

Polynesian chickens in the New World: a detailed application of a commensal approach

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ABSTRACT

In 2007, based on direct radiocarbon dates, we presented evidence that chickens were introduced to Chile before Europeans first made contact with the New World. The pre-Columbian age of the chicken bones and their mtDNA affinities with one of two prehistoric Pacific chicken haplogroups (E) led us to conclude that Polynesia was the most likely origin for these pre-Columbian chickens. Subsequently, the mtDNA and radiocarbon evidence provided has been applied to a range of studies and occasionally reinterpreted. This has revealed issues related to the brevity of the initial report in 2007. Here, we provide a full discussion of the evidence, including the relevant archaeological, historical and biological information necessary to provide the context for interpreting genetic analyses and understanding their implications for addressing archaeological questions. We include a comprehensive analysis of the isotope data within a geographical and temporally relevant dataset to verify the pre-Columbian age of the El Arenal chickens. In addition, we provide longer DNA sequences obtained from some of the ancient Chilean chicken remains to address objections raised by critics and to demonstrate that longer sequences do not change the observed affinities of the mtDNA sequences, nor their interpretation. In this analysis, historical information is used to critically evaluate the results of phylogenetic analyses. This comprehensive approach demonstrates that the examination of modern chicken DNA sequences does not contribute to our understanding of the origins of Chile's earliest chickens. Interpretations based on poorly sourced and documented modern chicken populations, divorced from the archaeological and historical evidence, do not withstand scrutiny. Instead, this expanded account will confirm the pre-Columbian age of the El Arenal remains and lend support to our original hypothesis that their appearance in South America is most likely due to Polynesian contact with the Americas in prehistory.

Keywords: chicken, *Gallus gallus*, Polynesian voyaging, pre-Columbian America, mtDNA, El Arenal-1.

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INTRODUCTION

Examining the human-mediated dispersal of animals by documenting their introduction to new areas, broad geographical distributions, density in archaeological sites and DNA signatures allows archaeologists to build richer reconstructions of prehistoric activities and relationships

(Matisoo-Smith 2007, 2009; Matisoo-Smith & Robins 2004). Known to archaeologists as a *commensal model*, this analytical technique may include the application of a phylogeographical approach to reconstruct the dispersal of plant and animal species outside of their natural range by humans (Matisoo-Smith 1996; Matisoo-Smith *et al.* 1998; Storey *et al.* 2013). In the Pacific, this analytical approach

may be applied to any non-native organism that cannot disperse naturally but that co-occurs in island environments with evidence of human activity or settlement.

The employment of a DNA-based commensal approach requires several steps to ensure that DNA sequence variation can provide reliable information for reconstructing aspects of prehistoric human behaviour. One must examine the characteristics of the organism under consideration, including its ability to self-disperse in specific environments. In addition, the association between humans and specific commensals must be established prior to a review of the genetic data, to identify suitable markers for determining relationships between populations. Any observed genetic links must be critically evaluated within the established archaeological and historical context of the samples (Storey *et al.* 2013). If the reconstruction of prehistoric behaviour is predicated on modern DNA data alone, an additional step is required. The context for interpreting the sequences and the relationships between them must include in-depth research in order to understand the dispersal of animals in the region of interest from their first appearance in the archaeological record to the day on which the modern samples were acquired. This is a much more complex process, involving overlapping waves of introductions from a global base for trade and exchange. This essential step, required to substantiate a link between ancient and modern groups of animals, is often missing from the literature due to the constraints placed on manuscript length and background in many publications. However, examples of appropriate links between historical events and genetic signatures have become more common in recent years (e.g. Haile *et al.* 2010; Robins *et al.* 2003). With respect to the prehistoric Pacific chickens, many of these research steps have been undertaken and previously published (Storey *et al.* 2008a, 2010, 2011b, 2012) and will only be briefly reviewed in the next section. This paper will instead focus on contested aspects of dating the chicken remains from El Arenal, Chile, as well as on exploring the contributions that ancient and modern DNA evidence have to make in reconstructing the first introduction of chickens to the Americas.

THE DISPERSAL OF CHICKEN IN PREHISTORY AND ITS IMPORTANCE TO HUMANS

The first undisputed domestic chicken (*Gallus gallus*) remains were recovered from deposits dating to around 8000 BP at the Chinese sites of Chishan, in the Hebei Province, and Peiligang, in the Henan Province (Chow 1984; Rodwell 1984–1985). Archaeological and historical evidence attests to the dispersal of the chicken beyond its natural range by 5000 years ago (Serjeantson 2009; West & Zhou 1989). This included dispersal to Remote Oceania by 3000 BP. The bones of domestic fowl have been recovered in association with the Lapita occupation

periods in several Pacific archipelagos (Storey *et al.* 2008a), the earliest recovered from Nenumbo site in Santa Cruz Islands and directly dated to 3150–2850 calBP at 2σ [3047 ± 25 years BP (NZA26177); ΔR of -81 ± 64] (Beavan-Athfield *et al.* 2008; Jones *et al.* 2007). Chickens have also been recovered from archaeological sites in islands and archipelagos occupied by Polynesians, including the remote regions of Hawai'i and Easter Island (Storey *et al.* 2008a). The earliest reported chicken bones from Hawai'i were recovered from contexts at the Kualoa site dated to between 910 and 690 calBP (at 2σ) (Carson & Athens 2006). Similarly, those from the Anakena site on Easter Island were associated with charcoal dates of between 920 and 680 calBP (at 2σ) (Steadman *et al.* 1994). The route by which chickens were introduced to the Pacific is not yet known (Storey *et al.* 2008a) but the domestic fowl is not native to the Pacific. It has been established that *Gallus gallus* is not a migratory species, cannot sustain prolonged flight and lacks the biological adaptations for swimming (Giles 1931). Therefore the introduction of chickens to the Pacific must have been due to human-mediated transport.

The suite of plant and animal species purposefully introduced to the Pacific islands varied in response to environmental, economic and other constraints. These factors informed a set of complex choices related to resource allocations, ease of transport, the utility of specific plants and animals, and a range of other considerations (Giovas 2006; Kirch 2000; White 2001; Wickler 2004). Butchering and discolouration of bone indicating cooking are good evidence that chickens were a primary food source on Pacific islands (Steadman *et al.* 2002). Their bones were also used for making flutes and whistles, beads, sewing and tattoo needles (Rorrer 1998; Steadman 1997; Steadman *et al.* 2002). Ethnographic evidence demonstrates that chicken were valued for their ritual/spiritual importance (Baldwin 1990) and that their feathers were used as important elements of costuming (Baldwin 1990; Rolett 1998). The symbolic value of chickens is suggested by their presence in cave paintings in Vatulele, Fiji and on several of the petroglyphs on Easter Island (Ewins 1995; Lee 1992). More recently, in-depth studies of the archaeological distributions and ancient DNA signatures of chickens in the Pacific have been used to reconstruct some aspects of human colonisation and later trade and exchange (Storey *et al.* 2007, 2008a, 2010, 2012).

In contrast to Oceania, in the Americas chickens were traditionally considered a late introduction to the continents, brought during voyages of discovery and conquest by Europeans carrying their own suite of commensals (Reitz & Scarry 1985). Yet some scholars have refused to accept this consensus view and have argued fervently for the existence of chickens in the Americas in the period before European contact. Generally, these pre-Columbian advocates have been of two schools. One group has supported the view that chickens were introduced to the New World as a result of

direct contacts between Asia and the Americas. Often defined as a hyperdiffusionist perspective, the hypothesis was that chickens were among a variety of plants, animals and ideas introduced by Asians in periods ranging from 3000 years ago to the early 1400s (Carter 1953, 1971, 1998; Gongora *et al.* 2008a; Johannessen 1981; Langdon 1989; Menzies 2002; Pearce & Pearce 2010). A second group of scholars have reasoned that it was far more likely that any pre-Columbian introductions of domestic fowl were the result of Polynesian contacts in the Americas (Austin 1961; Castello 1939; Caudill 1975; Crawford 1990; Ramírez 1990/1; Wilhelm 1978). However, until 2002 the debate remained moot, as no archaeological evidence had been recovered that could support the presence of chickens in the Americas prior to the arrival of Columbus in AD 1492.

The pre-Columbian chickens of Chile

The site of El Arenal-1 can be found in the Arauco Province of Chile, along the south bank of the Quidico estuary, south of the city of Concepción (Figure 1). The contemporary landscape is a small watershed situated on

the northern edge of the seasonal plain. Between 2002 and 2008, excavations at the site revealed a clear stratigraphic sequence composed of five levels (I–V), of which Levels III (Layers A and B) and IV (Layer C) corresponded to periods of human occupation sandwiched between culturally sterile deposits (Levels II and V). Fifty chicken bones were recovered during the 2002 excavation season and another 33 were recovered during subsequent excavations of Level III. Chicken remains were found across the site in all three excavated areas and in all strata, but the majority ($n = 62$) were recovered from Layer B. All of the chicken remains were contextually associated with remains from the El Vergel Cultural Complex which, broadly speaking, began *circa* AD 1000 and persisted until the arrival of Europeans. Very little cultural material was recovered from the Level I deposits at El Arenal and no indication of European contact was observed from any part of the site (Contreras *et al.* 2005). The exclusively pre-Columbian material culture and the associated thermoluminescence dates provided by the Laboratorio de Termoluminiscencia de la Universidad Católica de Chile (Table 1) also supported a non-European source for the chicken remains at El Arenal-1.

