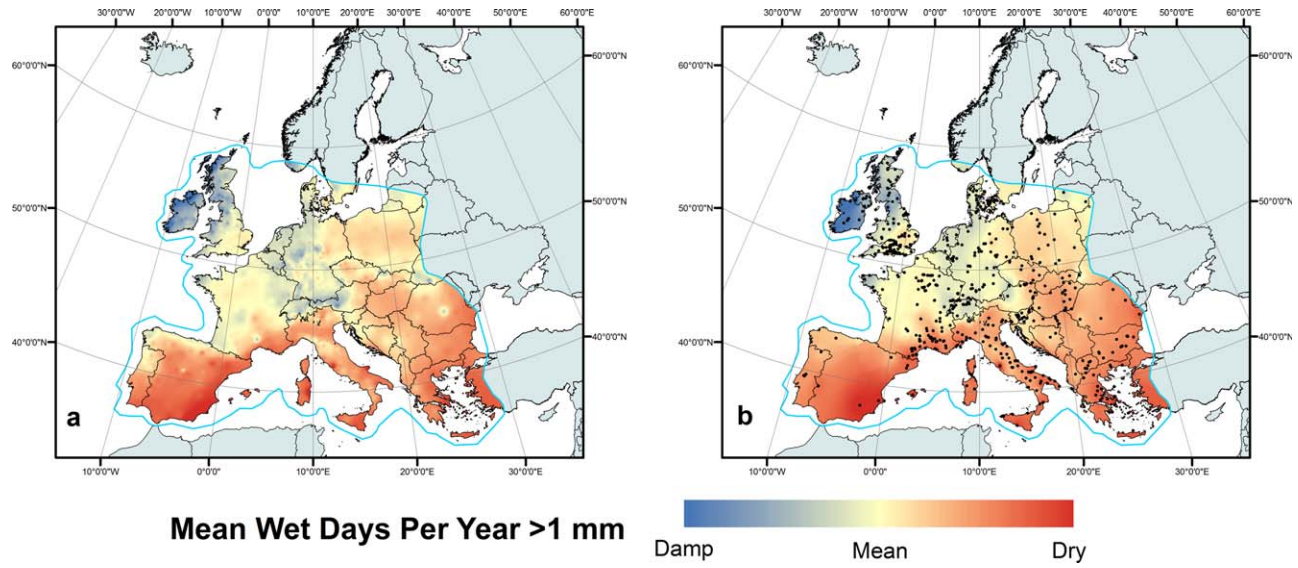

Fig. 5. Visual depiction of mean wet days per year >1 mm cline from northwest to southeast within our study area. Hard-blue represents the highest frequency of wet days per year (damp) while hard-red represents the lowest frequency of wet days per year (dry). **A**, Map represents wet days per year >1 mm for 2,326 climate stations within our study area. **B**, Map represents wet days per year >1 mm for 478 climate stations nearest Neolithic sites within our study area.

extremes. Here we demonstrate the differences between all European weather stations in our study area versus only those associated with Neolithic sites.

Figure 3 is the mean daily mean temperature map. All available station data in our study area are shown in Figure 3A, while only data associated with Neolithic sites are shown in Figure 3B and the difference is significant (two-tailed t-test: all stations, $n = 1{,}308$, mean = 9.53°C, SD = 3.08; Nearest Neolithic stations, $n = 370$, mean = 11.09°C, SD = 3.01; $t = -8.7$, $P < 0.001$).

Figure 4 is the mean daily maximum temperature map. All available station data in our study area are shown in Figure 4A, while only data associated with Neolithic sites are shown in Figure 4B and the difference is significant (two tailed t-test: all stations, $n = 1438$, mean = 13.81°C, SD = 3.32; Nearest Neolithic stations, $n = 457$, mean = 14.96°C, SD = 3.28; $t = -6.46$, $P < 0.001$).

Figure 5 is the mean wet days per year >1 mm map. All available station data in our study area are shown in Figure 5A, while only data associated with Neolithic

TABLE 2. Descriptive statistics for the dependent (C282Y) and independent variables

Variable	*N*	Minimum	Maximum	Mean	SD
Weighted C282Y allele (% frequency)	33	0	9.90	4.27	2.85
Mean daily $Temp_m$ (°C)	32	5.85	17.81	10.35	3.03
Mean daily $Temp_{mx}$ (°C)	31	9.89	21.45	14.57	3.55
Mean wet days	32	46.21	193.21	107.19	35.61

Mean $Temp_m$ = mean-of-mean daily temperature.
Mean $Temp_{mx}$ = mean maximum daily temperature.
Mean wet days = mean wet days per year >1 mm.

