An application of zooms to identify archaeological avian fauna from Teotihuacan, Mexico

AN APPLICATION OF ZOOMS TO IDENTIFY ARCHAEOLOGICAL AVIAN FAUNA FROM TEOTIHUACAN, MEXICO

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1. Abstract

The remains of aquatic birds often represent the best surviving evidence for prehispanic lake exploitation in highland Central Mexico, an important center of urban development with vast lacustrine resources. Yet unlike the sustained focus on turkey husbandry in Mesoamerican research, the economic importance of ducks and other lacustrine birds has received little attention. The diversity of birds in Central Mexico presents challenges to species identification from skeletal remains. To overcome these challenges, we present a new application of ZooMS, a collagen-based identification technique, to identify archaeological avian fauna from Teotihuacan. We develop the first database of avian biomarkers to include specimens across multiple taxonomic groups and apply ZooMS on 295 bone fragments to identify fragmentary and unidentified avian remains from the Tlajinga district of Teotihuacan. Our results indicate that ZooMS has good potential to identify

avian fauna to at least the family level and that the residents of Tlajinga exploited a range of aquatic birds.

Keywords: ZooMS, Teotihuacan, aves, birds, collagen, aquatic

2. Introduction

Postclassic (900-1519 CE) communities in Central Mexico heavily exploited aquatic birds, fish and insects from the expansive lacustrine system in the Basin of Mexico (Figure 1, De Lucia, 2021; de Sahagún et al., 1963; Hirth, 2016, p. 28; Parsons, 2010, 2008). However, there is little evidence for how these lake exploitation economies developed over time, or their role in supporting the large, densely settled city of Teotihuacan, which dominated the region during the Classic period (ca. BCE 100 – 550/600 CE) (Sugiyama et al., 2017; Valadez Azúa, 2013; Widmer and Storey, 2016). Aquatic birds provide the most direct evidence for the exploitation of lake resources, as the remains of small lake fish, insects and crustaceans are rarely recovered archaeologically. Yet traditional zooarchaeological identification of birds in Central Mexico is challenging, hindered by large groups of winter migratory birds (Ayala-Pérez et al., 2013; Gamboa et al., 2017; Peterson and Navarro Sigüenza, 2006) and high species diversity in this center of avian endemism

(Howell and Webb, 1995, p. 15). To address these challenges, we explore the potential of palaeoproteomics, and specifically ZooMS (Zooarchaeology by Mass Spectrometry), to identify archaeological birds from Teotihuacan while also developing new collagen peptide biomarkers for North American avifauna.

ZooMS is a collagen-based method for taxonomic identification of animals based on amino acid substitutions, called single amino acid polymorphisms (SAPs), within Type I collagen—the primary organic component of bone (Buckley et al., 2009; Richter et al., 2022; Welker et al., 2015). Collagen is extracted, digested with the enzyme, trypsin, and analyzed on a matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer. The resulting spectra are analyzed against lists of reference peptide markers that vary across taxa. In comparison to aDNA analysis, ZooMS is rapid, low-cost, and requires very little bone, allowing for high-throughput analysis and application to extremely small or fragmentary

remains (Buckley et al., 2016; Richter et al., 2011; Speller et al., 2016; Wang et al., 2021). ZooMS, therefore, provides taxonomic identification where traditional zooarchaeological methods could be limited, especially for fragmentary remains and morphologically similar species.

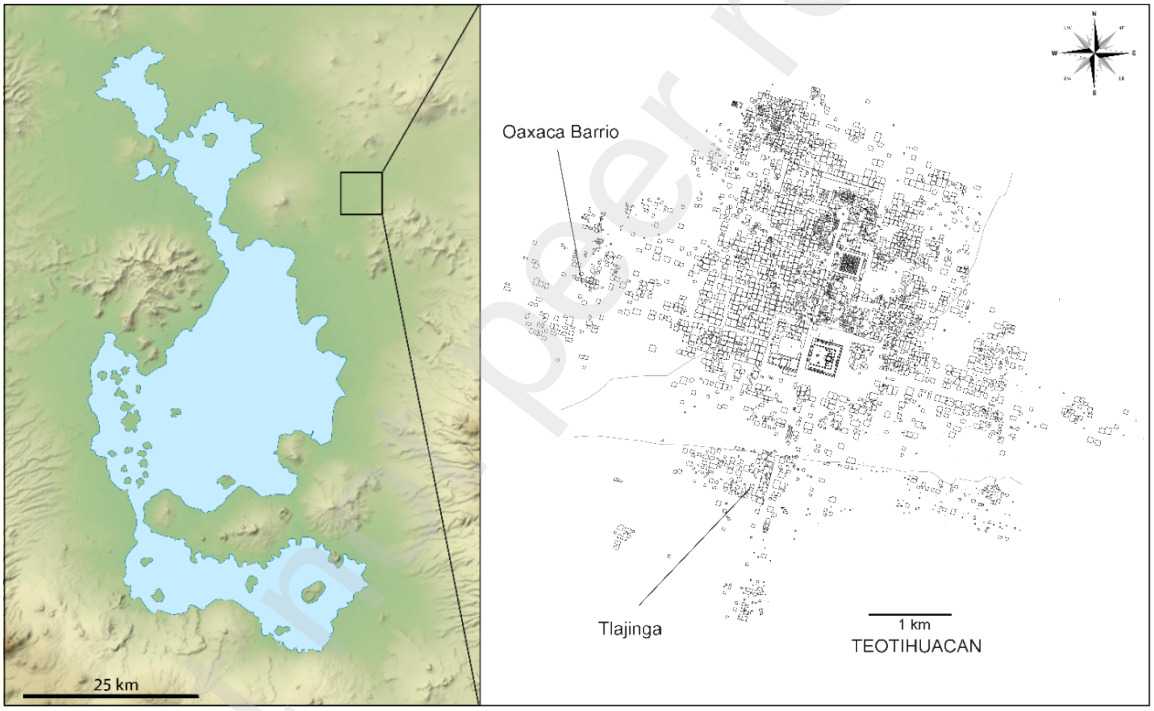

Figure 1. Location of Teotihuacan in the Basin of Mexico. Left: Lake system in Basin of Mexico prior to European contact (after *Lago de Texcoco Posclásico* 2007 by Yavidaxiu, and derivative work by historicair and Sémhur, CC BY-SA 4.0 via Wikimedia Commons). Right: Map of Teotihuacan (after Millon 1973).

While diagnostic markers have been established to discriminate among many types of mammals, fish and reptiles (Buckley and Kansa, 2011; Harvey et al., 2019b, 2018; Janzen et al., 2021; Peters et al., 2021; Richter et al., 2020; Speller et al., 2016; van der Sluis et al., 2014), the application of ZooMS for avian fauna has been minimal. As Eda et. al. (2020) note, this lack of interest stems largely from the slower collagen mutation rate in birds compared to mammals, which limits the number of mutations available to distinguish among taxonomic groupings (Buckley, 2018; Richter et al., 2022). However, despite the slow mutation rate, avian collagen has potential for taxonomic identification at the family (Horn et al., 2019) and sub-family levels (Eda et al., 2020). As of 2022, two peptide markers (COL1α2-502 and COL1α2-889, nomenclature after Brown et al. 2020) had been published that discriminate four domesticated fowl, including mallard duck (*Anas platyrhynchos*) and three members of the Phasianidae family: chicken (*Gallus gallus*), Japanese quail (*Phasianus coturnix*), and turkey (*Meleagris gallopavo*) (Buckley, 2018;

Buckley et al., 2009). Recently, a more detailed study of peptide COL1α2-889 noted two variations useful in discriminating archaeological chicken and indigenous pheasants in Japan (Eda et al., 2020), demonstrating the utility of ZooMS to address specific archaeological questions. However, two genera of indigenous pheasants of the Phasianidae family, *Phasianus* and *Syrmaticus*, were indistinguishable based on this peptide, and the marker used to identify *Phasianus* is also found in other families of birds (Eda et al., 2020). This demonstrates that relying on single peptide markers can limit the usefulness of ZooMS in areas with high avian diversity and highlights the need for further work on characterizing the collagen sequences of avian fauna.

