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Research Idea

Raising the Neanderthal (molecules) from the dead: a proposal for *in vivo* resurrection of Neanderthal haemoglobin for the investigation of biochemical adaptations for cold tolerance

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Abstract

Since the first discoveries of Neanderthal fossils, their derived characteristics, such as increased robusticity, have engaged researchers. Adaptation to cold environments has been hypothesised to explain such traits and this hypothesis has driven the majority of discourse. This proposal seeks to examine this hypothesis and locate evidence of Neanderthals being physiologically adapted to cold at the biomolecular level. Haemoglobin is a biomolecule that has been previously demonstrated to adapt to cold in some species, driven by the inhibition of the protein's function by low temperatures. Neanderthal haemoglobin is extinct; however, using pre-sequenced genomic data, I propose to resurrect Neanderthal haemoglobin so I can examine the consequences of lowered temperature on its function. This project could potentially detect signs of cold adaptation in the Neanderthal globin genes and provide empirical evidence for the cold adaptation hypothesis.

Keywords

Homo neanderthalensis, haemoglobin, molecular resurrection, adaptation, evolution

Project Aims

Neanderthals (*Homo neanderthalensis*) have long been hypothesised to have possessed thermoregulatory adaptations to their Ice Age environments due to traits such as skeletal robusticity and expanded ribcages (Ocobock et al. 2021). Alternative explanations to the cold-adapted hypothesis have been proposed, chiefly centred around morphological adaptation to a physically strenuous lifestyle (Rae et al. 2011; Stewart et al. 2019). However, morphological traits can appear beneficial for purposes other than those that drove their evolution (Clark and Thein 2004; Romagnoli et al. 2022). It is thus difficult to differentiate Neanderthal skeletal robusticity as a thermoregulatory adaptation from skeletal robusticity as an adaptation for an athletic lifestyle.

I propose shifting the focus to molecular genetics. I theorise that, if Neanderthals were biologically adapted to the cold, they will possess inherited molecular-level adaptations. To locate potential molecular adaptations in Neanderthals and diagnose their evolutionary purpose, I plan to utilise a method that has not yet been exploited in palaeoanthropology: the functional analysis of inferred ancestral gene products. The resurrection and functional analysis of extinct genes can help link molecular adaptation events to historical environmental changes (Huynen et al. 2012). This approach works only when there is:

1. clear evolutionary history of recent movement into a novel environment; and
2. living close relatives of the extinct species that evolved outside of that environment (Campbell et al. 2010).

The Neanderthal is thus an ideal subject.

Haemoglobin is a strong candidate molecule for this study as it is highly adaptable to environmental alteration and functionally dependent on temperature (Clementi et al. 1994). Haemoglobin binds and carries oxygen; however, its ability to offload this oxygen to cells is hampered at low temperatures (Clementi et al. 1994). Cold-adapted species thus often have inherited genetic haemoglobin alterations (see, for example, Weber and Campbell (2010); Signore et al. (2023)).

My research into Neanderthal haemoglobin cold adaptation would first involve identifying the globin genes using contemporary human genomes as a comparison. Observed differences may be indicative of evolutionary processes. After noting where the Neanderthal sequences differ from those of *Homo sapiens*, I would ascertain if nucleotide changes were likely to have been driven by positive selection. The globin genes for both species would be subcloned into expression vectors and the products expressed in cell culture. The now tangible Neanderthal haemoglobin could then be functionally tested under various environmental conditions.

If my hypothesis is correct, then I might expect to see amino acid alterations in the Neanderthal globin genes that lower the effect of temperature on haemoglobin-oxygen affinity and facilitate the release of oxygen at cold temperatures. Functional testing of the expressed molecule at various temperatures may differentiate increased oxygen affinity specifically for thermoregulation from increased oxygenation for alternate purposes. Ultimately, I aim to generate novel evidence of Neanderthal thermoregulatory mechanisms for cold adaptation by uniting palaeoanthropology and molecular resurrection.