TABLE 3. Pairwise Pearson correlations between dependent (C282Y) and independent variables

Variable	C282Y ($\%_f$)	$Temp_m$	$Temp_{mx}$	Wet Days
Weighted C282Y allele (% frequency)	1			
Mean daily $Temp_m$	−0.558[a]	1		
Mean daily $Temp_{mx}$	−0.637[a]	0.957[a]	1	
Mean wet days	0.782[a]	−0.729[a]	−0.753[a]	1

Mean $Temp_m$ = mean-of-mean daily temperature.
Mean $Temp_{mx}$ = mean maximum daily temperature.
Mean wet days = mean wet days per year >1 mm.
[a] Correlation is significant at the 0.01 level.

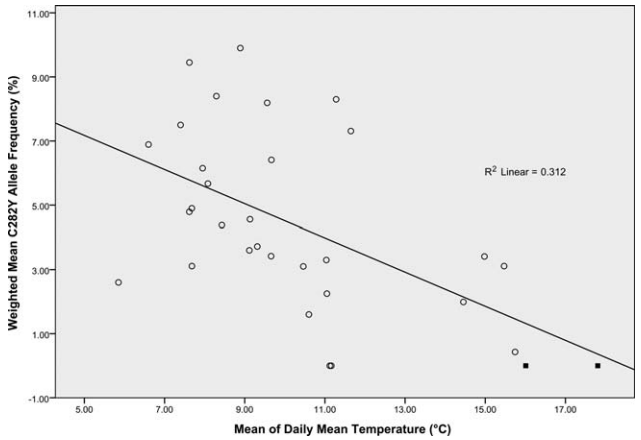

Fig. 6. Mean of mean daily temperature in °C by mean weighted C282Y allele frequency (%) (linear regression $r^2 = 0.312$, $t = -3.69$, $P < 0.005$). The solid black squares located on the lower right hand corner of the graph represent the Islands of Sardinia and Sicily where published studies show no occurrence of the C282Y allele.

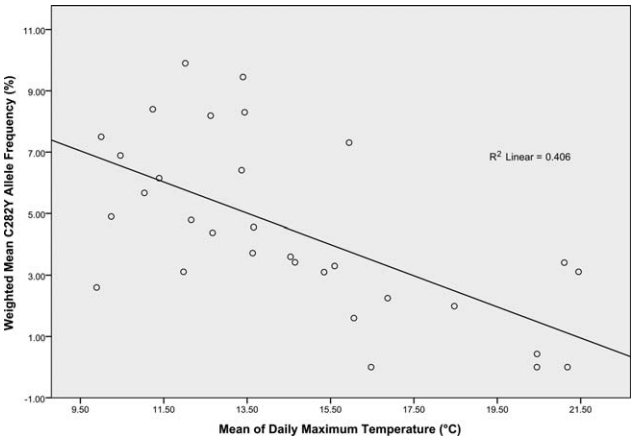

Fig. 7. Mean maximum daily temperature in °C by mean weighted C282Y allele frequency (%) (linear regression: $r^2 = 0.406$, $t = -4.46$, $P < 0.001$).

sites are shown in Figure 5B and the difference is significant (*t*-test: all stations, $n = 2327$, mean = 119.11 days, SD = 27.86; Nearest Neolithic stations, $n = 478$, mean = 106.94 days, SD = 30.75; $t = 8.00$, $P < 0.001$).

We kept only climate data associated with each Neolithic site after the GIS near analysis. This method preserved more of the variation between sites than a country centroid or countrywide statistics by eliminating the extreme values which did not reflect the conditions in which our study population had lived. Then, we averaged the data for each country/discrete region to avoid duplicating the dependent variable, C282Y allele weighted frequency, and to overcome variations in sampling density. The same climate station may be the nearest to several Neolithic sites; therefore, we eliminated the duplicate climate stations within each country or discrete region before averaging, to preserve variation in the climate data. This composite database, with one averaged measurement for each country/discrete region, resulted in a study population of 33 cases used for statistical analyses (see Supporting Information S2—Repeated Measures for results of raw point data analysis).

Analytical methods

The data were analyzed using SPSS with α of .05, unless otherwise indicated. In addition to descriptive statistics, we conducted a principle component analysis and a multicollinearity test among the independent variables. The independent variables (mean of mean daily temperature, mean maximum daily temperature, and mean wet days) are strongly correlated, making them unsuitable for the assumption of independence in a multiple regression analysis. Therefore, we created a climate index to test the predictions set out in our hypothesis.