We use these findings as a starting point to examine publicly available collagen sequences and reference modern samples to develop ZooMS markers that discriminate among 15 families, representing nine orders of birds that are frequently found in archaeological sites across the Americas (Figure 2).

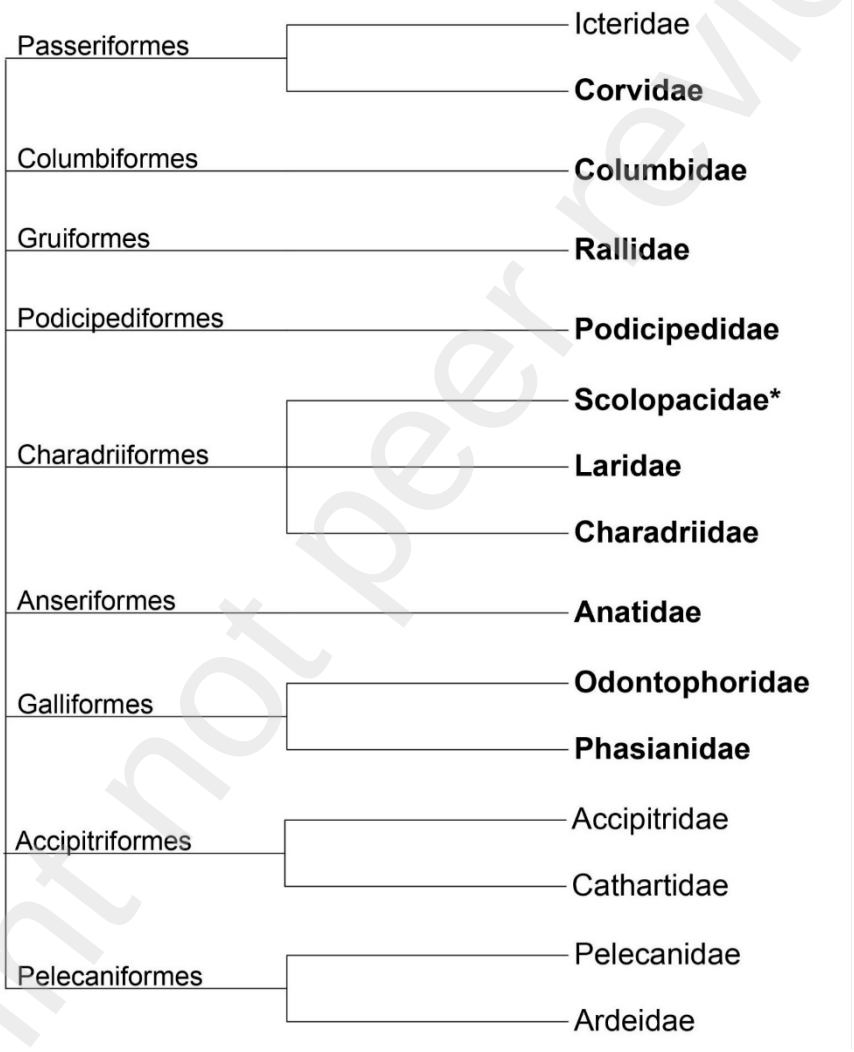

Figure 2. Phylogenetic tree of avian orders and families represented by modern and archaeological specimens (from itol.embl.de, v. 6.5.2, Letunic and Bork, 2021). Individuals from families in bold were analyzed by LC-MS/MS. *Scolopacidae family was identified through comparison of LC-MS/MS sequences to collagen sequences for this family.

3. Site Description: Teotihuacan, Mexico

Teotihuacan was a large, densely populated urban center, and one of the few cities in the Americas to have a population of 100,000 or more prior to European arrival (Figure 1). The city rose around 100 BCE and by its height in 250-550 CE had consolidated a large multiethnic population and presided over a network of communication corridors that connected the city to natural resources, trade goods, and other Mesoamerican cultures (Carballo, 2013; Hirth, 2020; Sanders et al., 1979). Parsons (2010) argued that lake exploitation in the Basin of Mexico would have intensified during this period to support growing urban populations and the expansion of settlements into landscapes that supported maguey (*Agave*) production, but otherwise had limited agricultural potential. Yet current estimates of aquatic resources at Tlajinga indicate waterfowl made up no more than 4% of the animals consumed in the city (Sugiyama et al., 2017, p. 66). On average, aquatic species make up 33% of the birds identified in residential areas of

Teotihuacan, while turkeys alone comprise 45% (Sugiyama et al., 2017, Table ESM1).

Recent excavations at Tlajinga, on the southern periphery of the city, provides new material to examine avian diversity and the importance of aquatic birds to urban subsistence (Carballo et al., 2019). The faunal analysis of these materials is described elsewhere (Codlin, in prep), but in brief, the assemblage contained 613 fragments of bird bone, only 282 of those were identified to a taxonomic group.

4. Materials and methods

4.1. Modern reference samples

Approximately 10-20 mg of bone was sampled from non-diagnostic skeletal portions, primarily ribs of 31 modern North American bird specimens (collections from Boston University and the American Museum of Natural History [AMNH]). The samples included 17 species of bird from 12 families commonly identified in bird checklists and archaeological sites in highland

Central Mexico: Accipitridae, Anatidae, Ardeidae, Cathartidae, Columbidae, Corvidae, Icteridae, Laridae, Odontophoridae, Pelecanidae, Podicipedidae, Rallidae (Table S1). Where possible, multiple individuals from the same species were sampled.

4.2. Archaeological samples

Two-hundred and ninety-five (n=295) archaeological specimens were sampled from Tlajinga, and from a nearby neighborhood, the Oaxaca Barrio (Tlailotlacan). We sampled 259 avian bones from two adjacent apartment compounds at Tlajinga (17:S3E1 and 18:S3E1), including 192 unidentifiable specimens and 67 specimens identified morphologically at least to taxonomic order (e.g. Galliformes). An additional 36 specimens, including five identified minimally to order, were collected from faunal material from salvage excavations at the Oaxaca Barrio (Ortega Cabrera, 2012, 2010, 2009). Approximately 25% of the samples selected from both sites were identified minimally to the level of family. Material from the Oaxaca Barrio is included in

ZooMS analysis, but the archaeological implications are not considered here. Faunal material from both excavations was recovered in the field using 5 mm screens, meaning that small birds, including quails, are likely to be under-represented in the dataset (Tellkamp, 2019). For all archaeological samples, small fragments of bone, weighing 10-50 mg, were removed from non-diagnostic portions of bone for analysis.

4.3. Bird collagen database

Avian collagen sequences were downloaded from UniProt and NCBI; these include data from avian genome sequencing (Feng et al., 2020; Jarvis et al., 2014; Zhang et al., 2014). The sequences were aligned and compared to the reference chicken sequences (COL1A1 - P02457, COL1A2 - P02467) using Jalview (see data at: 10.5281/zenodo.6363113) The sequence data was theoretically digested with trypsin using the Bacollite R package (v. 1.0, Hickinbotham et al., 2020) allowing the following post-translational

modifications: oxidation (+15.9949 Da) of proline and deamidation (+0.9840 Da) of asparagine and glutamine.

4.4. Collagen sequencing and peptide mass fingerprinting

Collagen was extracted and digested using established methods (Brown et al., 2020b; Buckley et al., 2009; Welker et al., 2015). Briefly, samples were incubated in 0.6 M hydrochloric acid (HCl) overnight, washed with 50 mM ammonium bicarbonate (NH~4~HCO~3~) pH 8.0 (AmBic), and incubated briefly in 0.1M of sodium hydroxide (NaOH) before gelatinization in AmBic at 65℃ for one hour and digestion with trypsin. Samples were then diluted and spotted 1:1 with a-cyano-4-hydroxycinnamic acid and analyzed on a Bruker Autoflex Speed LRF MALDI-TOF Mass Spectrometer located at the Max Planck Institute for the Science of Human History, Jena, Germany.

4.5. Marker ID and MS/MS confirmation

A list of candidate marker peaks was generated from visual comparison of the spectra using mMass (v 5.5.0, Strohalm et al., 2010) and differences in the theoretical peptide masses generated from Bacollite. One individual from each pattern of markers identified in the archaeological assemblage (n=14) was analyzed using LC-MS/MS (Lumos Orbitrap, Mass Spectrometry Facility, University of Massachusetts Medical School) to confirm the sequences of candidate markers. Where possible, the matching reference specimens were also analyzed (n=9).