Literature Review for Proposal Justification

The proposed focus is situated within the broader context of hominin adaptability. It is therefore necessary to discuss the nature of molecular adaptation in genus *Homo* and order Primates, as this will be intimately related to how haemoglobin may adapt and express in *Homo neanderthalensis*. To proceed, one must develop detailed understandings of three core concepts: the selective pressures experienced by Neanderthals, the known effects of such pressures on the haemoglobin protein, and the limitations and abilities of genome sequencing and gene expression technologies.

Adaptability of *Homo neanderthalensis*

Neanderthals shared swathes of Eurasia with *Homo sapiens* until approximately 40,000 years ago (Vaesen et al. 2021). The exact timing of their split from our lineage is uncertain (Gómez-Robles 2019), and the classification of the earliest Neanderthal remains is debated (Meyer et al. 2016), but a comprehensive fossil record exists from circa 130,000 years ago up until their disappearance. Neanderthals were, on average, more robust than modern humans (Gómez-Olivencia et al. 2018). Despite the equatorial origins of genus *Homo*, Neanderthals successfully colonised the high latitudes of Eurasia during the last Ice Age (Stewart et al. 2019). They occupied an environment characterised by glacial conditions and high aridity (Zimov et al. 2012).

Neanderthal Cold Adaptation

Neanderthals likely utilised behavioural adaptations to their new Arctic environment (Roebroeks et al. 2021; Zilio et al. 2021). Behavioural thermoregulatory strategies—namely fire, clothing, tool use, food storage, and dwelling construction—are widespread in humans (Ilardo and Nielsen 2018) and have even been observed in West African chimpanzees (*Pan troglodytes verus*) (Pruetz 2007, Pruetz 2018). In addition to behavioural cold buffers, Neanderthals likely possessed inherited biological adaptations to their cold environment. So far, morphological adaptations are those with the most robust support (Holliday 1997; Weaver 2003), but there is limited evidence for energetic thermoregulatory adaptations, such as high basal metabolic rates and diets designed to harness the thermic effects of digestion (Snodgrass and Leonard 2009). Most physiological adaptations remain chiefly theoretical, as it is challenging to obtain evidence for non-skeletal biology from the archaeological record (Ocobock et al. 2021).

In contemporary humans, biological thermoregulatory adaptations are not restricted to morphology and physiology. Inherited molecular-level adaptations, such as those that help Inuit cope with low-carbohydrate diets (Hale 2020), are also essential. I dissect pertinent examples in the section entitled *Haemoglobin Adaptation in Order Primates*. Molecular-level thermoregulatory adaptations in contemporary human populations imply that similar adaptations could have existed in the Neanderthals—our closest relatives.

Navigating Alternate Explanations

Not all research aligns with the cold-adapted hypothesis (Rae et al. 2011). One recent study by Stewart et al. (2019) proposed that morphological differences between Neanderthals and modern humans may be explained as adaptations for an active lifestyle and that Neanderthals' preferred environments were, in fact, more temperate than previously thought. However, climatic data strongly support glacial conditions for a majority of Neanderthal sites and palaeozoological evidence consistently associates them with glacial fauna (Bar-Yosef 2004). Additionally, no modern climate analogue exists for the mammoth steppe tundra that dominated the Eurasian landscape at this time (Zimov et al. 2012) and our ecological models, on which Stewart et al. (2019) rely, struggle when applied to such historical environments because they are parameterised using modern climate data.

Furthermore, the argument put forward by Stewart et al. (2019) that Neanderthals' morphological differences are adaptations for an active lifestyle largely depends on their identification of genomic variants associated with power and endurance in modern humans. They believe these alleles indicate that Neanderthals were proficient at athletic performance and adapted to an active lifestyle. However, alleles that support athletic prowess and power in modern humans may not necessarily have had the same phenotypic effects in Neanderthals.

