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High-throughput microCT and ZooMS collagen fingerprinting of Scombrid bone from the Marquesas Islands

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ABSTRACT

Collagen peptide mass fingerprinting of archaeofaunal remains, or Zooarchaeology by Mass Spectrometry (ZooMS), has increasingly established itself as a valuable tool for improving our understanding of highly-fragmented faunal assemblages. Although there have been developments in sampling strategies that have attempted to reduce damage to precious archaeological specimens, these tend to yield spectra inferior in quality to those yielded by destructive approaches. Also, in an effort to mitigate the impacts on faunal assemblages, researchers are beginning to turn to microCT for the digital preservation of specimens, as this enhances their value for further studies. Here we combine ZooMS and microCT, in application to over one hundred scombrid remains from the Hanamiai site in the Marquesas, which spans the time period from initial human colonization by Polynesians at ca. AD 1250 until around 1900. Almost all of the hypurals that yielded collagen fingerprints (71 of 73) were confirmed as skipjack. The results suggest striking continuity over time in fishing practices with an inferred emphasis on fishing strategies involving sailing canoes being present from the time of initial settlement. The focus appears to be on daytime fishing for skipjack rather than on night fishing for yellowfin, kawakawa and dogtooth tuna as might otherwise have been expected.

1. Introduction

The discovery and human settlement of islands in the Pacific Ocean counts among the world's most extraordinary achievements (Kirch, 2017). It was accomplished with sophisticated outrigger and double-hull sailing canoes guided by skilled seafarers (Howe, 2006). The same seafaring ability allowed Pacific Islanders to feed their populations by fishing, as is well documented by zooarchaeological studies investigating both the kinds of fishes exploited and fish capturing strategies (e. g. Butler 1994; Leach and Davidson, 1988; Weisler and Green 2013). Among the most demanding fishing strategies are those for capturing tunas, especially the large, fast pelagic species (Nordhoff, 1930). Because many of the tunas are far-ranging migratory species, the same zooarchaeological approaches developed for investigating ancient Pacific fishing cultures are also applicable on a global scale. Here we introduce one such approach involving an innovative combination of collagen peptide mass fingerprinting and X-ray imaging (microCT). We demonstrate the potential of this multidisciplinary method through a case study of the Hanamiai archaeological site (Rolett, 1998), which

documents continuous Polynesian occupation of the Marquesas Islands from initial human settlement until the time of European contact.

The Scombridae are a family of mackerel, tuna, and bonitos, including some of the largest bony fish in the world's oceans (Elliott and Ward, 1995). Scombrids live in tropical and temperate waters throughout the world. They comprise 54 carnivorous species divided into 15 genera and two subfamilies (Collette and Nauen, 1983). Pacific scombrids exist in three distinct habitats: oceanic, neritic (shallows), and reef waters (Anderson, 2013a). Oceanic scombrids including species such as yellowfin (*Thunnus albacares*) and skipjack tuna (*Katsuwonus pelamis*) usually live and hunt in deep offshore waters. By contrast, neritic scombrids, including mackerel and smaller tunas such as kawakawa (*Euthynnus affinis*), tend to live and hunt in shallow coastal waters (Froese and Pauly, 2017). Scombrids associated with reefs are, as the name suggests, fishes that predate reef habitats; only double-lined mackerel (*Grammatorcynus bilineatus*) and dogtooth tuna (*Gymnosarda unicolor*) are closely associated with Pacific reef environments (Bagnis et al., 1984; Tinker, 1978).

Isolation limited the natural introduction of neritic scombrids, while

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having a minimal effect on the presence of oceanic scombrids. Thirty-two species of Scombridae, in 14 genera, are found in the Pacific (Table 1), however regional biodiversity declines from west to east as a product of the increasingly remote landmasses (Kirch, 2017). This can be seen by comparing the biodiversity of West Polynesia and French Polynesia. West Polynesia has 17 species of Scombridae (eight neritic, two reef, and seven oceanic) while French Polynesia, which lies further east, has only seven (one neritic, one reef, and five oceanic) (Collette and Nauen, 1983; Froese and Pauly, 2017). The total number of species is diminished by more than one half, but the relative frequency of oceanic varieties is increased. Thus, while overall scombrid biodiversity drops from west to east, the proportion of oceanic to neritic species scales in reverse.

While scombrids are found in three habitats (oceanic, neritic, and reef), ecologically, they can be split into four groups reflecting differences in their predatory behavior: oceanic, partially oceanic, neritic, and “loner.” Oceanic scombrids are schooling fishes primarily found in offshore waters of the open sea. Typical examples of “pure” oceanic scombrids include the yellowfin and skipjack tunas (Collette and Nauen, 1983). In French Polynesia, skipjack occur in greater numbers and in larger schools than yellowfin (Bagnis et al., 1984:319). Partially oceanic and neritic scombrids are also schooling species. Although partially oceanic scombrids generally live offshore, they occasionally chase prey fish into lagoons and other inshore waters. Kawakawa is an excellent example of a partially oceanic scombrid (Bagnis et al., 1984:324). Neritic scombrids are typically smaller fishes, especially mackerels, inhabiting shallow, inshore waters (Collette and Nauen, 1983). Finally, the loosely labeled “loner” scombrids are typified by their predatory loner ecology; they tend to school in small groups of less than five fish

and are relatively rare finds. Wahoo (*Acanthocybium solandri*) is the best known loner scombrid (Bagnis et al., 1984:317), although others encountered in the eastern Pacific include the dogtooth tuna (Bagnis et al., 1984:325).