LC-MS/MS data was processed using Byonic (v. 3.4, Bern et al., 2012) allowing for oxidations of methionine, proline, and lysine, deamidation of asparagine and glutamine, and acetylation on N-terminal glutamine and glutamic acid. First, proteins present in the samples were identified using a database consisting of SwissProt (1/20/2022), whole proteomes from 13 species of bird, plus available avian proteins from UniProt (Table S2).

Focused databases for each sample were generated using a protein FDR of 2%. Second, to identify novel collagen peptides, assisted *de novo* sequencing was conducted using error tolerant searching in Byonic against a database containing 13 regions of interest from avian COL1α2-sequences. Third, marker confirmation was conducted against a database consisting of the sequences from the focused databases, excluding COL1 sequences, curated avian COL1 sequences, and novel collagen peptides from *de novo* sequencing. Markers were considered confirmed if there were 2 or more peptide spectral matches with PEP 2D scores below 0.001 with coverage of the SAP locations (Table S3).

4.6. Identification of archaeological samples and clustering

Clustering and sample identification was conducted using R version 4.1.1 (R Core Team, 2021) and the package MALDI-Quant (v 1.2, Gibb and Strimmer, 2012) after validation of the parameters used against mMass. Spectra underwent smoothing, baseline removal, calibration, peak picking,

and deisotoping before filtering for number of peaks between 50 and 90, as peak lists outside of this range correspond to poor quality spectral data. Technical replicates of the resulting spectra were then averaged and the averaged spectrum for each sample was peak picked and deisotoped. A binary matrix that recorded the presence or absence of markers (combining masses of amidated, deamidated, and oxidized versions where possible) was created from the markers confirmed by LC-MS/MS in the peak lists. The matrix was used for hierarchical clustering (stats package, R Core Team, 2021) with 20 groups. Spectra in these groups were then visually analyzed for homogeneity and taxonomic assignment. Low quality spectra which were previously filtered out were then visually inspected to see if taxonomic assignment was possible. The complete MALDI-quant workflow is available at 10.5281/zenodo.6366234. ZooMS data was the integrated with zooarchaeological data to generate Number of Identified Specimens (NISP) and Minimum Number of Individuals (MNI) (Lyman, 2008; Table S5).

5. Peptide biomarkers for avifauna

5.1. Peptide biomarkers and their taxonomic resolution

We confirmed 59 peptide sequence variations across 12 locations using LC-MS/MS, corresponding to 71 MALDI marker peaks for the identification of avian taxa (Table 1 and Table S3). These markers can successfully discriminate among 15 families based upon data from protein sequences and modern and archaeological samples (Table 2). Markers in the Anatidae, Charadriidae, Scolopacidae, Laridae, Podicipedidae, Rallidae, Phasianidae, Odontophoridae, Corvidae, and Columbidae families were confirmed using LC-MS/MS. Markers in Accipitridae, Cathartidae, Pelecanidae, Ardeidae, and Icteridae families are only candidate markers derived from ZooMS spectra and sequence data.

Not all the 71 markers identified are equally useful for identification. As reported in other taxonomic groups, some peptide variants are poorly visualized in the MALDI either for only modern species or both modern and

archaeological. Other variants overlap with each other, other collagen peptides, or common contaminants. For the marker peaks which overlap with common contaminants (*m/z* 1193.6, *m/z* 1566.7, and *m/z* 2108), LC-MS/MS analysis identified none of the contaminant peptides present in any of our samples. Moreover, no highly diagnostic marker peaks overlap with common contaminants, although care should be taken when interpreting the diagnostic peak for turkey, *m/z* 1622.6, which is 1 Da removed from peptide derived from the self-digestion of trypsin at *m/z* 1623.8. In addition, we identified several peptides which are consistently identified as deamidated in the MALDI and LC-MS/MS across all samples (e.g. COL1α2-175 and COL1α2-520). The most distinct peaks that can be used for identification are highlighted in bold in Table 1.

271 Table 1. Peaks for COL1α2 peptide markers identified for birds

Taxonomic ID	Sample #	10-42[b]		175-192[b]	292-309[d]	454-483[c]	502-519	520-555[b]	
Anatidae 1	MC148	3097.4	3113.4	1603.8	1609.7	**2777.3**	1578.8	**3152.4**	**3168.4**
Anas platyrhynchos	MC2	3097.4	3113.4	1603.8	1609.7	**2804.3**	1578.8	**3152.4**	
Anatidae 2	MC123	3097.4	3113.4	1603.8	1609.7	**2804.3**	1578.8	**3152.4**	**3168.4**
Anatidae 3	MC182	3097.4	3113.4	1603.8	1609.7	**2804.3**	1578.8	**3152.4**	**3168.4**
Oxyura jamaicensis	MC16	3097.4	3113.4	1589.8	1609.7	**2804.3**	1578.8	**3152.4**	**3168.4**
Anatidae 4	MC171	3097.4	3113.4	1589.8	1609.7	**2804.3**	1578.8	**3152.4**	**3168.4**
Podilymbus podiceps	MC37	**3106.4**	**3122.4**	1589.8	1596.8	**2804.3**	1578.8	**3152.4**	**3168.4**
Podicipedidae	MC187	**3106.4**	**3122.4**	1589.8	1596.8	**2804.3**	1578.8	**3152.4**	**3168.4**
Gallinula galeata	MC26	3097.4	3113.4	1589.8	1609.7	**2804.3**	1578.8	**3152.4**	**3168.4**
Fulica americana	MC30	3097.4	3113.4	1589.8	1609.7	**2804.3**	1578.8	**3152.4**	**3168.4**
Rallidae	MC110	3097.4	3113.4	1589.8	1609.7	**2804.3**	1578.8	**3152.4**	**3168.4**
Leucophaeus attricilla	MC28	3097.4	3113.4	1603.8	1609.7	**2777.3**	**1552.8**	**3206.5**	**3222.5**
Laridae	MC300	3097.4	3113.4	1603.8	1609.7	**2777.3**	**1552.8**	**3206.5**	
Charadrius semipalmatus	MC20		**3106.4**	1589.8	1609.7	**2777.3**	1578.8	**3156.4**	**3172.4**
Scolopacidae[a]	MC232	**3071.4**	**3087.4**	1589.8	1609.7	**2777.3**	**1608.8**	**3166.4**	**3182.4**
Meleagris gallopavo	MC39	3097.4	3113.4	1603.8	1609.7	**2777.3**	**1622.9**	**3192.5**	**3208.5**
Gallus gallus[a]	MC114	3097.4	3113.4	1603.8	1609.7	**2777.3**	1594.8	**3192.5**	**3208.5**
Colinus viginianus	MC32	3097.4	3113.4	1603.8	1609.7	**2777.3**	1594.8	**3182.4**	**3198.5**
Colinus viginianus	MC207	3097.4	3113.4	1603.8	1609.7	**2777.3**	1594.8	**3182.4**	**3198.4**
Odontophoridae	MC331	3097.4	3113.4	1603.8	1609.7	**2777.3**	1594.8	**3192.5**	
Zenaida macroura	MC23	3097.4	3113.4	1596.8	1579.7	**2804.3**	1578.8	**3150.4**	
Columbidae	MC129	3097.4	3113.4	1596.8		**2804.3**	1578.8	**3150.4**	**3166.4**
Corvus corax	MC349	**3111.4**	**3127.4**	1603.8	**1608.8**	**3166.5**	**1552.8**	**3166.5**	**3182.4**
				Unconfirmed markers					
Accipiter cooperii	MC9	3097.4	3113.4	1603.8	1580*	2804.3	1552.8*	3152.4*	3168.4*
Buteo jamaicensis	MC18	3097.4	3113.4	1603.8	1580*	2804.3	1552.8*	3152.4*	3168.4*
Cathartes aura	MC11	3097.4	3113.4	1589.8	1609.7	2804.3			
Ardea alba	MC13	3097.4	3113.4	1589.8	1596.8*	2804.3	1578.8*	3152.4*	3168.4*
Egretta thula	MC25	3097.4	3113.4	1589.8	1596.8*	2804.3	1578.8*	3152.4*	3168.4*

Quiscalus mexicanus	MC21	3111.4	3127.4	1603.8	1608.8	2777.3	1578.8	3166.5
Pelecanus erythrorhynchos	MC35	3097.4	3113.4	1603.8	1596.8*	2804.3*	1552.8	3152.4* 3168.4*

The most diagnostic and least ambiguous peaks are in **bold**. Non-bolded peaks may be identical to peaks from other peptide locations and should only be used to support identifications based on other markers. The unconfirmed markers are based upon MALDI and sequence data, but have not been confirmed by LC-MS/MS. Labelling of peptides follows Brown et al. (2020a). These markers are all on the COL1a2 chain.