Stewart et al. (2019) additionally presuppose that a species will not be driven to evolve cold-specific adaptions unless its environment is consistently extremely cold. They argue that, due to the warmer interglacial periods experienced by Neanderthals, selection would not drive them to develop such adaptations. The underlying assumption here is flawed—thermoregulatory adaptations can benefit an organism even if harsh lows only occur periodically (Campbell et al. 2010). So long as the trait does not disadvantage the organism and lower its fitness during times of warmth, then it will provide an overall selective advantage (Schou et al. 2022). All evidence considered, cold adaptation in Neanderthals remains plausible.

The Haemoglobin Molecule

One candidate I have identified as a plausible site for genetic evidence of cold adaptation in Neanderthals is the haemoglobin molecule. This is due to haemoglobin being:

1. dependent on temperature for proper function (Clementi et al. 1994; Weber and Campbell 2010);

2. highly adaptable in hypoxic conditions, including within short evolutionary timeframes (Storz and Moriyama 2008);
3. a known focus of adaptation in primates, both human and non-human (see, for example, Gassmann et al. (2019); Faust et al. (2020)) and
4. a known focus of cold-specific adaptation in a variety of vertebrate taxa (see, for example, De Rosa et al. (2004); Signore et al. (2023)).

Human Globin Genes

The human globin genes are organised into two clusters located on separate chromosomes, the alpha-globin cluster and the beta-globin cluster (Bahr and Ohls 2022). Neanderthal globin genes have not yet been identified or studied; however, due to the globin genes of the extant non-human great apes maintaining strong similarities to those of *Homo sapiens* (Goodman et al. 1983), I suggest that those of *H. neanderthalensis* will also be exceptionally similar. Additionally, genomic reconstructions and sequencing indicate that Neanderthals possessed 23 chromosome pairs—the same number as modern humans (Petr et al. 2020). This suggests that, not only may Neanderthal globin genes look largely identical to our own, they may occupy analogous locations. This theory is supported by other research: the Neanderthal melanocortin type 1 receptor gene (*MC1R*) was identified at the same locus as that of modern humans and was nearly indistinguishable (Lalueza-Fox et al. 2007). The investigation of human globin genes as a direct comparison to those of Neanderthals is hence justified.

Haemoglobin Function and Cold

Haemoglobin's ability to offload oxygen to respiring cells is inhibited at lower temperatures (Clementi et al. 1994; Weber and Campbell 2010). The deoxygenation of haemoglobin is an endothermic, or heat-dependent, process (Weber and Campbell 2010). Succinctly, haemoglobin requires sufficient heat to be present to successfully provide oxygen to the body's tissues. This means that haemoglobin-oxygen affinity increases and decreases in negative correlation with temperature changes, with higher temperatures favouring oxygen unloading and lower temperatures hampering unloading. Extremely cold temperatures thus restrict the body's ability to oxygenate tissue.

Adaptability of the Haemoglobin Molecule

Haemoglobin is a highly adaptable molecule. As the protein responsible for oxygen transport in almost all vertebrates (di Prisco et al. 2002), its functionality is imperative for respiration and tissue oxygenation (Baldwin 1976). When a species habitually endures hypoxic conditions, whether due to altitude, temperature, or diving, haemoglobin efficiency can adapt to the environment (Storz and Moriyama 2008).

Haemoglobin Adaptation in Order Primates. Haemoglobin adaptation has been observed in both humans and non-human primates. Over 60 human haemoglobin variants have amino acid replacements that increase oxygen affinity or blood concentration (Jones

and Shih 2009; Gassmann et al. 2019). Many of these alterations are for survival of hypoxic conditions of high-altitude living (Huerta-Sánchez et al. 2014; Zheng et al. 2023). Further human haemoglobin variants confer disease protection, for example, the sickle-cell anaemia and thalassaemia alleles for malaria resistance (Yuthavong and Wilairat 1993). Tibetan high-altitude adaptations were also found to have originated via admixture with Denisovans, proving the potential for adaptive genetic variation in extinct hominins (Huerta-Sánchez et al. 2014).