Figure 1. The location of the El Arenal-1 archaeological site, along the coast of Chile. Also plotted are the regions for which both ancient and modern isotope values have been obtained by Falabella *et al.* (2007), Tykot *et al.* (2009) and Hückstädt *et al.* (2007).

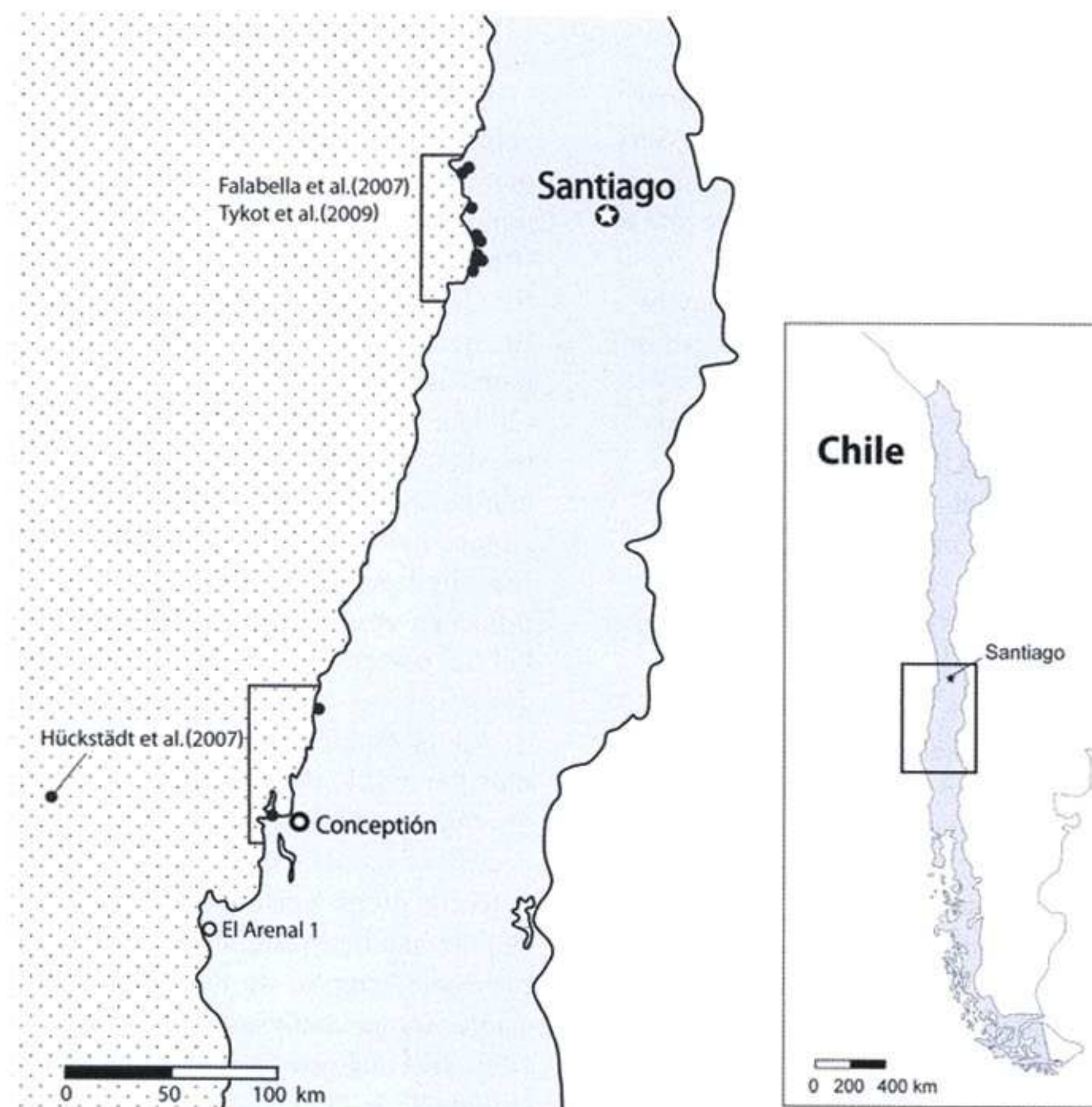


Table 1. Radiocarbon determinations and associated isotope data for three chicken bone samples from El Arenal. Conventional Radiocarbon Ages (CRA) were calibrated and calendar age ranges determined using CALIB 3.0 (Stuiver & Reimer 1993) and a Southern Hemisphere atmospheric curve [SHCal04; (McCormac *et al.* 2004)], and are reported at 2σ . All isotope values are represented as parts per mil. (‰). Atomic C:N ratios are within expected parameters for well-preserved protein. Included in the last two rows are the TL dates from the Laboratorio de Termoluminiscencia de la Universidad Católica de Chile and previously published in Storey *et al.* (2008b).

Sample number	Laboratory number	Conventional Radiocarbon Age (CRA)	Calibrated age (2σ)	$\delta^{13}\text{C}\text{‰}$	$\delta^{15}\text{N}\text{‰}$	$\delta^{34}\text{S}\text{‰}$	C:N
CHLARA001	NZA 26115	622 ± 35	650–500 calBP AD 1304–1424	–20.9	n.d.	n.d.	n.d.
CHLARA003	NZA 28271	510 ± 30	550–450 calBP AD 1427–1459	–19.85	2.6	2.16	3.2
CHLARA004	NZA 28272	506 ± 30	550–450 calBP AD 1426–1457	–19.45	3.5	n.d.	3.2

TL Dates		Date	
EA1-001	UCTL 1617	650 ± 65 BP	AD 1285–1415
EA1-002	UCTL 1618	610 ± 55 BP	AD 1335–1445

The direct radiocarbon dates from El Arenal chicken bones

Within a commensal model, the interpretation of any genetic sequence amplified from chicken bones must be considered within its archaeological context. Therefore, the first step in our analysis was to directly date the bones to ensure that they were not intrusive to the site and to confirm their pre-Columbian associations. The first sample sent for direct radiocarbon dating was a chicken bone designated CHLARA001, which produced a radiocarbon age of 622 ± 35 BP [650–500 calBP (NZA 26115) (Table 1)] (Storey *et al.* 2007). Consumption of marine proteins is known for its diminishing effect on measured concentrations of ^{14}C . Thus stable isotopes were necessary to assess the effect of any marine dietary contributions to the diets of the chickens, given the proximity of the site to the sea.

There are two sources of depleted carbon that may be considered. The first is the potential effect of shell grit on stable isotope values and the second concerns the consumption of marine invertebrates by individual birds. Chickens are known to utilise shell, including that of marine invertebrates, as grit to digest food, as well as to obtain calcium carbonate to use in egg production. Long *et al.* (1983) and von Schrinding *et al.* (1982) have established that the addition of ground oyster shell or other radiocarbon-depleted calcium carbonate in hen feed is dissolved in the digestion process by stomach acids and is lost as CO_2 . This demonstrated that the consumption of marine shell by chickens does not contribute to diet, as depleted ^{14}C is not incorporated into tissues, including bones. However, if chickens are also eating marine invertebrates as a source of protein, the ^{14}C signature of individual chickens may be depleted and the measured radiocarbon dates of those bones may have to be corrected.

Stable isotope analysis for $\delta^{13}\text{C}$ was also provided for sample CHLARA001 (cf. Beavan-Athfield *et al.* 2008).

Due to the small amount of material available for analysis after the requirements for mtDNA testing and replication, there was insufficient sample for a $\delta^{15}\text{N}$ measurement. However, the $\delta^{13}\text{C}$ value of –20.9‰ reported in 2007 sits well within the range expected for terrestrial omnivores (see Figure 2). Another interesting possibility suggested by the stable ^{13}C isotope value of CHLARA001 is that these chickens may have been eating maize. Maize was important in local diets during the late prehistoric period (Falabella *et al.* 2007) and maize cobs were recovered from excavations at El Arenal, in association with the chicken bones (Quiroz & Contreras 2002).

In controlled feeding experiments, the protein $\delta^{13}\text{C}$ values for flocks of chickens fed a maize-enriched diet typically had $\delta^{13}\text{C}$ values of –20.67‰, while non-maize-fed chickens had $\delta^{13}\text{C}$ values of –24.68‰ (Rhodes *et al.* 2010). Therefore, the carbon isotope value for CHLARA001 may be indicating that the chickens of El Arenal had a maize component in their diets. This is a more likely scenario than that proposed by Gongora *et al.* (2008a: 10305), who suggested a marine contribution to the diet of the chicken based on the co-occurrence of marine shells in the archaeological midden, rather than the isotope evidence. The available data from controlled feeding experiments shows that maize consumption may influence observed stable isotope values in bone collagen, but the use of shell as grit and a source of calcium does not.

Archaeological bone samples are finite resources and thus the first El Arenal bone sample CHLARA001 did not provide a sufficient amount of material for all the necessary mtDNA tests and the full set of stable isotope determinations. Once the pre-Columbian age of the first sample had been established by direct radiocarbon dating, two more samples were submitted for radiocarbon and stable isotope analyses. Samples CHLARA003 and CHLARA004 provided ages of 510 ± 30 BP [550–450 calBP (NZA 28271)] and 506 ± 30 BP [550–450 calBP

Figure 2. A plot of marine, lacustrine and terrestrial fauna by $\delta^{13}\text{C}$. Adjustments to isotope values collated from Supporting Table S1 for modern shellfish and *Otaria flavescens* (sea lion) are a +0.86‰ Suess effect for the marine environment and +1.5‰ for modern wild and terrestrial plants (Suess 1958). Archaeological chicken *Gallus gallus* (indicated by pink squares) clearly plots among other terrestrial and lacustrine plants and animals (green diamonds) and not among marine fauna (blue circles). All the data plotted in this graph can be found in Supporting Table S1.