* denotes markers not visible in the MALDI, but expected based on collagen sequences of related taxa.

[a] Taxonomic identification is based on comparison to publicly available collagen sequences. [b] Peak appears most strongly at deamidated version. [c] Flies poorly in MALDI modern specimens. [d] This marker is not consistently identified in LC-MS/MS analysis. However, this marker does fly in the MALDI and often present at identical masses to other markers. Peak appears most strongly at deamidated version. [e] Appears inconsistently in LC-MS/MS and MALDI when no proline oxidation is present but has identical masses to some other markers. [f] Appears inconsistently in LC-MS/MS, however most birds in available collagen sequences have the peptide sequence responsible for peak at *m/z* 1221.6. *m/z* 1225.6 is diagnostic in MALDI and was confirmed in LC-MS/MS.

Table 1. *continued*

Taxonomic ID	Sample #	604-618[f]	625-648[c]	625-653[c]	658-687	757-789	889-906[e]	978-990[b]
Anatidae 1	MC148	1221.6	2108	2466.2	**2511.3**	**2985.5**	1616.8	**1632.8**	**1192.6**
Anas platyrhynchos	MC2		2108	2466.2	**2511.3**	**2969.5**	1616.8	**1632.8**	**1192.6**
Anatidae 2	MC123	1221.6	2108	2466.2	**2511.3**	**2969.5**	1616.8	**1632.8**	**1192.6**
Anatidae 3	MC182	1221.6	2108	2466.2	**2511.3**	**2984.5**		**1632.8**	**1192.6**
Oxyura jamaicensis	MC16	1221.6	2108	2466.2	**2511.3**	**2927.5**		**1660.8**	**1192.6**
Anatidae 4	MC171	1221.6	2108	2466.2	**2511.3**	**2927.5**	1644.8	**1660.8**	**1192.6**
Podilymbus podiceps	MC37	1221.6	2108	2466.2	2497.2	**2927.5**	1550.8	**1566.8**	1220.6
Podicipedidae	MC187	1221.6	2108	2466.2	2497.2	**2927.5**	1550.8	**1566.8**	1220.6
Gallinula galeata	MC26	1221.6	2108	2466.2	2497.2	**2985.5**	1578.8	1594.8	1220.6
Fulica americana	MC30	1221.6	2108	2466.2	2497.2	**2985.5**	1578.8	1594.8	1220.6
Rallidae	MC110	1221.6	2108	2466.2	2497.2	**2985.5**	1578.8	1594.8	1220.6
Leucophaeus attricilla	MC28	1221.6	2108	2466.2	**2531.2**	**2927.5**	1550.8	**1566.8**	1220.6
Laridae	MC300	1221.6	2108		**2531.2**	**2927.5**	1550.8	1566.8	1220.6
Charadrius semipalmatus	MC20	1221.6	2108	2466.2	**2511.3**	**2955.5**	1550.8	**1566.8**	1220.6
Scolopacidae[a]	MC232	1221.6	2108	2466.2	**2559.2**	**2985.5**	1550.8	**1566.8**	1220.6
Meleagris gallopavo	MC39	1221.6	2108	2466.2	**2539.3**	**2927.5**	1578.8	1594.8	1220.6
Gallus gallus[a]	MC114	1221.6	2108		**2539.3**	**2927.5**	1604.8	**1620.8**	1220.6
Colinus viginianus	MC32	1221.6	2108	2466.2	**2539.3**	**2927.5**	1550.8	**1566.8**	1220.6
Colinus viginianus	MC207	1221.6	2108	2466.2	**2539.3**	**2927.5**	1550.8	**1566.8**	1220.6
Odontophoridae	MC331	1221.6	2108	2466.2	**2539.3**	**2927.5**	1550.8	**1566.8**	1220.6
Zenaida macroura	MC23		2108	2466.2	2497.2	**2881.5**	1592.8	**1608.8**	**1192.6**
Columbidae	MC129	**1225.6**	2108	2466.2	2497.2	**2881.5**		**1608.8**	**1192.6**
Corvus corax	MC349	1221.6	**2135**	**2493.2**	**2525.3**	**2927.5**		**1580.8**	1220.6
			Unconfirmed markers						
Accipiter cooperii	MC9	1221.6	2108*	2466.2*	2511.3	2913	1578.8	1594.8	1220.6
Buteo jamaicensis	MC18	1221.6	2108*	2466.2*	2511.3	2913	1578.8	1594.8	1220.6
Cathartes aura	MC11	1221.6			2497.2	2913	1578.8	1594.8	1220.6

Ardea alba	MC13	1221.6	2108*	2466.2*	2511.3	2939	1550.8	1566.8	1220.6
Egretta thula	MC25	1221.6	2108*	2466.2*	2511.3	2939	1550.8	1566.8	1220.6
Quiscalus mexicanus	MC21	1221.6			2511.3	2927.5			1220.6
Pelecanus erythrorhynchos	MC35	1221.6	2108*	2466.2*	2497.2	2927.5	1578.8	1594.8	1220.6

See notes on previous page

Table 2. Summary of taxa analyzed and comparison of morphological and ZooMS identifications

Order	Family	ID type	Morphological identification	ZooMS identification
Anseriformes	Anatidae	Modern	*Anas platyrhynchos*	Anatidae 2
Anseriformes	Anatidae	Modern	*Oxyura jamaicensis*	*Oxyura* sp. (Anatidae 4)
Anseriformes	Anatidae	Archaeological	Anatidae	Anatidae 1
Anseriformes	Anatidae	Archaeological	Anatidae	Anatidae 3
Anseriformes	Anatidae	Archaeological	Anatidae	Anatidae 5*
Anseriformes	Anatidae	Archaeological	Anatidae	Anatidae 6*
Columbiformes	Columbidae	Modern	*Zenaida macroura*	Columbidae
Columbiformes	Columbidae	Modern	*Columbina talpacoti*	Columbidae+
Columbiformes	Columbidae	Archaeological	Columbidae	Columbidae
Charadriiformes	Scolopacidae	Archaeological	Charadriiform	Scolopacidae*
Charadriiformes	Charadriidae	Modern	*Charadrius semipalmatus*	Charadriidae
Charadriiformes	Laridae	Modern	*Leucophaeus attricilla*	Laridae
Charadriiformes	Laridae	Archaeological	Laridae	Laridae
Passeriformes	Corvidae	Archaeological	*Corvus corax*	Corvidae
Passeriformes	Icteridae	Modern	*Quiscalus mexicanus*	Icteridae[2]
Podicipediformes	Podicipedidae	Modern	*Podilymbus podiceps*	Podicipedidae
Podicipediformes	Podicipedidae	Archaeological	*Podiceps nigricollis*	Podicipedidae
Galliformes	Odontophoridae	Modern	*Colinus virginianus*	*Colinus virginianus*
Galliformes	Odontophoridae	Archaeological	Odontophoridae	Odontophoridae
Gruiformes	Rallidae	Modern	*Fulica americana*	Rallidae
Gruiformes	Rallidae	Modern	*Gallinula galeata*	Rallidae
Galliformes	Phasianidae	Archaeological	Phasianidae	*Gallus gallus* *
Galliformes	Phasianidae	Archaeological	*Meleagris gallopavo*	*Meleagris gallopavo*
Accipitriformes	Accipitridae	Modern	*Accipiter cooperii*	Accipitridae+
Accipitriformes	Accipitridae	Modern	*Buteo jamaicensis*	Accipitridae+
Accipitriformes	Cathartidae	Modern	*Cathartes aura*	Cathartidae+
Pelecaniformes	Pelecanidae	Modern	*Pelecanus erythrorhynchos*	Pelecanidae+
Pelecaniformes	Ardeidae	Modern	*Egretta thula*	Ardeidae+