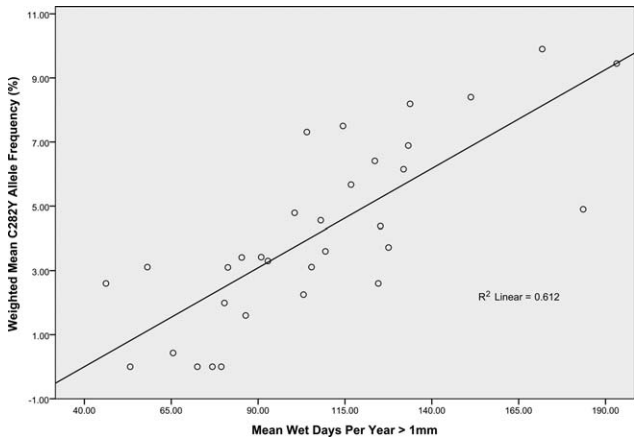

Fig. 8. Mean wet days per year >1 mm by mean weighted C282Y allele frequency (%) (linear regression $r^2 = 0.612$, $t = 6.89$, $P < 0.001$).

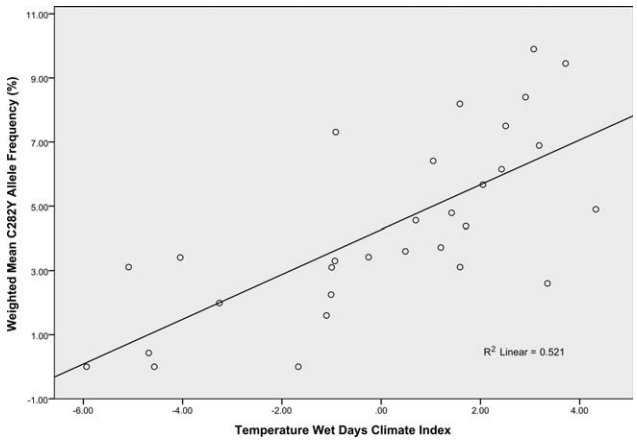

Fig. 9. Climate index of combined variables (linear regression $r^2 = 0.521$, $t = 5.52$, $P < 0.001$). The climate index is a composite of annual wet days, mean temperature, and maximum temperature. A high climate index corresponds to a high number of wet days, low mean temperature, and low maximum temperature (see Materials and Methods section for details).

Since more than one climate factor ultimately impacts human physiology, indices based on multiple climate variables have long been used to predict human physiological outcomes (Jendritzky et al., 2012). To create our climate index, the following climate data were transformed to *Z*-scores: mean of mean daily temperature, mean maximum daily temperature, and mean wet days. The *Z*-scores were then summed, inverting the temperature scores, to produce the climate index, thus giving each data type equal weight. For the visual aid maps, the point data were projected in ArcView GIS 10.1. The continuous raster surface of the data was then computed using inverse distance weighting.

RESULTS

Descriptive statistics

Our database represents 33 cases, comprised of 24 countries and nine discrete regions. Each data point has a corresponding mean C282Y allele frequency as the dependent variable and the independent variables: mean of mean daily temperature, mean maximum daily temperature, and mean wet days. We conducted a Principal Component Analysis (PCA) to determine if a smaller number of related factors could account for the variability. The analysis resulted in only one component extracted, including all the variables, with an Eigenvalue of 2.6 accounting for 87.9% of the cumulative variance.

The descriptive statistics for these variables are presented in Table 2. The averages for both the mean and maximum daily temperature are below 16°C, a critical threshold for thermoregulation. The means of the independent variables fall within our expectations for the adaptability of the C282Y allele to Europe's novel environment for the Neolithic diet—chilly and damp. Table 3 presents the pairwise Pearson correlations of the variables, all of which are significantly correlated.

Hypothesis testing

We argue that the spread of the C282Y allele was an adaptive response to the Neolithic diet moving into the *chilly and damp environments* of Europe, where thermoregulation is dependent on sufficient iron status. The first prediction, that the weighted C282Y allele frequency will have an inverse linear relationship with mean of mean daily temperature, is verified, and the association is significant as seen in Figure 6 (linear regression $r^2 = 0.312$, $t = -3.69$, $P < 0.005$). Of the 32 countries reporting mean of mean daily temperature, only two, the Islands of Sardinia and Sicily, had mean temperatures greater than 16°C, the threshold temperature for thermoregulation. Moreover, published studies show no occurrence of the C282Y allele in either Sardinia or Sicily.