Most of these human haemoglobin adaptions are single-base point mutations, which can occur in short evolutionary timeframes (Clark and Thein 2004). Consequently, the known human haem-adaptations appear evolutionarily recent and have not been identified in ancient samples. Any ancient globin gene modifications in Neanderthals are thus likely to be analogous, but non-identical, to those in *Homo sapiens*. These types of mutation are hard to track due to their sizeable genotypic diversity despite phenotypic similarity, similar to the diverse thalassaemia haemoglobinopathies (Clark and Thein 2004).

Haemoglobin adaptation has also been described in a non-human primate. Researchers identified mutations in the haemoglobin sequences of *Macaca fascicularis* (Faust et al. 2020). These were associated with the same genes responsible for many human haemoglobin adaptations—*HBA1* and *HBA2*. Researchers concluded that this variation likely evolved to confer protection against malaria via a similar process to that seen in humans (Faust et al. 2020). This study reinforces the likelihood of finding adaptive haemoglobin mutations in primates other than *Homo sapiens*—namely Neanderthals.

Haemoglobin Adaptation to Cold. Cold-adapted haemoglobin has been identified in various taxa, from fish to mammals. The haemoglobins of several Arctic mammals, including polar bears and reindeer, have been found to possess lowered enthalpy values, decreasing the effect of temperature change on haem-oxygen affinity (De Rosa et al. 2004). Notably, the average summer temperature in the reindeer's habitat, circa 10°C (Grayson and Delpech 2005), is on par with the theorised average of 8–10°C in the Neanderthals' range (Zimov et al. 2012). Reconstructions of the woolly mammoth's (*Mammuthus primigenius*) globin genes also identified substitutions that minimised heat loss associated with haem-oxygenation (Campbell et al. 2010).

In an alternative approach to the low-temperature oxygenation problem, polar fishes developed haemoglobins with increased oxygen-binding properties (Anderson et al. 2009). The base sequences of these Arctic fish haemoglobins show closer similarity to Antarctic fish haemoglobins than those of temperate fish, implying analogue patterns and processes in cold adaptation (Verde et al. 2008). This recurrence emphasises the plausibility of finding thermoregulatory adaptations in Neanderthal haemoglobin. In another episode of convergent cold adaptation, the specific molecular mechanisms of haemoglobin-oxygen binding in Arctic cod (*Arctogadus glacialis*) are shared with high-altitude avians (Anderson et al. 2009).

Whilst cold-adapted haemoglobin has not yet been identified in humans, signals of strong natural selection for other cold-relevant alleles, specifically those that limit hepatic fatty acid

oxidation, have been found in Inuit populations (Hale 2020). However, research has concentrated primarily on diet-driven vulnerabilities to iron-deficiency anaemia (see, for example, Appel et al. (2018)). There is, thus, a possibility of Arctic human populations also having undiscovered cold-adapted haemoglobin.

Resurrection of Extinct Molecules

The same evolutionary processes have influenced development throughout time and environmental change consistently leads to evolutionary innovation (Wood et al. 2019). Hypotheses about ancient evolutionary events can be tested by resurrecting ancient genes via contemporary genomes, characterising their functions, and analysing their expressed products (Huynen et al. 2012). The functional analysis and expression of extinct genetic loci can help create a clearer picture of palaeospecies by identifying molecular adaptations to prehistorical environmental conditions.

Current Methodologies in Ancient DNA Work

Ancient DNA (aDNA) refers to any DNA isolated from palaeo- or archaeological specimens (Orlando et al. 2021). aDNA is often more degraded than contemporary material, making it challenging to sequence and analyse (Huynen et al. 2012). Early aDNA work only recovered short sections of DNA from large amounts of well-preserved material. However, the development of next-generation sequencing and capture methods increased the information we can extract from fragments and enabled genomes to be sequenced at unprecedented speeds (Quail et al. 2012; Shapiro and Hofreiter 2014). Next-generation techniques are ideal for aDNA, as they can exploit tiny fragments. Full palaeogenomes have now been sequenced, including the mammoth to 0.1x coverage and Neanderthal to over 50x coverage (Campbell et al. 2010; Green et al. 2010). Next-generation techniques have also extended the upper temporal boundary for useable samples to ~1.65 million years (van der Valk et al. 2021).