Pelecaniformes	Ardeidae	Modern	*Ardea alba*	Ardeidae[+]

[*] Taxonomic identification is based only on sequence data and similarities to other analyzed taxa, not MALDI reference data. [+] ZooMS identification is estimate as markers are not confirmed by LC-MS/MS

5.1.1. Galliformes

Galliformes includes two families important for urban subsistence at Teotihuacan, Phasianidae and Odontophoridae. Reference samples were collected from *Colinus virginianus* (northern bobwhite) while *Meleagris gallopavo* (turkey) and *Gallus gallus* (chicken) are available in collagen sequence data. While no peptides can clearly discriminate the two families, COL1α2-889, COL1α2-520, and COL1α2-502 allow us to discriminate among *Gallus, Meleagris,* and *Colinus,* as well as other quails of the Odontophoridae family that are present in archaeological data. COL1α2-757 may have the potential to further discriminate among other quail genera present in Central Mexico (*Callipepla*, *Dendrortyx* and *Cyrtonyx*), but we have not been able to unambiguously confirm the marker without further reference material.

5.1.2. Charadriiformes

Charadriiformes is a diverse order of aquatic birds, and many species are found within the five families of this order common in Central Mexico:

Laridae, Scolopacidae, Charadriidae, Recurvirostridae and Jacanidae (Peterson and Navarro Sigüenza, 2006). Reference samples were collected for species from Laridae and Charadriidae, while collagen sequences are available for species in Charadriidae and Scolopacidae. The samples from these three families are distinguished by variations across six peptide locations: COL1α2-10, COL1α2-175, COL1α2-502, COL1α2-658, COL1α2-757, and COL1α2-520. Given that only one species from each group was sampled, these peptide markers may be specific to genus or species within these families, and there does not appear to be any peptide marker specific to this order of birds.

5.1.3. Anseriformes

Anatidae (duck, geese and swans) are the only family from Anseriformes present in Central Mexico (Howell and Webb, 1995) and we identified the greatest diversity of markers among this family. The most common genera in our study region are *Anas*, *Oxyura*, *Spatula*, *Aythya*, and

Mareca, while *Anser*, *Mergus*, and *Bucephala* may have been more common in the past (Ayala-Pérez et al., 2013; de Sahagún et al., 1963, pp. 26, 34–38, 57; Howell and Webb, 1995). Reference samples were acquired from *Anas* and *Oxyura* and sequence data are available for *Anas*, *Oxyura*, and *Anser*. Four main marker locations are important for Anatidae: COL1α2-658, COL1α2-889, COL1α2-454, and COL1α2-757. We identify six archaeological duck groups that are distinguishable based on various combinations of these four peptides (Figure 3). *Anas* and *Oxyura* are distinguished by differences in COL1α2-889 and COL1α2-757. *Anser* and *Anas* are distinguished at peptides COL1α2-658 and COL1α2-889.

The peptides identified in Anatidae 2 closely match *Anas platyrhynchos*. Archaeological specimens in this group likely include the closely related *Anas diazi* (Mexican duck), which was common in the region (Ayala-Pérez et al., 2013), and may also include other taxa in the *Anas* genus or dabbling ducks of the Anatini tribe, such as *Spatula* and *Mareca*. Markers

for Anatidae 4 are identical to *Oxyura jamaicensis* (ruddy duck) which is the only species of its tribe found in Central Mexico (Howell and Webb, 1995, pp. 172–3). It is likely, then, that all archaeological specimens identified as Anatidae 4 belong to *Oxyura jamaicensis*. Further reference samples are required to identify Anatidae groups 1, 3, 5, and 6, although the presence of peak *m/z* 2497.5 for COL1α2-658 in Anatidae 5 and 6 suggests they could be types of geese, based on sequence data for *Anser.*

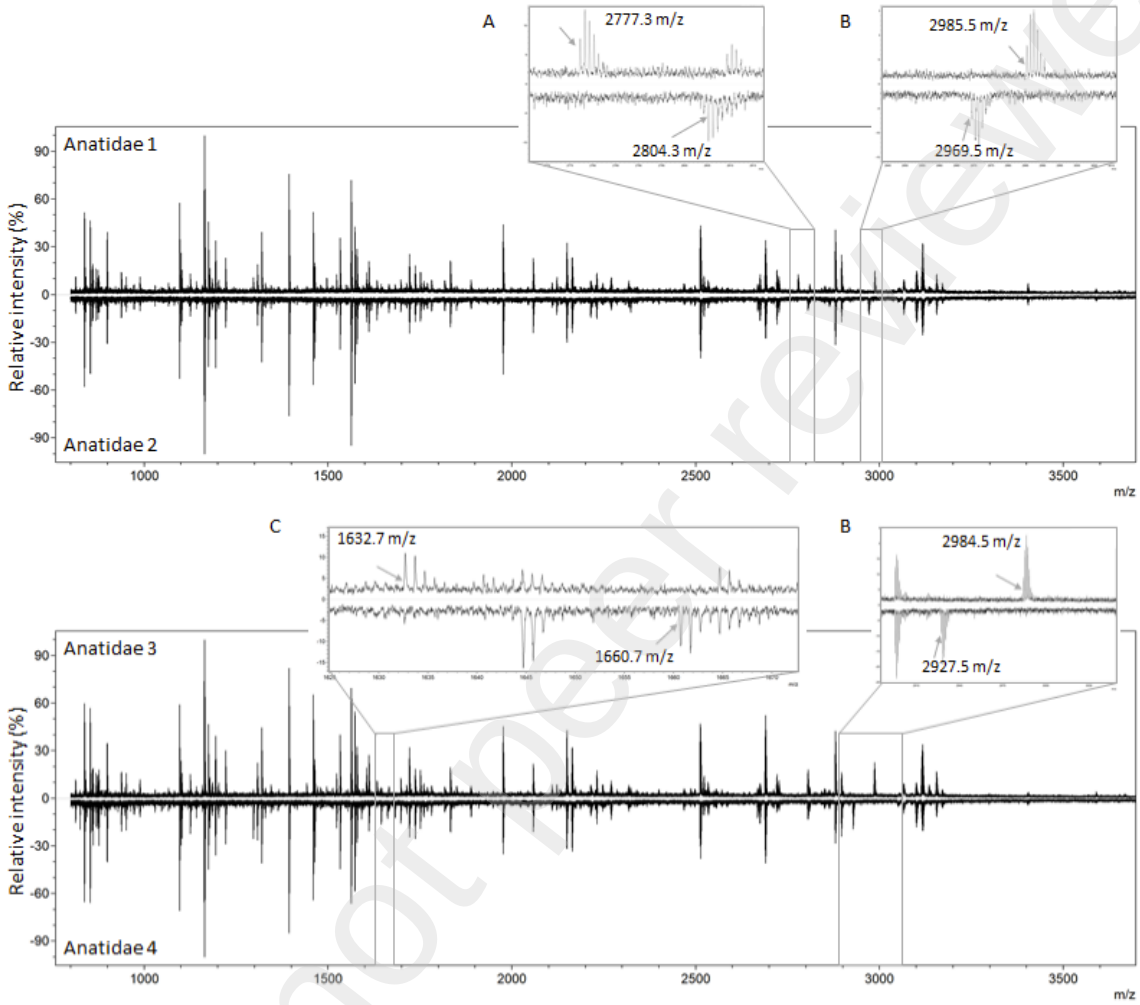

Figure 3. Examples of peptide markers that distinguish four groups of Anatidae. Anatidae 1 and 3 are not identified to taxonomic group, while Anatidae 2 is identical to *Anas platyrhynchos* and Anatidae 4 is identical to *Oxyura jamaicensis*. A) the difference between the COL1 α 2-454 marker at *m/z* 2777.3 for Anatidae 1, and *m/z* 2804.3 found in the other Anatidae groups. B) unique peaks for each Anatidae group among the COL1 α 2-757 marker—Anatidae 1: *m/z* 2985.5, Anatidae 2: *m/z* 2969.5, Anatidae 3: *m/z* 2984.5, and Anatidae 4: *m/z* 2927.5. C) the difference between the COL1 α 2-889 marker at *m/z* 1660.7 in Anatidae 4 and *m/z* 1632.7 that is present in other ducks of the Anatidae family.