The second prediction, that the weighted C282Y allele frequency will have an inverse linear relationship with mean maximum daily temperature, is also verified and significant as seen in Figure 7 (linear regression: $r^2 = 0.406$, $t = -4.46$, $P < 0.001$). It is worth noting that those regions above the critical threshold of 16°C ($n = 9$) have an average C282Y allele frequency of only 1.42 while the remaining regions ($n = 22$) have an average C282Y allele frequency of 5.71.

The third prediction, that the weighted C282Y allele frequency will have a positive linear relationship with the mean number of wet days per year, is verified and the association is significant as seen in Figure 8 (linear regression $r^2 = 0.612$, $t = 6.89$, $P < 0.001$).

As seen in Figure 9, we combined these three predictive variables to form a Climate Index (see Materials and Methods for details). The linear relationship with the weighted C282Y allele frequency is significant (Linear Regression: $r^2 = 0.521$, $t = 5.52$, $P < 0.001$). Figure 10 depicts the geographic distribution of the Temperature Wet Days Climate Index.

DISCUSSION

Humans are tropical animals and climate is a major selective force on geographic variation in allele frequencies (Hancock et al., 2008; Laland and O'Brien, 2010; Veeramah and Novembre, 2014). Here we have shown that the geographic distribution of the C282Y allele for iron retention in contemporary Europe is associated with increasingly chilly and damp environments of human occupation during the Neolithic. Furthermore, dietary iron is necessary for effective

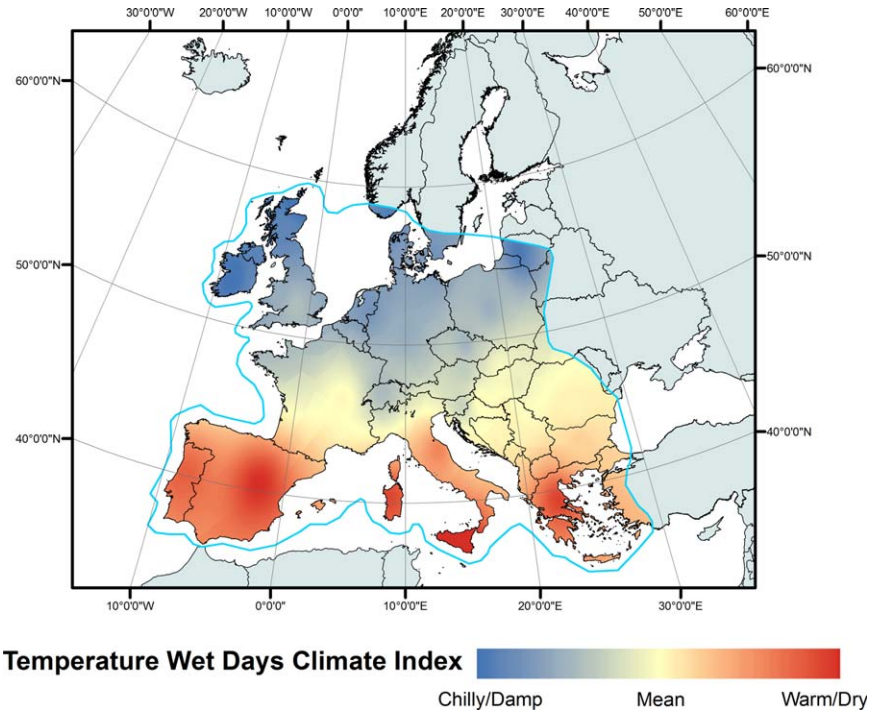

Fig. 10. Visual depiction of the temperature-wet days climate index cline from northwest to southeast averaged for nearest Neolithic sites of 31 European countries within our study area. Hard-blue corresponds to a composite of low mean temperature, low maximum temperature and high number of wet days (chilly & damp), while hard-red corresponds to a composite of high mean temperature, high maximum temperature and low number of wet days (warm & dry) (processed in ArcView GIS, see Materials and Methods section for details).

thermoregulation—an important component of homeostasis (Lukaski et al., 1990; Beard et al., 1990, 1996; Brigham et al., 1996). When the near-eastern Neolithic farmer migrated into Europe an iron-poor diet was no longer simply a physiological stressor, it had become a separate source of selection. Under such conditions, natural selection would favor carriers of the C282Y allele, as those individuals would have higher survival rates and greater reproductive success than non-carriers.