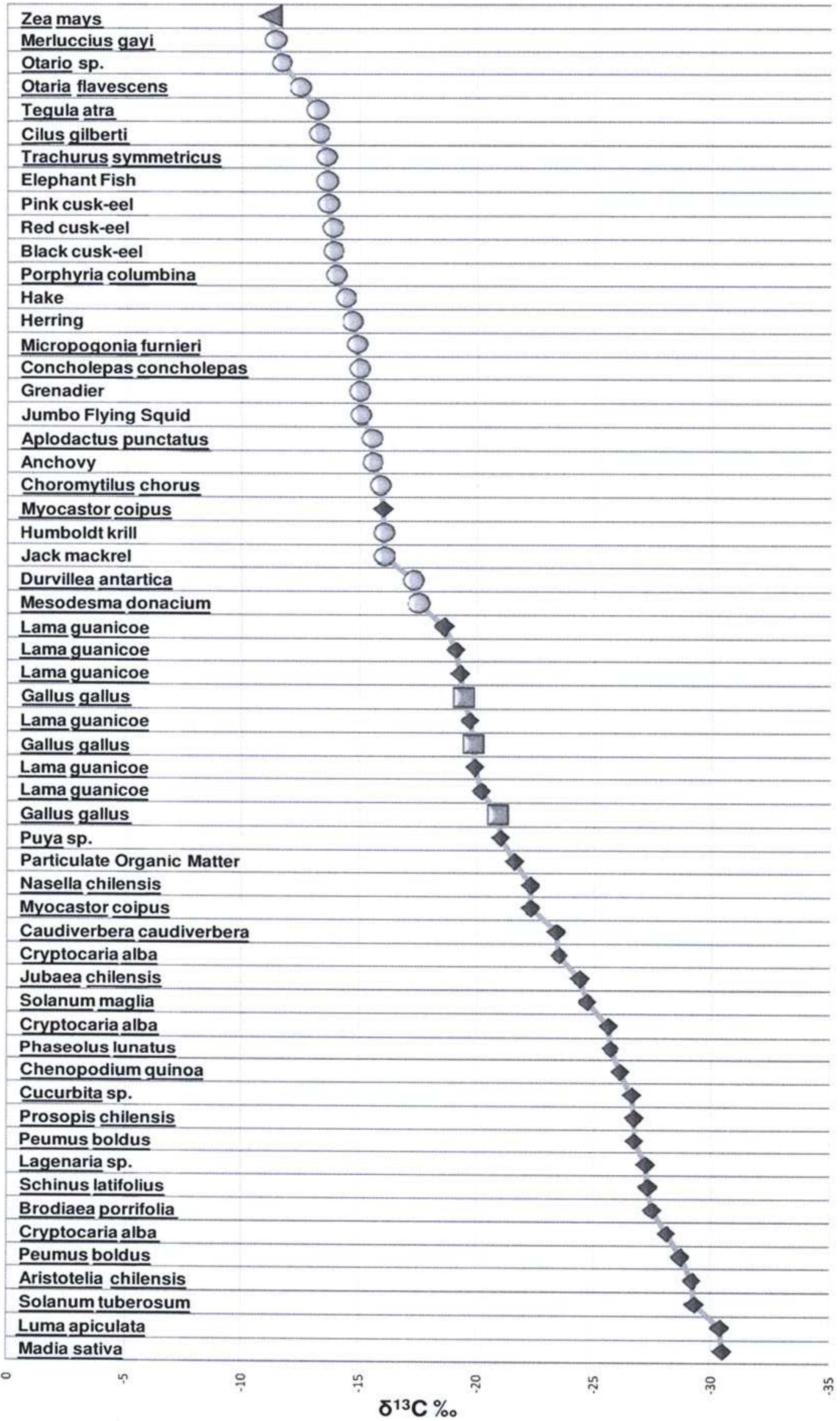
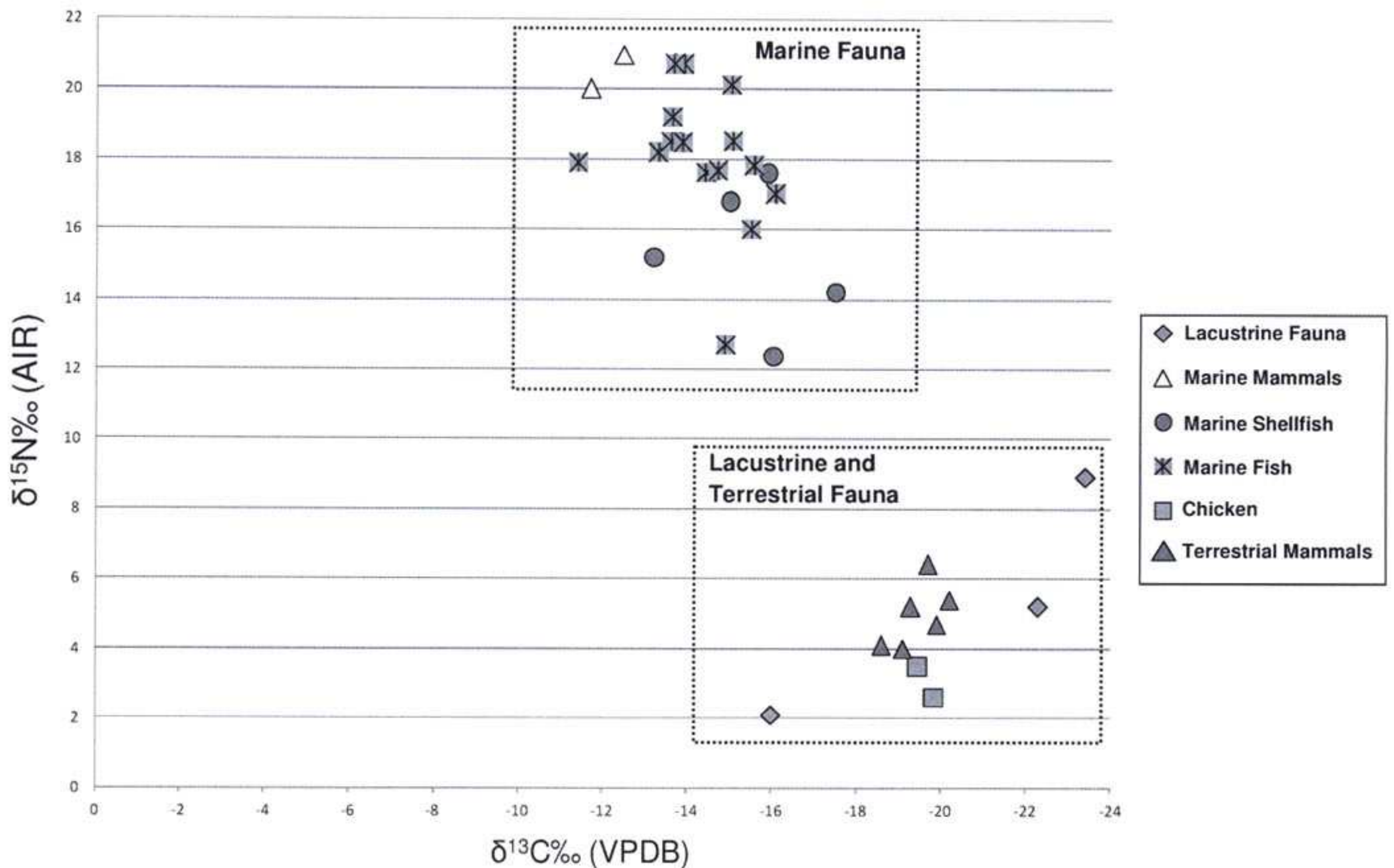


Figure 3. A plot of marine, lacustrine and terrestrial fauna with archaeological *Gallus gallus* to illustrate ancient terrestrial and marine diet relationships. Archaeological chicken *Gallus gallus* clearly plots among other lacustrine terrestrial herbivores and omnivores. As a major portion of the data for organisms are from modern samples, adjustments to isotope values collated from Falabella *et al.* (2007) and Hückstädt *et al.* (2007) are made to normalise all values to ancient flesh and produce an illustration of how various species would isotopically relate to an environment. Isotope values for modern shellfish and *Otaria flavescens* (Sea Lion) from Supporting Table S1 have had a +0.86‰ Suess effect adjustment in $\delta^{13}\text{C}$ to reflect what the carbon flesh values would have been in antiquity (Suess 1958). Archaeological bone from *Myocastor coipus* (nutria), *Caudiverbera caudiverbera* (Helmeted toad), *Micropogonia furnieri* (White Croaker), *Gallus gallus* (chicken) and *Lama guanicoe* (Guanaco) have had a -3.7‰ (Keegan and DeNiro 1988) bone to flesh conversion for $\delta^{13}\text{C}$ and a -0.6‰ bone to flesh conversion factor (Beavan-Athfield *et al.* 2008) for $\delta^{15}\text{N}$. All the data plotted in this graph can be found in Supporting Table S1.



(NZA 28272)], respectively (Storey *et al.* 2008b). Additional measurements of stable isotopes were undertaken using these samples in order to establish the reliability of the direct radiocarbon dating. These included nitrogen and sulphur isotope analyses (Storey *et al.* 2008b).