5.1.4. Gruiformes, Podicipediformes, Columbiformes, and Passeriformes

Gruiformes, Podicipediformes and Columbiformes are each represented by two species from one family: Rallidae, Podicipedidae, and Columbidae respectively (Table 2). While Podicipedidae and Columbidae are the only families from these orders present in the Basin of Mexico, Rallidae is the most diverse family of three Gruiformes (Peterson and Navarro Sigüenza, 2006). Although specimens in each order are distinguished from other birds based on multiple markers, species within each family are indistinguishable from each other. Based on the large numbers of markers that distinguish families within other orders studied here, the samples from Rallidae are likely to be distinct from other families in the order Gruiformes.

Passeriformes are the largest order of birds with upwards of 140 families. An archaeological specimen for *Corvus corax* (raven) of the Corvidae family is similar to *Quiscalus mexicanus* (Mexican grackle), except for the marker at COL1α2-658. Compared to other taxa in the study, however, *Corvus*

and *Quiscalus* have the highest number of unique markers and many more variations among passerine families are visible in the available collagen sequences.

5.1.5. Pelecaniformes and Accipitriformes

Pelecaniformes and Accipitriformes are represented by multiple reference samples but were not submitted for LC-MS/MS analysis as the peptide marker patterns were not observed in any archaeological samples. MALDI peaks observed in these samples are presented in Table 1, but these unconfirmed peaks are not included in the biomarker list for identification of taxa. Accipitridae is the largest of three families of Accipitriformes in Central Mexico, including Cathartidae and Pandionidae (Peterson and Navarro Sigüenza, 2006). While *Buteo* and *Accipiter* from Accipitridae cannot be separated by MALDI marker, Accipitridae and Cathartidae are distinguished from each other by markers at COL1α2-175 and COL1α2-658. Similarly, among the Pelecaniformes, Ardeidae is the largest of three families common

in Central Mexico, also including Pelecanidae and Threskiornithidae (Peterson and Navarro Sigüenza, 2006). *Egretta* and *Ardea* in the Ardeidae family are indistinguishable, while Ardeidae are distinct from Pelecanidae at multiple markers.

5.2. Comparison to established peptide markers

The overall mutation rate of avian collagen is lower than mammals. However, several peptide regions appear to have particularly high variability, meaning avian taxonomic groups can be identified more effectively than predicted by the slower mutation rate. Peptides COL1α2-520 and COL1α2-757, for example, both have at least five locations of SAPs in their respective 36- and 33-number amino acid sequences. These combinations of variants correspond to seven unique peptide sequences for each marker location that was confirmed with LC-MS/MS. While COL1α2-889 is a shorter peptide with only 18 amino acids, SAPs occur in three locations and correspond to eight unique peptide sequences. Overall, the taxonomic resolution achievable in

birds appears to be only slightly less than that of mammals. For example, five markers have been identified that distinguish taxa within the family Bovidae (Janzen et al., 2021), while we identified four peptide markers that distinguish taxa within the family Anatidae.

COL1α1 508-519 has been previously noted a useful marker to discriminate broad groups of mammals (Buckley et al., 2014). This peptide appears conserved across all birds in this study, present at *m/z* 1162. However recent research suggests this marker and amino acid sequence is also shared with Australian marsupials and some reptiles (Harvey et al., 2019a; Peters et al., 2021).

As in mammals, most of the diagnostic bird biomarkers are identified from COL1 α 2. Some potential markers were identified on COL1 α 1, but these are not presented as they could not be consistently confirmed by LC-MS/MS. Of the avian markers identified here, all but two are homologous to locations where biomarkers are identified in the collagen sequences of mammals or

fish: COL1α2 454, COL1α2 502, COL1α2 757, and COL1α2 978 (Pep E, C, G and A, Buckley et al., 2009); COL1α2 292 (P2, Buckley et al., 2014), COL1α2 10 (Pep 9, Buckley et al., 2016), COL1α2 889 (Janzen et al., 2021, although first described as a bird marker by Buckley, 2018 and Eda et al. 2020), COL1α2-604 (Harvey et al., 2019a), COL1α2-625 (Harvey et al., 2018), COL1α2-658 (Richter et al., 2020). These locations seem to have good discriminant ability across animal types. While Wang et al. (2021) noted COL1α2 175 as a non-diagnostic marker shared by mammals, this marker does discriminate among birds, although it overlaps with identical masses from other markers. One novel marker presented here, COL1α2-520-555, is highly diagnostic for avian identification, but should be confirmed through visual inspection of spectra. This marker is often missing in modern specimens and as the peak is broad with a large isotope distribution, the correct peak in the distribution is often not properly identified during

automatic peak picking and deisotoping, resulting in being incorrectly identified 1-2 Da off.

6. Analysis of archaeological samples

6.1. Taxonomic discrimination via clustering

All 31 modern reference specimens produced high quality spectra and the peaks picked were consistent across replicates and individuals. Modern and archaeological samples were assigned to one of 20 groups based on hierarchical clustering of marker peaks (Figure 4, see also Figure S1 and Table S1). Two-hundred eighty-five of the 295 archaeological samples clustered into groups, while 10 samples were removed by the screening process. In contrast, only 5 of the 31 reference specimens passed initial screening due to higher rates of noise in spectra for modern samples. However, these parameters were kept due to the excellent discrimination of archaeological samples in the clusters. Four of the five reference specimens clustered where expected, while one sample clustered separately from archaeological

samples. Identification of samples within each group was independently verified by visual inspection of marker peaks and further identification of taxa within clusters was possible.

Six of the 20 clusters contained a total of 30 archaeological samples matching established mammal biomarkers, while Corvidae, Columbidae, Rallidae, Podicipedidae, and Scolopacidae each clustered separately. Laridae clustered apart, with the only erroneous clustering of a non-related sample (MC306, identified as passeriform). Galliformes clustered together with one distinct sub-grouping of *Meleagris*, and another distinct group comprising both Odontophoridae and *Gallus*. Three clusters contained Anatidae. Cluster 1 largely contained specimens from Anatidae 1, while Cluster 9 grouped all Anatidae 4 samples. Cluster 5 contained two sub-groups, which broadly separated Anatidae 1 and 3.

Eleven samples produced high-quality spectra but were not identifiable to a taxonomic group by visual inspection. Each of these samples presented

the peak at *m/z* 1162, common to all birds and additional avian biomarkers. However, these 11 samples represent ten unique peak lists and group across multiple taxonomic clusters (Figure 4). This suggests there could be ten distinct taxa yet to be identified in this assemblage. Of the ten archaeological samples that were excluded prior to clustering, one was identified as *Meleagris*, and nine were too poor to identify.