However, we suspect that the adaptive advantage for the C282Y allele began to decline as new culturally constructed niches emerged during the Iron Age. By 400 BCE iron largely replaced bronze and ceramic as the principle material for cooking vessels (Wells, 1984). Cooking in iron pots results in some iron uptake in the food, particularly when cooking dairy products (Kröger-Ohlsen et al., 2002; Geerligs et al., 2003; Charles et al., 2011; Adeniyi and Ajayi, 2013; Kulkarni et al., 2013). As milk begins to sour, lactic acid bacteria can accidently or purposefully be added to the mixture to begin the fermentation process (Kunji et al., 1996). Lactate acid chelates increasing the iron uptake from the vessel to the food as much as nine times the normal iron leaching (Kröger-Ohlsen et al., 2002). Following the Iron Age, the ancient Romans introduced indoor climate control (Cunliffe, 2013) further reducing thermoregulation stress. Recently an iron-rich diet with a surfeit of flesh and other iron-enriched food and supplements became economically available to Europeans (Stoltzfus et al., 1998). Therefore, the C282Y allele once advantageous in the chilly and damp environments of Neolithic Europe is now a residual echo of the past or has even become deleterious to some through hemochromatosis.

Our study demonstrates that about 52% of the variation in present day C282Y allele frequencies across Europe is associated with the climatic variables of temperature and wet days associated with Neolithic habitation areas. However, surfing on the crest of a migrating wave (Edmonds et al., 2004; Klopfstein et al., 2006; Excoffier and Ray, 2008) may also have generated the geographical patterning of the C282Y allele seen in Europe. To date, computer simulations on gene surfing have primarily considered neutral alleles and have not taken into account the effect of culture nor migration into novel environments. Culture increases genetic selective pressures as evidenced by the lactase persistence mutation that co-evolved with dairying during the Neolithic (Hawks et al., 2007; Rogers, 2011; Jobling et al., 2013) and shows a similar SE-NW cline as the C282Y allele in Europe (McCullough et al., 2015). As Neolithic farmers arrived from the Near East and moved across Europe, they would be encountering novel environments where selection pressures would favor either genetic or cultural adaption.

Similarly, the C282Y allele frequency may be a product of gene flow from a founder population. However, the temporal footprint for the C282Y mutation does not correspond to a founder population for continental Europe. The LBK, for example, reached its height between 7500 and 6900 BP (Whittle, 1996), roughly 900 years before C282Y is thought to have mutated (Raha-Chowdhury and Gruen, 2000; Symonette and Adams, 2011). As mentioned in the Introduction, the expansion of farming stalled in continental Europe for at least 1,000 years before crossing the channel into Britain and Ireland (Shennan, 2009). Therefore, genetic drift by founder effect may have influenced the initial spread of the C282Y allele during the expansion of the Neolithic into the British Isles. However, the distribution of the Y-

chromosome haplogroup sub-lineage, R-S145, is almost completely concentrated in Celtic Britain, especially in Ireland (Busby et al., 2012). This is the same area where the C282Y allele is most common making endogenous selection of C282Y more plausible.

Integrated Haplotype Score (iHS) analysis of the phase three data from five European populations in the 1000 Genomes Project partially supports our findings that the C282Y allele was the target of selection. Although iHS statistics for the region surrounding C282Y do not show evidence for a recent strong selective sweep; the presence of some extreme values in this region suggest that the iHS scores are consistent with a softer signal (Harris n.d.). If a beneficial allele was present in non-negligible frequency before a sweep, existing variation around that allele is maintained. This is referred to as a "soft sweep," and cannot be detected by iHS (Voight et al., 2006). This is unlikely to be the case for C282Y because selection would have taken place shortly following the initial mutation. Weak selection requires more time to increase the frequency of a beneficial allele to intermediate frequencies, allowing variation around an advantageous site to be maintained by recombination (Slatkin, 2008). iHS signals also decay with time as diversity is reintroduced to the region via recombination, even if selection was relatively strong (Voight et al., 2006). The benefit gained by C282Y carriers is not sufficient for selection to act strongly. In addition, the allele would lose its benefit once iron was introduced into diet via iron cooking pots, allowing variation to accumulate around the region. This would explain the insignificant iHS signals around C282Y (see Supporting Information S3—iHS).

Continued research in ancient and contemporary DNA will definitely further our understanding of human genetics. However, more data alone will not result in improved interpretations of these data. Models need to take into account the role of culture, human life history, niche construction and climates associated with migration routes and local habitation. A massive effort is underway in health and genetics to identify potentially deleterious alleles for a myriad of contemporary human diseases (Craddock et al., 2010), but we may miss the mark by only assuming contemporary cause and effect. Understanding the origins, history and possible ancestral adaptive value of alleles will shed more light on the relationship between genes, culture and the environment thereby advancing genetic research and in the process contributing to our understanding of the genetic basis of human health.

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