The stable isotope results, as shown in Table 1 and plotted in Figures 2 and 3, demonstrate no marine component in the diet of the ancient El Arenal chickens analysed. To confirm this hypothesis, a sulphur stable isotope measurement was also requested for CHLARA003 (Storey *et al.* 2008b). Sulphur can be used, in conjunction with stable isotopes of carbon and nitrogen, to differentiate marine and terrestrial organisms within ecosystems and/or specific food webs (Richards *et al.* 2003). Modern marine organisms typically have $\delta^{34}\text{S}$ values of around +20‰, closely resembling the value of marine ^{34}S . A broad range of sulphur isotope values have been observed for terrestrial

plants, between -20 and +20‰: these values reflect both geology and proximity to the sea. Organisms that have derived a significant portion of their diet from the sea are expected to have enriched sulphur values much closer to the +20‰ value of marine organisms. Richards *et al.* (2003) reported that terrestrial birds and mammals typically have sulphur isotope values of around +10‰, whereas mammals, such as polar bears, have values of around +16 to +18‰ due to their high intake of marine mammal proteins. The El Arenal chicken bone sample CHLARA003 had a measured $\delta^{34}\text{S}$ value of +2.6‰. The sulphur result also adds an important independent measure of potential marine contributions to the diet and does not indicate a marine component in the diet of the chicken. This confirms our interpretation of the stable isotopes of carbon and nitrogen: no marine correction is required for the radiocarbon dates derived directly from the El Arenal chicken remains.

The El Arenal isotope data were also compared with a contemporary, geographically relevant dataset. Falabella *et al.* (2007) analysed faunal remains from an archaeological site in central Chile, between 33° and 34°15' latitude in a comparable era (900–1400 BP) to those from El Arenal (see Figure 1 for locations). Combined with isotopic evidence from Tykot *et al.* (2009) and Hückstädt *et al.* (2007) the El Arenal chicken samples were plotted against a regional and, in several cases, temporally relevant dataset. The ancient chickens from El Arenal fell well within the range of Chilean terrestrial herbivores eating C₃ plants (Table 1, Supporting Table S1, and Figures 2 and 3). Figure 2 shows a plot of only $\delta^{13}\text{C}$ values, in order to include all three archaeologically associated chickens from El Arenal in direct comparison with a broad range of plant and animal resources that would have been available during the occupation of the site (for a complete list of the flora and fauna used in this analysis, see Supporting Table S1). It is clear that the El Arenal chicken remains, plotted as pink squares in both figures, sit firmly amongst the terrestrial resources in terms of their $\delta^{13}\text{C}$ isotope values. It is also particularly interesting to note the location of maize in Figure 2, represented by an orange triangle at the top left-hand corner of the graph, having stable carbon isotope values that are above those of both marine fish and mammals. In Figure 3, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are plotted for a range of terrestrial, lacustrine and marine fauna. The prehistoric chicken remains are firmly associated with the former and create a distinct group from the marine fauna, as reported in Tykot *et al.* (2009) and Hückstädt *et al.* (2007). The pre-Columbian age of the El Arenal samples is therefore supported by stratigraphy, contextually associated TL dates from Layer B ceramics (Table 1), and directly and securely dated chicken bones from the site that require no additional corrections for dietary offset.

Ancient DNA evidence

Having established that the chicken bones recovered from the archaeological site of El Arenal were indeed pre-Columbian, the next step in the employment of a commensal approach was to obtain mtDNA sequences. Due to the uniqueness of the samples, only four chicken bones of the 88 recovered from the site of El Arenal were provided by the excavators for destructive ancient DNA analysis and direct radiocarbon dating. Laboratory methods are provided in the Methods section at the end of the paper.

Three of the chicken bone samples produced reliable, replicable ancient DNA sequences of two haplotypes; named ch7 and ch8 in this analysis, both of which fit within the E haplogroup as defined by Liu *et al.* (2006) (see Table 2). The samples provided sequences of varying lengths resulting from the peculiarities of biomolecular preservation of individual bones (Miloš *et al.* 2007). We were able to obtain a total of 583 base pairs (bp) of mtDNA sequence from sample CHLARA001 (GenBank accession number EF535241.2), a total of 183 bp from

CHLARA003 (JF433983) and 373 bp of sequence from CHLARA004 (JF433992). See Supporting Table S2 for all sequences, their lengths and GenBank Accession Numbers.

The Chilean chicken mtDNA sequences have been compared with other ancient DNA sequences of samples derived from archaeological contexts across the Pacific representing chicken populations dating from 3000 BP (from Vanuatu) to the early historical period (from Easter Island) (Storey *et al.* 2012). These sequences were examined to ascertain whether the same types observed in the Pacific were also observed in Chile. The Chilean remains had identical sequences to two haplotypes documented in the Pacific, and previously identified as ah2 and ah3 (Storey *et al.* 2012; see also Table 2). Having classified the ancient sequences within an established typology, two issues then had to be addressed before the Chilean sequences could reliably be applied to the reconstruction of prehistoric human behaviour. The first was to establish the authenticity of the DNA results, and the second was the critical assessment of the affinities and origins of the ancient DNA sequences.

Issues of authenticity

Contaminants from a range of potential sources, including pre- and post-excavation contamination of samples and laboratory consumables, are a serious concern for ancient DNA studies (Leonard *et al.* 2007). Therefore, a set of internationally recognised protocols have been put in place to authenticate ancient DNA results (Cooper & Poinar 2000; Pääbo *et al.* 2004). The research undertaken in Storey *et al.* (2007, 2010, 2012) adhered to these protocols, as is further described in the Methods section of the current paper. It is important to stress that ancient DNA extractions were undertaken in two separate laboratories and over a period of three years, during which time no chicken sequences were ever obtained when negative extractions or PCR blanks were sequenced. It is, therefore highly unlikely that the E type sequences, of two different haplotypes, observed for the Chilean chicken remains represent laboratory contamination, despite previous suggestions to the contrary (Gongora *et al.* 2008a).

In a set of controlled experiments, Leonard *et al.* (2007) found that that contaminating DNA sequences from pig, cow and chicken appeared in blank extractions at rates of between 2 and 5%. This in turn led to the suggestion that the same contamination could be introduced into ancient sample extractions, resulting in false positives in the laboratory. In the case of cow and chicken, it was hypothesised that contamination may derive from specimen handling or from laboratory equipment, rather than in the production of consumables and reagents used in the extraction and amplification of ancient DNA. Leonard *et al.* (2007) concluded that up to 5% of sequences amplified for domestic animal samples have the potential to be contaminating sequences. The success rate of ancient DNA amplification for our chicken material is 52% (48/92) (Storey *et al.* 2012), suggesting that

Table 2. Haplotype affinities determined by this study and the major Haplogroups identified by Liu *et al.* (2006) to which they belong and are reflected in phylogenetic analyses. Haplotypes can only be confidently assigned to sequences of the same length, and shorter Pacific sequences previously published by Storey *et al.* (2012) are assigned provisional affiliations based on sharing an identical sequence over a shorter stretch of the hypervariable region than was used in this analysis. PAQHAN001 was assigned a unique haplogroup in this analysis due to the identification of a unique SNP at position 331 (Supporting Figure S1).

Haplogroup	Haplotype – this analysis	Ancient sequences	Gongora <i>et al.</i> (2008a)	Provisionally affiliated ancient samples and country of origin	Storey <i>et al.</i> (2012) haplotype
A	ch1		CHL31		
B	ch2		CHL1 CHL 17 CHL6 CHL36 CHL37		
D	ch3	HWIPLR001		HWIKIP003, Hawai'i, US HWIKIP004, Hawai'i, US HWIPLK002, Hawai'i, US HWIPLR002, Hawai'i, US HWIWAI001, Hawai'i, US HWIWAI002, Hawai'i, US HWIWAI003, Hawai'i, US PAQANA004, Rapa Nui, Chile PAQANA006, Rapa Nui, Chile PAQANA009, Rapa Nui, Chile PAQANA010, Rapa Nui, Chile	ah10
E	ch4	PAQHAN001			ah10
	ch5	HWIKIP005			ah11
	ch6		CHL23	VUTTEO003, Vanuatu	ah1
	ch7		CHL26	CHLARA003, Chile HWIKUA001, Hawai'i, US NIUPKI009, Niue Tonga TD, Tonga VUTTEO006, Vanuatu	ah2
	ch8	CHLARA001	CHL2 CHL21 CHL8 CHL25 CHL9 CHL27 CHL11 CHL28 CHL12 CHL30 CHL13 CHL33 CHL18 CHL35 CHL43	CHLARA004, Chile FSMFSP003, Fais, Federated States of Micronesia HWIKIP002, Hawai'i, US PAQANA011, Rapa Nui, Chile SLB33001, Santa Cruz Group, Solomon Islands SLBTKP001, Tikopia, Solomon Islands SLBTKP002, Tikopia, Solomon Islands	ah3
	ch9		CHL10 CHL22 CHL29		
	ch10		CHL32		
	ch11		CHL24		

contamination of the samples, due to handling, would have to be significantly more common than that reported by Leonard *et al.* (2007). In addition, the ancient chicken sequences of the Pacific fall into six distinct haplotypes, and the three samples from El Arenal are of two haplotypes. Therefore, contamination through handling seems unlikely, as samples of the same haplotype are derived from different researchers working on different sites in different archipelagos. However, some remains from the same site, such as those from El Arenal, or the Teouma site in Vanuatu, are of distinct haplotypes (Storey *et al.* 2012).