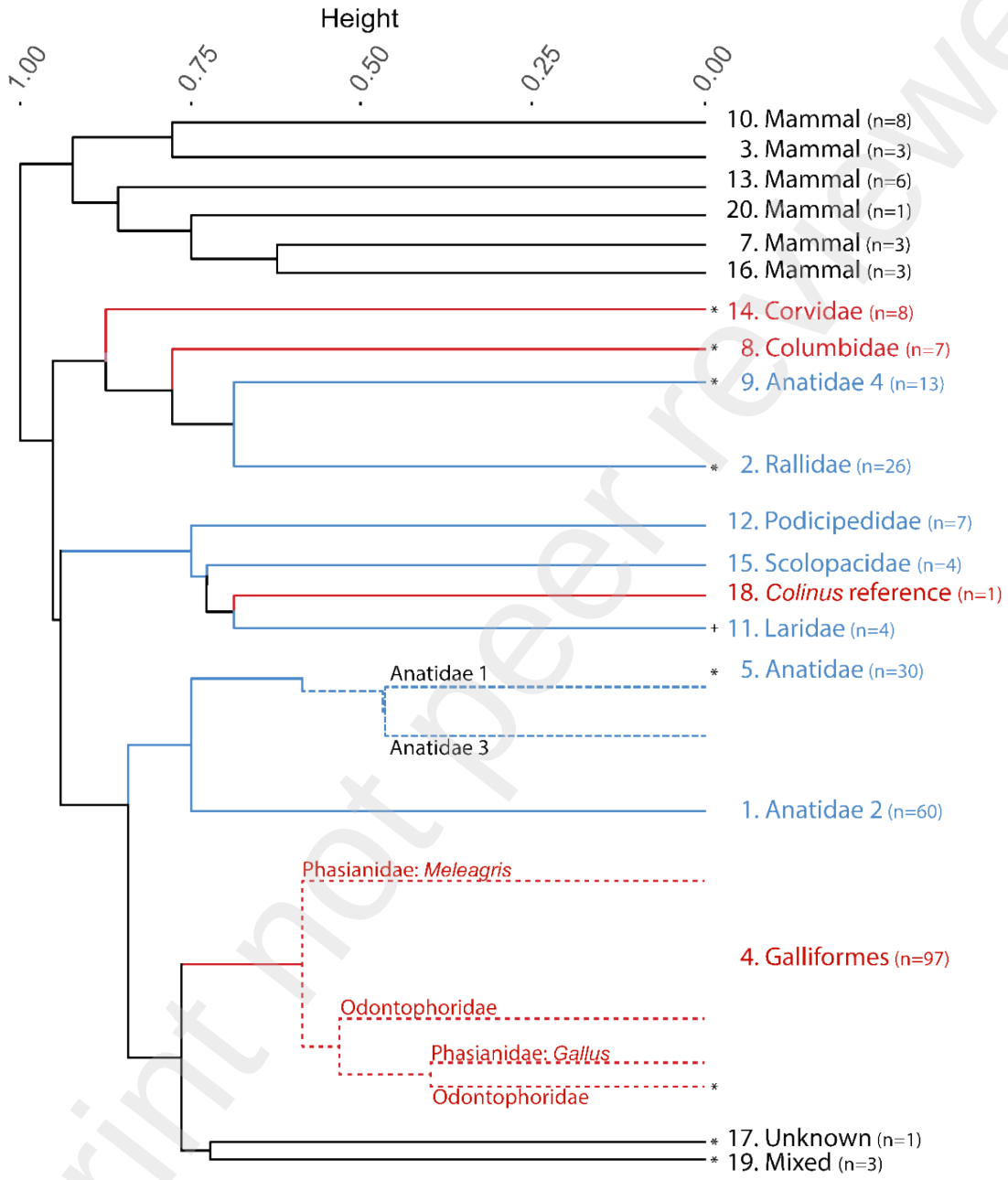

Figure 4. Schematic of hierarchical clustering results for archaeological samples. Solid lines represent assigned clusters while dashed lines indicate where additional groupings were observed within assigned clusters. Aquatic birds are shown in blue and terrestrial birds in red. * denotes presence of unknown taxa, while + denotes incorrect classification at order or family level. See detailed clustering diagram in Figure S1.

This clustering achieved the highest accuracy when limiting variation among spectra. Averaging replicates analyzed at multiple dilutions and removing poor spectra prior to clustering greatly improves assignment of samples to the correct taxonomic group. Moreover, both accuracy and precision is improved by matching to carefully curated peak lists and combining the presence of markers with deamidated peaks and varying number of proline oxidations. Our clustering demonstrates the utility of this workflow to rapidly assign a large number of archaeological samples, each with multiple replicates, to broad taxonomic groups.

6.2. Identifications and collagen preservation at Tlajinga

Some differences were observed between the morphological and collagen-based identifications (see Table S1). Thirty specimens were identified as mammals and an additional nine had avian identifications that differed from their morphological identification. Given the difficulties in

identifying small mammals and birds from fragmented specimens, these are certainly errors in morphological analysis (Driver, 2011; Wolverton, 2013). Nevertheless, the archaeological sample was not selected randomly, and the high number of erroneous morphological identifications is partly due to sampling bias towards less confidently identified fragments. Moreover, these discrepancies highlight, as others have argued, the importance of checking the quality of zooarchaeological identifications using molecular methods, particularly for difficult to identify taxa (Driver, 2011; Horsburgh et al., 2016; Speller et al., 2016).

Nine carbonized bone specimens produced low quality spectra, likely because collagen begins to break down around 70℃. However, at least 24 other samples with evidence of burning or partial carbonization produced identifiable spectra, suggesting that heat exposure does not completely rule out ZooMS analysis because of differential temperatures reached at different parts of the bone during burning. As ZooMS requires intact peptides, but less

overall collagen than isotopes or radiocarbon analyses (Harvey et al., 2016; Wang et al., 2021), more research is needed to establish the level of heat exposure tolerance. That all non-carbonized samples (n=262) produced spectra suitable for taxonomic identification suggests that the collagen preservation at Tlajinga is very good and that archaeological avian bones can readily produce successful spectra using ZooMS.

6.3. The archaeology of birds at Tlajinga

ZooMS identifications of elements were incorporated into the existing zooarchaeological data from Tlajinga to calculate site NISP and MNI for bird taxa (Figure 5, see also Table S5). Overall, 18 avian taxa were identified in the Tlajinga assemblage, while an additional 10 taxa remain unidentified. The total number of identified bird elements at Tlajinga increased from 280 to 384. While the MNI increased from 33 to 44, 10 of these additional birds represent the 10 unidentified taxonomic groups. Therefore, the MNI pre and

post ZooMS analysis changed very little, highlighting that the new MNI is more representative of taxonomic diversity rather than taxonomic abundance.

The integrated results from ZooMS and morphological identifications (Figure 5) confirm that waterfowl are the most common type of bird identified at Tlajinga and, together with other aquatic birds, make up almost 50% of the assemblage by NISP and 36% by MNI. Overall, these results are consistent with elsewhere at Teotihuacan, were aquatic birds make up roughly a third of the avian assemblage by MNI (Sugiyama et al., 2017). Turkeys, however make up a much smaller proportion of the avian assemblage at Tlajinga compared to the Teotihuacan average. Together, this suggests that aquatic taxa may have been more important to urban subsistence at Tlajinga than other areas of the city, which may have placed greater reliance on terrestrial resources like turkeys.

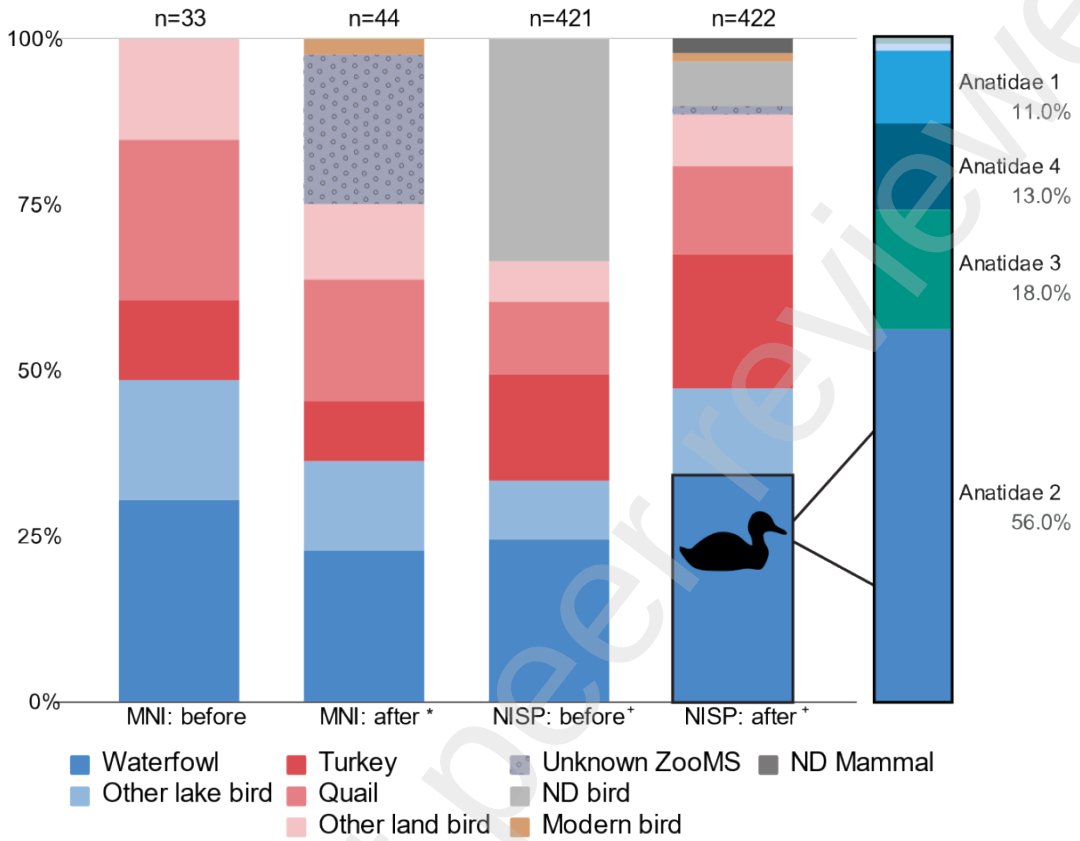

Figure 5. Relative abundance of all avian taxa identified at Tlajinga before and after ZooMS analysis. Call out presents relative proportions of the six Anatidae distinguished by ZooMS. * Includes taxa identified from shaft fragments, + includes only bones identified to element.