Expressing Extinct Gene Products

The next step in palaeogenetics was to identify regions of potential evolutionary importance and analyse their functional consequences (Huynen et al. 2012). This molecular-level de-extinction enables us to travel back in time and observe characteristics of extinct organisms. If reconstructed genomes are compared with those of related extant species, it is possible to identify the differences responsible for target phenotypes (Shapiro 2017). Genome editing can then alter the living species' genome and insert relevant portions from the extinct material. Once reconstructed, it is possible to create living cells containing extinct genes. If focusing on a single gene product, researchers can subclone the gene into an expression vector and express the long-extinct molecule in living cell culture (Thornton 2004).

Research is now producing results that would have once been considered science fiction. Extinct gene products have been expressed from a range of species (see, for example,

Huynen et al. (2012)). In the plant kingdom, molecular de-extinction synthesised scent molecules from extinct flowering plants (Dorado et al. 2019). In the animal kingdom, a transcriptional enhancer from the extinct thylacine was activated *in vivo* and found to affect chondrocyte expression (Pask et al. 2008). Two separate studies also sequenced the *MC1R* gene, an influencer of hair colour, in the woolly mammoth (Römpler et al. 2006) and Neanderthal (Lalueza-Fox et al. 2007).

A study by Campbell et al. (2010) on the woolly mammoth further expanded this approach to include structural functional analysis of the resurrected molecules, i.e., they investigated potential selective pressures that could have driven the evolution of differences. Campbell et al. used recovered DNA to resurrect the species' globin genes. After expressing the genes *in vitro*, testing of the resurrected molecule demonstrated improved oxygen offloading when exposed to cold temperatures. The woolly mammoth was already assumed to be adapted to its high-latitude Ice Age environment, but the work of Campbell et al. put weight behind this theory.

Detecting Positive Directional Selection

Simply identifying differences between extinct genes and living analogues does not inform whether selection caused these differences. Discerning whether ancient differences were caused by positive directional selection is integral to linking potential adaptations to causative environmental changes. Neutral evolutionary theory must be excluded to claim that an observed mutation is adaptive (Kimura 1983): to demonstrate that a given sequence has been subjected to selective pressure, one must first reject the null that it evolved neutrally, i.e., was driven by genetic drift and stochastic processes alone (Koonin 2016).

Detecting positive selection can be achieved by searching for signatures left behind by selective sweeps (Koropoulis et al. 2020), where a beneficial mutation's increase in frequency and subsequent fixation leads to the reduction or elimination of genetic variation in nearby sections. This condition is observed when strong selective pressures force allele frequencies to change rapidly; for example, in early Arctic populations, a selective sweep followed sudden extreme selection for alleles associated with metabolic adaptation to low carbohydrate diets (Hale 2020). During recombination, other genes can "hitch-hike" on to the focal allele and increase in frequency alongside it, reducing nearby variation. Such sweeps are primarily identified by measuring linkage disequilibrium (LD), where alleles in close proximity are non-randomly associated (Slatkin 2008). High LD indicates that an allele has recently increased in frequency under strong selection. Measurement of LD has already been used in studies on extant human populations (see, for example, Hale (2020)). Statistical methodologies used to ascertain the likelihood of given nucleotide changes occurring by chance range from binomial tests to Bayesian approaches (Koropoulis et al. 2020), with different methodologies befitting different circumstances (Slatkin 2008).