Despite suggestions to the contrary, although a majority of sequenced commercial-type chickens are of haplogroup E, this small global sample of modern commercial chickens does not provide sufficient evidence to exclude

ancient samples of any haplogroup. Domestic chickens examined by Liu *et al.* (2006: Supporting Table S2) possessed haplogroups A, B, D, E and I. A more recent survey of contemporary chicken populations by Miao *et al.* (2013) revealed that sampled individuals in commercial chicken populations predominantly fell into haplogroup E (73%), with the remainder in haplogroups A (17%) and B (10%). However, it is unclear how well the diversity in these 351 domestic chicken sequences available in the literature approximates the diversity found in the 40 billion birds produced every year by commercial operations (Muir *et al.* 2008). Commercial chickens have been developed from a relatively small pool of inbred strains, but the commercial poultry industry is often reluctant to allow the publication of sequences associated with their specialised lines (Guan *et al.* 2007). It is likely that as more

commercially bred chickens are genotyped, the proportions of observed haplotypes will change and new haplotypes will be discovered.

Authenticity criteria include documentation of small fragment size for amplified DNA, independent amplification and the preservation of other ancient biomolecules (Brown & Brown 2011). Purposeful attempts to amplify fragments longer than 350 bp were unsuccessful, suggesting that the short amplifiable fragment lengths were consistent with the size range expected in ancient DNA studies (between ~200 and 300 bp). Ancient DNA sequences were only considered authentic if they were derived from at least two amplicons from distinct PCR reactions, generally obtained on different days. Furthermore, the sequence from CHLARA001 was independently replicated using a different set of protocols at Massey University, which included cloning as opposed to direct sequencing (Storey *et al.* 2007). Finally collagen obtained for dating from the same samples was consistent with good organic preservation in the sample, lending further independent support to the probable preservation of other ancient biomolecules such as DNA (Götherström *et al.* 2002).

Examining genetic affinities

The next step was to examine the affinities of the ancient DNA sequences to determine whether they could provide evidence for the origins of the Chilean chicken remains. Haplogroup E has been identified in eight distinct archipelagos in the Pacific since the Lapita period (Storey *et al.* 2012) and descendants of lineages ch7 and ch8 were transported prehistorically to both Hawai'i and Easter Island. Thus it stands to reason that the relative proximity of Polynesia to South America and the current dates for the eastward expansion of the Polynesians point to the renowned sailors of the Pacific as the agents for the transportation of the pre-Columbian chickens of El Arenal. Other potential agents of introduction, including European sailing ships and pre-Columbian contacts from Asia, have been proposed (Gongora *et al.* 2008a: 10311). Those possibilities have been investigated using evidence from documentary sources, archaeology and DNA (Storey *et al.* 2011a,b). In our view, these sources have not provided a more compelling alternative to Polynesian introductions.

EVALUATING THE CONTRIBUTIONS FROM MODERN DNA ANALYSES

In 2007, we hypothesised that the E haplotypes observed in both ancient and modern Chilean chicken populations may represent a link between fowl introduced by Polynesians in prehistory and contemporary flocks (Storey *et al.* 2007). This hypothesis was challenged on the basis of an analysis of DNA from contemporary populations of Chilean chickens, particularly sequences obtained from the Araucana breed (Gongora *et al.* 2008a). This led to the formulation of an alternative hypothesis by Gongora *et al.* (2008a) that the E haplotype observed in the El Arenal

chickens was not of a Polynesian but an Indo-European type (Gongora *et al.* 2008a). However, as discussed at the outset, in a commensal paradigm, the application of modern DNA sequences to address aspects of prehistoric behaviour requires an additional historical research step. Without this, the phylogenetic analysis provides only a grouping of sampled types that provide a snapshot of contemporary diversity. This may contain a signal of a prehistoric event of interest, but is more likely to reflect more recent events, such as population replacement. This is analogous to using seriation to organise artefacts collected from a conflated surface assemblage in a new or understudied geographical area where the cultural chronology is unknown. Both systems of categorising individuals provide an opportunity to identify groups containing types that are related in some defined way, but without stratigraphic or chronological information and a regional culture history to compare the types with, all that is produced is a set of clusters. It is incredibly difficult to understand the appearance and disappearance of types and the duration of their use.

Our initial study, which included modern mtDNA of Chilean chickens, also lacked this crucial historical context in drawing links between ancient chicken DNA and modern blue-egg laying chickens (Storey *et al.* 2007). Therefore, it is of critical importance that, prior to the development of conclusions about the potential links between modern and ancient birds, the documented history of the Araucana breed should be thoughtfully examined. This provides a necessary context in which to evaluate the potential of modern DNA evidence to reconstruct prehistoric events, and to establish direct relationships between ancient and modern flocks.

Documenting the historical dispersal of chickens to the Pacific and the Americas

The transport of the domestic fowl both to and within the Pacific did not cease in the period after first European contact. Chickens were an important food and trade item aboard European sailing ships, and abundant accounts exist of European sailors trading livestock with Pacific Islanders. Chickens were often acquired by Europeans in one archipelago and weeks later traded to another. Examples of such trades are well documented by Cook *et al.* (1784), Dillon (1829) and Edwards *et al.* (1915). In addition, after initial colonisation of South-East Asian and American ports, chickens were regularly moved back and forth across the Pacific. This means that there are three important processes that must be considered when comparing modern and prehistoric sequences in these regions: (1) hybridisation between Pacific chickens and those introduced by foreigners in the post-contact period; (2) the translocation of Pacific varieties around the Pacific by Europeans; and (3) the post AD 1500 introduction of chickens from Asia to the Pacific, the Americas and Europe. These events have undoubtedly obliterated any affiliation that once existed between genetic signatures and specific geographical locales in prehistory. This is

illustrated by the fact that ancient DNA studies in the Pacific revealed that haplogroup E was the dominant lineage in early sites (Storey *et al.* 2012), yet studies of modern Pacific chickens show a population dominated by haplogroup D (Dancause *et al.* 2011; Miao *et al.* 2013).

A full account of the historical translocation of chickens by explorers, missionaries, whalers and other traders operating in the Pacific is still to be assembled.

Observations about crosses between commercial breeds and Indigenous chickens in Rarotonga and Hawaii have been recorded by Cornwallis (2002). In addition, a small case study tracing the history of chicken introductions in the Santa Cruz Islands of the Solomon chain was recently assembled by Harris *et al.* (2013) and demonstrates the complexity of documented post-contact chicken translocations into and around the Pacific. Indeed, it has been argued these long-term global interaction networks have had a substantial effect that has not yet been adequately accounted for in the majority of studies that utilise modern chicken DNA to understand prehistoric human behaviour (Storey *et al.* 2012).

The paucity of archaeological evidence for pre-Columbian chickens in the Americas strongly suggests that while present, chickens were not common and may have existed in limited numbers or in specific geographical locations. Thus, European or other introductions of chicken after AD 1492 would have quickly overwritten most or all of the DNA and morphological signatures of ancient flocks. The first well-documented introduction of chickens to the Americas was in AD 1500, when the Portuguese explorer Pedro Álvares Cabral gave a hen to a Brazilian guest on board his ship (Greenlee 1939: 12). Along the west coast of South America, the written records indicate that chickens were first introduced to Peru by Alonso de Molia in 1528 (Hemming 1970). The domestic fowl was a well-established component of Peruvian village life by the early 1600s, as evidenced in the chronicle of Don Felipe Guaman Poma de Ayala (1615). This was likely facilitated by the importance placed on chickens and eggs by the Spanish. In both the Americas (Caudill 1975) and the Philippines (Newson 2009), chickens and, in particular, their eggs were a well-regarded tribute item, equivalent to a Spanish *real* in value.

Although it has yet to be intensively researched, the Manila galleon trade was likely also a significant source of Asian domestic fowl introduced to the Americas after AD 1500. Chickens could have been obtained through trade with China and India through ports in the Philippines. Specific examples are rare but telling. Espinosa picked up chickens from Halmahera in Indonesia in AD 1533, before setting sail for Panama via the Marianas (Levesque 1992a). In AD 1596, a ship filled with Chinese merchandise arrived in Peru (Levesque 1992b: 21). In the early years of the 1600s, trade flourished between Japan and Acapulco (Levesque 1992b: 324). The specific cargo of these Chinese and Japanese ships is not yet documented, but one cannot discount the possibility that

chickens were aboard the ships and were introduced to the Americas in the first 100 years of recorded contact in the region.

The Araucana chickens of Chile

In 2007, we suggested that E haplotype signatures observed in blue-egg laying chickens in Chile may indicate a persistence of ancient Polynesian lineages in modern breeds of chickens. Gongora *et al.* (2008a,b) countered that not only was the E haplogroup not ancient, but that it was related to an Indo-European dispersal of chickens, unrelated to the transport of chickens to the Pacific. This conclusion was based solely on the novel analysis of 41 modern Chilean birds. In order to evaluate either argument, it is important to understand why Chilean chickens, which are in some cases morphologically unique, are part of the argument.

In the 1920s, a morphologically distinct breed of chicken was observed in the Arauco region of Chile. Castello (1921) unveiled the new breed at the World's First Poultry Congress and described it as a bird that laid blue eggs, had ear tufts and lacked a tail. The chicken was so unique that it was designated a new species, *Gallus inauris*, from the Latin "inarius", meaning earring (Castello 1921). The breed had been developed by Dr Ruben Bustos, through careful crossbreeding of roosters with earrings and tailless/rumpless hens that laid blue eggs. The combination of all three traits in a single bird was completely novel to the observers in the early 1900s but most, if not all, of the individual characteristics of the Araucana breed were likely introduced to the Americas in a variety of imported fowl after AD 1500 (for an extended discussion, see Storey *et al.* 2011a).