6.3.1. Taxonomic diversity at Tlajinga: aquatic taxa

At Tlajinga, eleven aquatic bird taxa were identified by ZooMS and morphological analyses, including six members of the Anatidae family, two members of the Rallidae family, *Fulica americana* (American coot) and *Gallinula galatea* (common gallinule), as well as examples of Laridae (gulls),

Scolopacidae (sandpipers), and Podicipedidae (grebes). Four Anatidae groups are represented by multiple specimens (Figure 5). Anatidae 2, which includes taxa in the *Anas* genus, is the most abundant of the ducks identified by ZooMS at Tlajinga (56% of Anatidae by NISP), followed by Anatidae 3 (18%), Anatidae 4, identified as *Oxyura jamaicensis* (13%), and Anatidae 1 (11%). Anatidae 1 and 3 make up 11% and 18% respectively of the ducks identified with ZooMS. Anatidae 5 and 6 are rare, each represented by a single specimen.

As Anatidae are typically not identified beyond family level at Teotihuacan (Sugiyama et al., 2017, Table ESM1), the identification of at least six Anatidae taxa provides additional information into exploitation of lake resources at the city. Understanding taxonomic diversity of aquatic birds in urban and rural settlements provides an opportunity to examine changes in specialization or diversification of the production and distribution of lake resources throughout Central Mexico. Our results demonstrate that the

residents of Tlajinga were exploiting a wide range of aquatic birds, including multiple types of ducks whose habitats and behaviors vary across species. This suggests that the production and distribution systems that contributed to the faunal assemblage at Tlajinga may have employed a diversified strategy, exploiting a wide range of animals, rather than specializing in the acquisition of particular taxa. This also supports the argument that other lake resources, such as small fish and insects, may also have played an important role in urban subsistence during the Classic period (e.g., Parsons, 2010; Widmer and Storey, 2016).

Moreover, many aquatic birds are migratory, while others, including *Oxyura jamaicensis* and *Anas diazi,* breed in Central Mexico year-round. Careful examination of the relative abundance of Anatidae species via ZooMS alongside other paleo-proteomic techniques such as peptide-based identification of eggshells (Demarchi et al., 2020; Presslee et al., 2017; Stewart et al., 2014), provides new opportunities to examine the seasonality

of lake exploitation and the implication of intensive lake exploitation on avian populations in the past.

6.3.2. Taxonomic diversity at Tlajinga: terrestrial taxa

Seven types of non-aquatic birds were identified at Tlajinga from the combined ZooMS and morphological analysis, including Columbidae (pigeons and doves), Odontophoridae (New World quails), *Colinus virginianus*, *Meleagris gallopavo*,*Corvus corax, Gallus gallus,* and *Accipiter cooperii* (Cooper's hawk). Compared to morphological identifications, ZooMS provided additional discrimination potential for Phasianidae and Odontophoridae families. In addition to *Meleagris gallopavo*, we identified specimens that closely match theoretical peaks for *Gallus gallus*. Domestic chickens were introduced to Central Mexico after European arrival and these specimens derive from an area of the site with early colonial features. The discovery of this taxon in deposits below these features provides additional information to assess the location and extent of colonial period deposits at Tlajinga.

ZooMS also provides the ability to discriminate between *Colinus virginianus* and other quails of the Odontophoridae family. While this result was unexpected and deserves further analysis, the ability to discriminate between these taxa presents an opportunity to investigate the exploitation of quails in Central Mexico. Some Central Mexican quails prefer more open woodland or grassland environments (*Colinus* and *Cyrtonyx*), while others prefer denser forested environments (*Dendrortyx*) (Howell and Webb, 1995, pp. 226, 229, 231). Greater taxonomic precision through ZooMS could be employed to understand environmental and agricultural shifts within the valley, while integration with proteomic techniques for eggshell identification would provide a means to examine the hypothesis that *Colinus virginianus* were raised for urban consumption at Teotihuacan (Widmer and Storey, 2016).

Finally, the ten unidentified taxa highlight that rare avian taxa are underrepresented in traditional zooarchaeological analysis. Rare taxa may not

have major implications for understanding urban subsistence but can provide additional insight into birds acquired for other reasons, such as their colorful plumage, bird song, or symbolic meanings especially when combined with pictographic or historical references such as the murals recently excavated at civic-ceremonial structures in Tlajinga (Carballo et al., 2021).

To address these new avenues for research, the integration of zooarchaeological and ZooMS data needs to be considered carefully in project planning. Initially, we did not expect to discriminate among Anatidae, and so specimens that were identified confidently to Anatidae were not selected for ZooMS analysis. Consequently, the relative abundances of Anatidae groups shown in Figure 5 are estimates of abundance, as morphological identifications in the non-ZooMS assemblage may be biased towards one taxon or another. For larger projects that require confident assessments of relative abundance, it may be productive to choose fewer skeletal elements (i.e., the most robust limb bones) and to analyze all

identified and unidentified specimens in the collection. With further research on reference materials, we expect that our ability to discriminate among birds with peptide mass fingerprinting will increase and some of the less specific identifications of taxa in this study may be refined.

7. Conclusion

With the confirmation of a 71-MALDI-marker panel across 12 peptides, we have demonstrated that ZooMS is suitable for identification of avian taxa from archaeological contexts to at least the level of family. The slower mutation rate of avian collagen appears to be offset by high variability in particular regions, meaning that ZooMS can also identify birds to sub-family levels. While Buckley (2018) and Eda et al. (2020) previously noted differences that discriminate among members of the Phasianidae family, we demonstrate that sub-family differences are also possible among Odontophoridae and Anatidae.

Using ZooMS we revealed higher avian taxonomic diversity from the faunal assemblage at Tlajinga, Teotihuacan during the Classic period including the exploitation of a range of aquatic birds. Combining ZooMS with morphology allowed for an increased recovery of rare taxa which are often underrepresented in reference collections and thus less frequently identified compared to commonly exploited taxa. To fully take advantage of ZooMS, the ability to integrate ZooMS into traditional zooarchaeological analyses such as NSIP and MNI should be considered. Going forward, the enhanced ability to identify avian remains to the family and subfamily level has great potential to elucidate a wide range of societal topics, from urban provisioning systems, seasonality of settlements or hunting activities, and past environmental changes.

8. Data accessibility

MALDI raw data and MS2s for confirmed biomarker sequences is available through Zendo at https://doi.org/10.5281/zenodo.6363114. MS/MS data is available through ProtoemeXchange (PXD034547) through MassIVE (MSV000089660) at DOI: https://doi.org/10.25345/C5N29PB27. R code for MALDI analysis and clustering is available through Zenodo at https://doi.org/10.5281/zenodo.6366234. All other data are included in the manuscript and/or supplemental materials.

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Author contributions:

M.C.: Conceptualization, Writing - Original Draft, Formal Analysis, Visualization, Investigation, Funding Acquisition

K.D.: Writing, Writing - Review & Editing, Funding Acquisition

K.R.: Conceptualization, Writing - Original Draft, Writing - Review & Editing, Visualization, Investigation

Competing Interest Statement: The authors declare that they have no competing interests.

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