Anthropological De-Extinction

Neanderthal adaptation, haemoglobin genomics, and molecular resurrection are disparate subjects. However, when brought together, a gap in the literature appears. While the expression of extinct genes and gene products has become increasingly common in the biological sciences, palaeoanthropologists have remained focused on reconstructing anatomy and energetics. Researchers have not yet resurrected extinct hominin gene products for any purpose, as the relevant technologies have only been recently developed —let alone for testing hypotheses about ancient evolutionary events. I plan to unite these subjects to test my hypotheses that: 1) Neanderthals were indeed a cold-adapted species; 2) this cold adaptation included inherited genetic traits; and 3) haemoglobin is one of the Neanderthal genetic products affected by this adaptation.

Proposed Materials and Methods

My proposed methods consist of seven steps:

1. procuring genomic data for both Neanderthals and *Homo sapiens*;
2. identifying and isolating loci that code for the Neanderthal globin gene clusters;
3. identifying regions of difference between the two species' globin gene sequences;
4. utilising statistical testing and empirical structure-function analyses to distinguish selection from neutral processes;
5. expressing haemoglobin for both species in cell culture;
6. testing the functional characteristics of the expressed proteins; and
7. constructing molecular models to search for structural bases of functional differences.

Data Acquisition

I will obtain annotated copies of human globin gene sequences via a genomic database, most likely from the databases run by the United States National Center for Biotechnology Information (NCBI). I will obtain an annotated copy of the Neanderthal genome via the Neandertal Genome Project Browser, run by the Department of Evolutionary Genetics at the Max Planck Institute for Evolutionary Anthropology. The Neanderthal data will be a compilation of four primary specimens along with various fragments (Green et al. 2010; Prüfer et al. 2014).

Ethical Considerations

Due to the secondary nature of my data, I will not need to conduct extraction, amplification, or sequencing myself and, thus, will not need to apply for approval from my institution's relevant ethics committees. I will, however, need to verify that all human genetic information I use was previously obtained with appropriate ethical approval from equivalent institutions.

As I am working within an Aotearoa New Zealand context, my research must be performed in accordance with the 1940 Treaty of Waitangi (Boulton 2018), a formal agreement that Indigenous Māori interests would be recognised and protected in perpetuity (Hudson and Russell 2009). The Treaty remains an integral part of New Zealand's legislative and organisational systems and honouring the Treaty in research is both a legal and ethical requirement (Health Research Council of New Zealand 2010). An important step will be ensuring that appropriate *tikanga* (Māori ethical protocol) for working with biological samples is consistently followed (Health Research Council of New Zealand 2010). As researchers often neglect cultural considerations (Boulton 2018), it may be impossible to obtain complete information on the nature of available data. At a minimum, I will ensure that no known breaches of ethics have been committed and that accepted international standards have been met.

Identification of Neanderthal Globin Genes

I will next move to identify the Neanderthal alpha- and beta-globin gene sequences. These will consist of a series of loci that need to be individually located. Due to the strong similarities between human globin sequences and those of other great apes, along with the equivalence between Neanderthal and *Homo sapiens* chromosomes (Goodman et al. 1983 ; Petr et al. 2020), I expect to locate the Neanderthal alpha-globin gene cluster on the short arm of chromosome 16 and the beta-globin cluster on the short arm of chromosome 11 (Bahr and Ohls 2022). The sequences will likely resemble those of modern humans.

DNA Sequence Comparisons

I will align the alpha- and beta-globin sequences for Neanderthals and modern humans and produce consensus sequences that represent the most frequent bases at each locus —eliminating sub-species-level variation. Finalised consensus sequences will be compared and I will note the position of any mutations. Since template damage can affect aDNA sequencing (Hofreiter et al. 2001), I will independently verify the position of each mutation using Römpler et al. (2006)'s approach for targeting single nucleotide polymorphisms in aDNA. Due to the dynamic nature of the beta-globin gene cluster (Hardison 2012), I anticipate a greater likelihood of identifying novel alterations in this complex.