Castello (1924) himself suggested that the characteristics of the breed may have resulted from the crossbreeding of Spanish/European types and a pre-Columbian chicken breed. He also suggested the latter may have come to the Americas from Polynesia (Castello 1939). In the 1930s, "pure" Araucana hens that had not been extensively crossed with American and European breeds were nearly impossible to find (Vosburgh 1948; Wilhelm 1978). Of course, this assumption presupposes that there had not been any crossbreeding to produce the distinct Araucana characters in the first place. Therefore, the origins of the Araucana breed have yet to be resolved, and while the breed has been linked to pre-Columbian birds by several scholars, its complex origins preclude its use as a direct link between the pre-Columbian fowl of El Arenal and modern Chilean flocks.

On the basis of the historical evidence for post AD 1500 livestock introductions and the traits of the Araucana breed, it is clear that the study of modern Chilean chicken genetics will be limited by the maternal inheritance of mtDNA and the numerous sources that may contribute to signatures of individual chickens. In addition, since all chicken breeds are ultimately Asian derived (Crawford 1990; Serjeantson 2009), this may result in confusion between the identification of immediate and ancient

ancestors in a conflated phylogenetic typology. Bearing this in mind, and in critical consultation with the extensive literature on the modern phylogeographical distribution of chickens, we re-examined the conclusions of Gongora *et al.* (2008a) through a critical re-analysis of the data.

RE-ANALYSING THE CHILEAN mtDNA DATA, ANCIENT AND MODERN

At least 550 years separate the mtDNA signatures of the archaeological chicken bones from El Arenal and the modern chicken sequences generated by Gongora *et al.* (2008a). Given the complex history of trade and exchange that has been documented in the post-contact period (see Storey *et al.* 2011b), it is valid to question whether in this particular case the comparison of ancient and modern sequences has any value.

In order to assess the conclusions presented by Gongora *et al.* (2008a), we thoughtfully re-examined the genetic evidence. To do this, we included the haplotype-defining mtDNA signatures from a study by Liu *et al.* (2006), modern sequences from the Gongora *et al.* (2008a) dataset, which had a least one of the recognised Araucana breed traits (see Methods), and one of the ancient El Arenal chicken haplotypes that was of sufficient length to be included. To bring in a Pacific component, we also included longer sequences obtained from ancient chickens from the Hanga Hahave site on Easter Island (PAQHAN001), as well as two sites in Hawaii: Kahikinui on Maui (HWIKIP005) and Puu Lanai Ranch on Kona (HWIPLR001). Despite the fact that most of the variability in the chicken mtDNA hypervariable region can be found in the 201 bp fragment previously studied, longer ancient samples were used in this analysis to address the concerns of critics that the DNA sequences provided in the 2007 study were too short to reliably compare them with longer modern sequences (Gongora *et al.* 2008a: 10310). The use of longer sequences did not change the affinities of sequences that had previously been observed in shorter stretches of mtDNA (Storey *et al.* 2012).

RESULTS

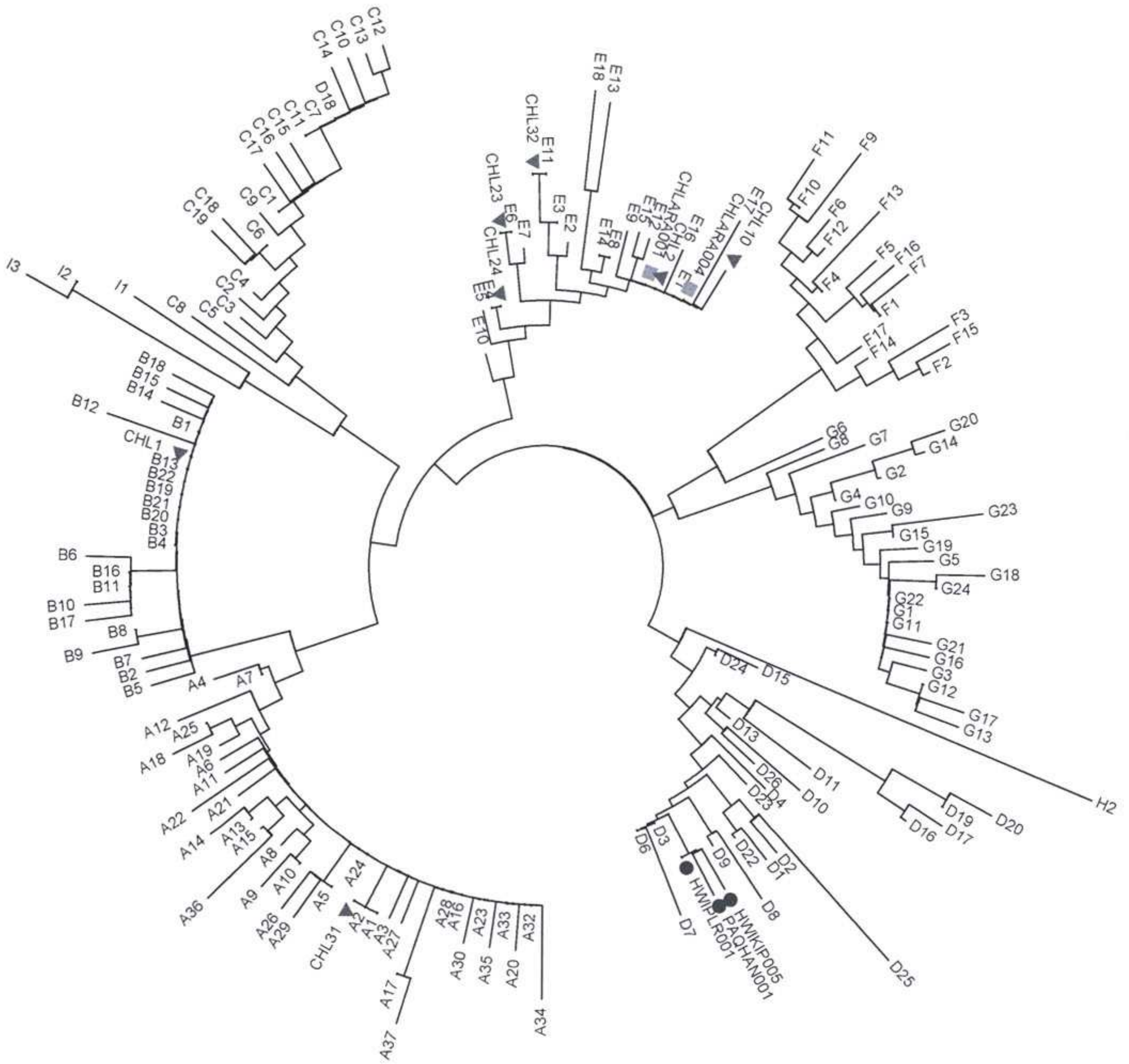
In order to understand the evolutionary relationships amongst the sequences, they were compared and then assigned to haplotypes and haplogroups. Haplotypes are defined as groups of sequences that share base changes called single nucleotide polymorphisms (SNPs). The longer the sequence, the more likely it is that multiple haplotypes will appear, as each additional base pair analysed has the potential to reveal a new SNP. The similarities and differences amongst the sequences in this study were immediately apparent in the multiple alignment due to the limited variability observed in the 169 mtDNA haplotypes, the ancient and the modern samples. Two modern Chilean chicken haplotypes from the Gongora *et al.* (2008a) study were affiliated with a haplotype observed in ancient Pacific material (Table 2 and Figure 4).

Two individuals were part of ch7, previously identified by Storey *et al.* (2007, 2010, 2012) and in those analyses designated ah2. Another 15 modern Araucana-type sequences from the Gongora *et al.* (2008a) study were of haplotype ch8, which was also observed in ancient individuals from the Pacific and from the pre-Columbian site of El Arenal (ah3); all of which fell into Liu *et al.*'s (2006) haplogroup E. The modern Chilean chicken sequences also contained five individuals of haplogroup E that were distinct from ancient Oceanic sequences. Another five modern Chilean individuals belonged to haplogroup B and a final one to haplogroup A. Neither haplogroups A nor B have been detected in samples from prehistoric Pacific archaeological contexts, but are documented commercial mtDNA haplogroups (Miao *et al.* 2013). Also of interest was the fact that no modern Chilean chickens with Araucana-type traits were of haplogroup D (ch3, ch4 and ch5), which was detected in several of the archaeological samples from Polynesia (Storey *et al.* 2007) and one from early historical Peru (Storey *et al.* 2012).

As outlined in the previous section, the Araucana chickens of Chile were developed by crossing at least two, and possibly three, distinct breeds (Castello 1924; Caudill 1975). Thus the distribution of modern Chilean birds into three distinct haplogroups is not unexpected. These may represent the three different types of chickens that were crossbred in the late 1800s to develop the recognised Araucana breed (American Poultry Association 2001) or later introgressions with commercial broilers and layers. It is notable that tailless and ear-tufted birds do not sort into distinct haplogroups in either analysis, indicating that the transmission of these particular traits is unlikely to be female mediated. If this is the case, then mtDNA data will not be a suitable means by which to determine the origins of these traits.

In order to confirm the grouping of sequences determined by directly comparing SNPs, phylogenetic methods were employed (for the specifics, refer to the Methods section at the end of this paper). As predicted by our sequence alignments (Supporting Figure S1), the Neighbour Joining (NJ) analysis showed the same haplotype and haplogroup affiliations outlined above, shown in Table 2 and Figure 4, and supported by previous analyses (Storey *et al.* 2010, 2012). Unfortunately, the results cannot be directly compared with the MP consensus tree presented by Gongora *et al.* (2008a: Figure S6 in the Supporting Information), as only 2 of their 41 samples (CHL10 and CHL44) are present on their published tree. Sample CHL44 is classified by Gongora *et al.* (2008a) as a passion fowl, and its relationship to either modern Araucana chickens or ancient Polynesian breeds was unclear. As a result, it was not included in our re-analysis. The NJ analysis does confirm the previously illustrated phylogenetic affinity of CHL10 with haplogroup E type chickens. A comparison with the network presented by Gongora *et al.* (2008a: 10309, figure 1) broadly suggests the same affinities observed in our analysis;

Figure 4. An Unrooted Neighbour Joining Tree, showing the relationships between modern Araucana chickens, haplotypes and haplogroups classified by Liu *et al.* (2006), and ancient samples from archaeological sites in the Pacific. Pink triangles represent Gongora *et al.*'s (2008a) modern samples from Chile, which had at least one of the distinguishing traits of Araucana chickens, green squares are ancient Chilean chickens from El Arenal, and blue circles represent ancient Pacific chicken samples from Hawai'i and Easter Island. All of the modern Chilean chickens fall into the A, B and E haplogroups. These confirm the haplogroup affinities reported by Gongora *et al.* (2008a) and those observed in this and previous studies (Table 1).



however, the 27 samples with Araucana traits from their study are not distinguished in their network, and thus no direct comparison can be made between this analysis and that undertaken in 2008. We are confident, however, that the longer Chilean sequences would fall in the E cluster of a Median Joining Network due to their clear affinities observed in the multiple alignments and the Neighbour Joining Tree (Figure 4). In this endeavour, we concur with

the results of Gongora *et al.* (2008a); however, we strongly disagree with their interpretations of these results.

Critically evaluating the results and conclusions based on modern mtDNA

Gongora *et al.* (2008a: 10308) concluded that the modern Araucana chickens that they sampled were predominantly of European origin, and their analyses provided “no

support for a Polynesian introduction of chickens to South America". This is a large intuitive leap to make from ancient to modern evidence, without any critical review of the historical or morphological evidence. The attribution of a European origin by Gongora *et al.* (2008a) was based on the fact that 19 of their samples were observed to be of the same haplogroup as 56 modern European samples. These European samples were wholly derived from the work of others (see Liu *et al.*'s (2006: Supporting Information Table S1). Of those, 53 are described only as "European", lacking both the specific geographical affiliation of the samples and identification of their breed. The other three European chickens used in the Gongora *et al.* (2008a) analysis were drawn from a study by Akishinomiya *et al.* (1996), which included two White Leghorns and a Barred Plymouth Rock. One of the Leghorns was procured from a flock kept at the Hiroshima Animal Husbandry Experimental Station in Japan and the origin of the second is unclear. The Barred Plymouth Rock chicken was an individual sampled at the Domestic Fowl trust in Worcestershire, United Kingdom, but is, as the name suggests, an American breed (American Poultry Association 2001). It is important to note that it is Liu *et al.* (2006) who refer to these as European, and not Akishinomiya *et al.* (1996). The study of Gongora *et al.* (2008a: 10310) carries on this misattribution error, describing several industrial breeds, including Plymouth Rocks and New Hampshires, as European. These are types developed in the United States, through deliberate crosses between a range of birds of international origin in the late 1800s and early 1900s (American Poultry Association 2001). While these breeds may be derived in part from the bloodlines of established European breeds such as White Leghorns, the "hen craze" of the 1850s that led to the development of named American and European breeds relied heavily on crossbreeding with birds obtained from unknown Asian markets. Therefore, the use of these currently recognised national breeds is not relevant to understanding prehistoric dispersals or the historical movements of chickens preceding AD 1800, or for grounding phylogeographical analyses.

Gongora *et al.* (2008a) have identified an affinity between some of their modern Chilean birds and American breeds of chicken, but do not discuss the possibility that the observed relationship may be a remnant of crossbreeding within the past 100 years. Extensive crosses between Rhode Island Reds, Barred Plymouth Rocks and Araucanas were well documented in the years leading up to the 1930s (Vosburgh 1948). Instead, Gongora *et al.* (2008a) attributed the observed genetic affinities of particular samples to ultimate origins, but these are not supported by the evidence. Historical sources reveal that many European/Mediterranean types of chickens, including Leghorns, were either developed or improved through crossbreeding with Asian stock in the Americas (Bennett 1851; Dixon 1850). These scholars also list the islands of the Pacific as a significant source of breeding

stock for North and South American chicken flocks. Such birds were introduced by sailors who regularly travelled back and forth to the European trade centres in Island South-East Asia, the Americas and Europe (McGrew 1921).

Perhaps one of the most oft cited conclusions of the Gongora *et al.* (2008a) paper is that haplogroup E chicken have Indo-European origins. They based this largely on the Liu *et al.* (2006) dataset, which included only 27 domestic chickens from India, and Gongora *et al.* (2008a) did not add any new Indian DNA sequences to their analysis. It is true that a small majority of those Indian domestic fowl analysed by Liu *et al.* (2006) were of haplogroup E (15/27); however, all but one of the rest were of haplogroup D (11/27). Liu *et al.* (2006) tentatively opined that the origin of haplogroup E may be India, but Oka *et al.* (2007) suggested that it may have a South-East Asian origin and that it was introduced to India before its widespread foreign dispersal. Modern chicken flocks in India consist of a significant number of foreign breeds (80%) (Pirany *et al.* 2007), suggesting that identifying an Indian-specific signature using modern DNA will be difficult, if not impossible. Contemporary flocks of Indian chickens have several mtDNA lineages, reflecting this cosmopolitan population (Kanginakudru *et al.* 2008). This suggests that the data used by Gongora *et al.* (2008a) is insufficient to identify definitive Indian mtDNA signatures. Regardless, Gongora *et al.* (2008a: 10308) claimed that the D signature observed in ancient Pacific remains is "an uncommon haplogroup from Indonesia, Japan, and China and may represent a genetic signature of an early Polynesian dispersal". This conclusion appears to overlook the 41% of Indian chickens in the D haplogroup (Liu *et al.* 2006: 15, table 1), as well as dismissing the E haplogroup signatures in ancient material as historical introductions or laboratory contamination. A more comprehensive analysis by Miao *et al.* (2013) concluded that haplogroup D could be found in modern populations in Africa, South Asia, South-East Asia and East Asia, and that the most likely source of the D haplogroup in the Pacific is a South-East Asian domestication centre.

Although a great deal of modern chicken mtDNA data exists in GenBank, it is not appropriate for the prehistoric phylogeographical assumptions it has been used to make by Gongora *et al.* (2008a). The claim that E haplotypes are necessarily Indian in origin is not supported by the available evidence, ancient or modern, and yet this attribution now appears in the literature (for examples, see Dancause *et al.* 2011; Tixier-Boichard *et al.* 2011). The use of groupings based only on – often poorly documented – modern DNA evidence, divorced from its historical and archaeological context, will create serious problems if applied to reconstructing the origins and affinities of the people who carried those chickens into the Pacific.

India is unquestionably a candidate as a major chicken domestication centre, but Burma, Thailand, China (both north and south) and several other regions are also

potential sources for Island South-East Asian and Pacific chicken stocks. At this stage, there is insufficient data available to link specific modern chicken haplogroups to definitive ancient domestication centres or individual lineages to Oceania specifically, as Dancause *et al.* (2011) attempt to do with haplogroup D. It had already been shown that the haplogroup is also often encountered in modern chicken flocks in Zimbabwe and Malawi (Muchadeyi *et al.* 2008). The global chicken sample available on GenBank is currently less than 5000 sequences (Miao *et al.* 2013) and is not sufficient to support such conclusions. Therefore, ancient DNA will be essential to test hypotheses built on the analysis of modern DNA sequences alone (Storey *et al.* 2012).

A final problem with the Gongora *et al.* (2008a) analysis is that the chickens used are not representative of the breed standard for Araucanas. Of 41 individuals, one had two Araucana traits and 27 had only one – either taillessness or ear tufts. There is no mention made of blue-egg laying, a critical diagnostic criteria for the attribution of the breed. The other chickens were identified as Creole ($n = 7$), Passion fowl ($n = 5$) and Japanese Long Tail ($n = 1$). It is not made clear in the text or the supporting information how these other three types of chickens are relevant to the study, or to the question of the potential contribution of ancient Polynesian chickens to modern Araucana flocks.

Therefore, it is not possible to determine, on the basis of the current evidence, whether the modern Chilean chickens of ch7 and ch8 type are descended from the pre-Columbian chickens of El Arenal. They may indicate later historical-era introductions from other areas, including the Pacific. This does not invalidate in any way the findings of the Storey *et al.* (2007) paper that the archaeologically associated El Arenal chicken bones are pre-Columbian, nor the hypothesis that domestic fowl were introduced by Polynesians. The observations of Gongora *et al.* (2008a) do suggest that the potential influence of pre-Columbian chickens on modern Araucana stocks cannot be determined using modern mtDNA sequence data from the hypervariable region alone. Therefore, future studies must be extended to include other genetic markers, and perhaps to utilise the recent advances in next-generation sequencing technology to generate whole mitochondrial genomes and target specific nuclear genes for traits such as blue-egg production, in order to resolve the issue.