Identifying Natural Selection

Statistical Methodologies

I will utilise statistical tests to ascertain whether observed nucleotide differences were likely driven by positive directional selection. Neutral evolutionary theory will function as my null hypothesis. To search for the signatures left behind by selective sweeps, I will utilise a binomial test to compute summary statistics on the likelihood of my observations occurring in the absence of selection. A variety of other approaches to identifying LD and positive

selection may also be beneficial, including a Bayesian framework (Koropoulis et al. 2020). If my functional difference analyses do not yield the hoped-for results, being able to report that Neanderthal haemoglobin shows signs of selection for an adaptive purpose is interesting in its own right—even if that purpose remains unclear. However, since the dataset available for Neanderthals is ancient and extremely small and most potential tests require samples of a minimum size to produce certainty, my tests are vulnerable to low statistical power. I thus plan to explore structure-function analysis as an alternate method of identifying selection.

Structure-Function Analyses

I will conduct empirical structure-function analyses to investigate whether observed changes alter protein behaviour in a manner consistent with selection. I will obtain amino-acid sequences for the globin genes of other mammalian species via a cross-organism database, align the sequences, and note shared, deleted, or substituted amino acids. This will establish whether Neanderthal alterations are common across species and identify conserved regions. Nucleotide alterations at points that fundamentally alter a protein's physiochemical properties rarely occur by chance and, if they do, are promptly eliminated from populations if they provide no fitness benefit (Perutz 1983). Thus, if I identify an alteration that affects the amino-acid sequence in a highly conserved region of structural importance, then the likelihood of that change being driven by positive selection is increased.

Expressing Extinct Proteins

I will use the normal human adult haemoglobin-expression plasmid pHE2 to express modern human haemoglobin (Shen et al. 1993). I will express this plasmid in *Escherichia coli* cells to produce synthetic human haemoglobin—isolation and purification will follow the methods of Shen et al. (1993). For the expression of Neanderthal haemoglobin, I will create a Neanderthal expression plasmid by cloning the Neanderthal-specific alpha- and beta-chain substitutions into the human plasmid pHE2 via site-directed mutagenesis. The procedures for expression, isolation, and purification will follow those described above for humans. The result will be the resurrection of functional Neanderthal haemoglobin. I know of no relevant limitations for these methodologies, as, despite not being previously employed on Neanderthal material, the techniques have been utilised across a variety of mammalian taxa (Huynen et al. 2012).

Molecular Function Analysis

I will measure the oxygen binding equilibria for the expressed proteins while exposing them to stepwise increases in temperature at various pH levels. Analysis of measured cooperative binding will utilise the Hill–Langmuir equation, an essential tool for determining the degree of cooperativity (Gesztelyi et al. 2012). Corrected values for the enthalpy of oxygen will be calculated from absolute temperatures and gas constants as described by

Clementi et al. (1994). This will enable me to differentiate between alterations in oxygen binding characteristics for thermoregulatory purposes versus for high activity levels.

Identifying Structural Bases for Functional Differences

Finally, molecular models will be constructed to locate potential structural causes of observed functional differences. Park et al. (2006) have refined crystal structure models of human oxygenated and deoxygenated haemoglobin to an extremely high resolution—I will use these as a foundation. The Neanderthal amino acid substitutions will be introduced into Park et al. (2006)'s models of oxy- and deoxy-human haemoglobin. I will then look for interactions between the functional groups of amino acids, variations in the number and type of ion bindings, and alterations in the ion binding sites. Structural alterations like increased Cl- binding would lower enthalpy values, decreasing the effect of changes in temperature on haem-oxygen affinity (De Rosa et al. 2004; Campbell et al. 2010).

This identification of structural bases for observed functional differences is predicated upon finding differences between the Neanderthal and human haemoglobins. Regardless, this project would still be the first expression of an extinct hominin gene product. Even if I fail to identify thermoregulatory adaptations in Neanderthal haemoglobin, this work would be a stepping-stone for further research into palaeoanthropological gene resurrection.

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Conflicts of interest

The authors have declared that no competing interests exist.

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