CONCLUSION

The study of ancient DNA enhances the interpretative power of phylogenies by providing the means by which the appearance or disappearance of types may be dated, either directly or by association, in the same way that a stratigraphic excavation provides a chronology for a seriated regional dataset. However, sufficient global sampling exists for few, if any, DNA datasets, making it difficult to definitively identify the origin of specific

DNA signatures. Like the artefact classes in archaeological sites whose novel appearance signals a new arrival, the new type cannot be traced back to its homeland until a well-documented assemblage from its place of origin is recovered. These issues plague archaeology in the same way that they affect genetics, with two very important differences. The first is that people have been studying archaeology much longer than they have been generating and analysing genetic sequences. The second is that archaeologists understand the limitations of a seriated assemblage of surface-collected artefacts, without realising that most conclusions drawn from modern DNA datasets about ancient human behaviour use a similar methodology. This means that archaeologists are very well equipped to evaluate and critique the application of DNA phylogenies to archaeological questions.

The myriad introductions of chickens to the Americas that occurred from a range of geographical sources after AD 1500 and the later import of Asian varieties in the 1850s have led to a great deal of confusion in the application of modern chicken mtDNA datasets to reconstruct ancient events. The historical literature from the early contact period has always been contentious in regards to the earliest introductions of chickens to the Americas (Storey *et al.* 2011a). Thus the direct radiocarbon dating of three samples from El Arenal, with isotopic analyses, and the established site stratigraphy represents the first definitive evidence for pre-Columbian introductions of chickens to the Americas. The evidence supporting the authenticity and interpretation of the radiocarbon dates has been presented here and confirms their antiquity. With or without mtDNA evidence, the most likely source for chickens in pre-Columbian Chile is through prehistoric Polynesian contacts.

The re-evaluation of the El Arenal evidence within the established methodology of a commensal approach also supports our previous hypothesis for a Polynesian introduction of chickens to Chile. The mtDNA signatures of these prehistoric chickens belong to one of two ancient haplogroups of chickens also present in archaeologically associated fowl bones from the Pacific. That same haplogroup (E) can be securely dated to the Lapita period in Vanuatu. The circumstantial evidence for Asian introductions has been evaluated and, as yet, no compelling evidence has been revealed to substantiate direct contacts between pre-Columbian South America and Asia (Storey *et al.* 2011b). Therefore, the most reasonable explanation is that the direct ancestors of the archaeologically associated remains of *Gallus gallus* at El Arenal are Polynesian. It has been clearly shown that the Araucana breed, with its myriad and modern origins, does not provide mtDNA signatures that are suitable for answering questions related to the origins of ancient stocks. The evidence from modern mtDNA from chickens with some of the traits associated with Araucana chickens certainly cannot invalidate the conclusions from well-attested archaeological samples.

METHODS

Laboratory methods

Ancient DNA work was undertaken in the Ancient DNA Facility in the Anthropology Department at the University of Auckland. Three physically separated task-dedicated laboratories were used in the course of the experiments. One-way traffic was strictly observed and no researchers entered the ancient laboratory on the same day on which they had been in the modern lab. The protocols used for laboratory work, particularly with reference to PCR protocols, visualisation and sequencing, are available in other publications (Storey 2009; Storey *et al.* 2007, 2010, 2012); however, parameters unique to this study – or to clarify our adherence to strict contamination controls – are listed here in full, including primers, some of which are published for the first time as part of this study (Table 3).

Ancient DNA extractions were carried out using a modified guanidine thiocyanate silica suspension technique (Matisoo-Smith *et al.* 1997). The Ancient DNA Facility follows the standards to monitor for contamination and establish the authenticity of ancient DNA set out by Cooper and Poinar (2000) and Pääbo *et al.* (2004). Work is conducted in a physically isolated work area, to which access is carefully controlled, and all occupants dress in disposable gowns and wear hairnets, face masks, disposable booties (or shoe covers) as well as latex gloves. All stages of work from extraction to PCR amplification included negative controls in which no sample or template aliquots were added. Ancient DNA sequences were required to have been derived from at least two amplicons originating from distinct PCR reactions, generally obtained on different days. The sequence from CHLARA001 was independently replicated in another laboratory, using a different set of protocols at Massey University that included cloning (Huynen *et al.* 2003; Storey *et al.* 2007).

Primers were developed to target DNA templates under 300 base pairs (bp) in length. Primer sets were designed to overlap to assemble longer sequences when possible (see Table 3). Ancient DNA template molecules were monitored for appropriate molecular behaviour. Some samples (such as CHLARA001) were run with primer sets specifically

designed to amplify longer target molecules (over 350 bp), as a test to determine whether longer templates could be amplified. These trials were unsuccessful.

Phylogenetic analysis

To allow direct comparison between the ancient samples and the modern sequences of Gongora *et al.* (2008a), all sequences were aligned using MUSCLE (Edgar 2004), within Geneious Pro 5.0.3 (Drummond *et al.* 2010). The sequences for comparison from the Gongora *et al.* (2008a) dataset were chosen critically based on the established criteria for the identification of Araucana chickens. All of the Chilean sequences used in our re-analysis were required to have at least one of the traits associated with the Araucana breed, including the laying of blue eggs, the absence of caudal vertebrae, resulting in taillessness, and/or the presence of ear tufts (American Poultry Association 2001). Given the potential presence of these individual characters in the ancestral stocks used to develop the breed, sequences from individuals with any of these traits in the Gongora *et al.* (2008a) dataset were included. Of those, 16 were identified in the Supporting Table S1 as tailless, 11 had ear tufts and one had both traits. While blue-egg laying is included in their introduction, Gongora *et al.* (2008a) do not clearly link that information to any of the samples used in their analysis, despite the fact that this trait was considered very important by both Castello (1924) and Latham (1922).

To address the concerns of Gongora *et al.* (2008a) about the potential issues with short sequences (i.e. 201 bp), in our re-analysis a Neighbour Joining tree was generated using 350 base pairs (bp). This was accomplished using a reduced version of the full Muscle Alignment. It contained one representative of each of the unique modern Chilean chicken haplotypes from individuals listed as having Araucana-like morphological features ($n = 7$), 161 of the 169 haplotypes identified by Liu *et al.* (2006), and ancient sequences CHLARA001 (EF535241.2), CHLARA004 (JF433992), HWIKIP005 (JF434015), HWIPLR001 (JF343009) and PAQHAN001 (EF535247.2). A multiple alignment of the major haplotypes represented in the Gongora *et al.* (2008a) analysis and several longer sequences of distinct Pacific haplotypes can be found in Supporting Figure S1.

Table 3. Primers used in the amplification of ancient DNA sequences used in this study.

Name of primer	Direction	Sequence	Binding site on reference sequence NC_01323
GG144	Forward	5' ACCCATTATATGTATACGGGCATTAA	138
GG313R	Reverse	5' AACCATTCATAGTTAGGAGACTTGTT	313
GG218F	Forward	5' CATTACCCCTCCCCATAGACAG	218
G387R	Reverse	5' CGAGCATAACCAAATGGGTTAGA	387
GG316	Forward	5' AACAAGTCACCTAACTATGAATGGTTAC	313
GG582R	Reverse	5' ACAGATAATCCACAGATGACTTCGTG	582
GG582F	Forward	5' CACGAACTCATCTGTGGATTATCTGT	582
G747R	Reverse	5' GGACGCAACGCAGGTGTAGTCCAGGC	747

Sequences were trimmed to the longest shared sequence length. This necessitated cutting the first 171 bases of the Gongora *et al.* (2008a) and Liu *et al.* (2006) datasets. The trimming resulted in the loss of only one SNP from the modern Chilean chickens (CHL31); a C at position 168 (relative to the reference sequence NC_013223): the remaining Chilean chickens all differed from the reference sequence at this position, with a transversion from C to T. This transversion was also observed in 132 of the 169 Liu haplotypes.

The low diversity of chicken mtDNA sequences makes the use of traditional phylogenetic analyses questionable (Soltis & Soltis 2003). However, Liu *et al.* (2006) and Gongora *et al.* (2008a) used Neighbour Joining and Maximum Parsimony methods, respectively, and in samples with low diversity both of these methods have essentially the same rate of misclassification (Woolley *et al.* 2008). To provide comparable phylogenetic results for critical analysis, aligned sequences were uploaded to MEGA 5.1 (Tamura *et al.* 2011) to construct a Neighbour Joining (NJ) tree using a Maximum Likelihood model, and including analysis of transitions and transversions. Using this method, the Liu *et al.* (2006) haplogroups retained their previous haplotypes when reduced by more than 150 base pairs (bp) from that of the original analysis. Previous analyses using shorter datasets and Maximum Parsimony analyses had already proven this to be true when using Liu haplogroups to classify ancient DNA data from the Pacific (Storey *et al.* 2010, 2012).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Alignment of sequences representing the distinct haplotypes in modern Chilean and ancient Pacific/Chilean chickens used in the phylogenetic analysis. The last 15 bases included in the analysis (not shown in this alignment) were identical in all 13 sequences. Numbers are based on the Reference Sequence NC_001323 (Desjardins & Morais 1990). Modern sample CHL31 is identical to the Reference Sequence in this 350 bp region and thus is a suitable standard by which to compare the other sequences in this alignment.

Table S1. Isotope values for modern and archaeological samples of Chilean marine, lacustrine and terrestrial plants and animals. Symbols are used to indicate individual references for the data as follows: •, Falabella *et al.* (2007); ‡, Hückstädt *et al.* (2007); †, Storey *et al.* (2008b); !!, Storey *et al.* (2007).

Table S2. Sample names, geographical and/or morphological provenience, length of sequence and GenBank Accession Number for Chilean and Pacific sequences listed in Table 2 and utilised in the analysis.