1.1. Scombrid remains for archaeological research in the Pacific

Archaeological research in the Pacific increasingly draws upon the analysis of fish vertebrae (Lambrides and Weisler 2015). For Scombridae, diagnostic morphological features of the ural vertebrae – the last eight to twelve vertebrae in the scombrid vertebral column – can be used to identify bones to the family level. In addition to being morphologically unique, the ural vertebrae are unusually robust, reflecting their role in facilitating the powerful swimming motion produced by the scombrid tail (Jawad et al., 2013:104). This robustness makes them resilient to taphonomic processes that, over time, might otherwise destroy or fragment the vertebrae.

Among the ural vertebrae, the hypural (also called the ultimate vertebrae) is particularly valuable, both for identifying the taxonomic family and for estimating the minimum number of individuals (MNI) represented. The hypural is the last vertebra in the scombrid vertebral column (Fig. 1); it has a centrum on the anterior side and flattens out into a fan-like plate on the posterior side to facilitate the connection of the tail’s caudal rays (Froese and Pauly, 2017). Several characteristics of the hypural contribute to its value in the analysis of archaeologically excavated fish bone assemblages: 1) as with other ural vertebrae, the robustness of the hypural allows it to survive a broad scope of taphonomic scenarios; 2) unlike the other ural vertebrae, the hypural is a non-repeating skeletal element, making it an ideal indicator of MNI; 3)

Table 1
Habitat types and regions of known scombrids across the Pacific.

Habitat	Name: Scientific - Common	Region:								
		IS	SA	MI	WP	FP	EI	HI	NZ	
Neritic	<i>Auxis rochei</i> - Bullet tuna ^{2,3,6}	✓	✓	✓	✓	–	–	–	–	✓
	<i>Auxis thazard</i> - Frigate tuna ^{2,3,6}	✓	✓	✓	✓	–	–	–	✓	✓
	<i>Cybiosarda elegans</i> - Leaping bonito ³	✓	✓	–	–	–	–	–	–	–
	<i>Euthynnus affinis</i> - Kawakawa ^{1,3,4,7}	✓	✓	✓	✓	✓	–	–	✓	–
	<i>Rastrelliger brachysoma</i> - Short mackerel ³	✓	✓	✓	✓	–	–	–	–	–
	<i>Rastrelliger faughni</i> - Island mackerel ³	✓	✓	✓	–	–	–	–	–	–
	<i>Rastrelliger kanagurata</i> - Indian mackerel ³	✓	✓	✓	✓	–	–	–	–	–
	<i>Sarda australis</i> - Australian bonito ^{3,6}	–	✓	–	–	–	–	–	–	✓
	<i>Sarda orientalis</i> - Striped bonito ^{3,7}	✓	✓	✓	✓	–	–	–	✓	–
	<i>Scomber australasicus</i> - Spotted chub mackerel ^{3,6}	✓	✓	–	–	–	–	–	–	✓
	<i>Scomber japonicus</i> - Chub mackerel ^{3,7}	✓	✓	✓	✓	–	–	–	✓	–
	<i>Scomberomorus commerson</i> - Narrow-barred Spanish mackerel ³	✓	✓	✓	✓	–	–	–	–	–
	<i>Scomberomorus guttatus</i> - Spotted seerfish ³	✓	–	–	–	–	–	–	–	–
	<i>Scomberomorus koreanus</i> - Korean seerfish ³	✓	–	–	–	–	–	–	–	–
	<i>Scomberomorus lineolatus</i> - Streaked seerfish ³	✓	–	–	–	–	–	–	–	–
	<i>Scomberomorus multiradatus</i> - Papuan seerfish ³	–	✓	–	–	–	–	–	–	–
	<i>Scomberomorus munroi</i> - Australian spotted mackerel ³	–	✓	–	–	–	–	–	–	–
	<i>Scomberomorus queenslandicus</i> - Queensland school mackerel ³	–	✓	–	–	–	–	–	–	–
	<i>Scomberomorus semifasciatus</i> - Broad-barred king mackerel ³	–	✓	–	–	–	–	–	–	–
	<i>Scomberomorus sinensis</i> - Chinese seerfish ³	✓	✓	–	–	–	–	–	–	–
<i>Thunnus tonggo</i> - Longtail tuna ³	✓	✓	–	–	–	–	–	–	–	
Oceanic	<i>Acanthocybium solandri</i> - Wahoo ^{1,3,4,5,6,7}	✓	✓	✓	✓	✓	✓	✓	✓	✓
	<i>Allothunnus fallai</i> - Slender tuna ^{3,6}	✓	✓	–	✓	–	–	–	–	✓
	<i>Gasterochisma melampus</i> - Butterfly kingfish ^{3,6}	–	–	–	–	–	–	–	–	✓
	<i>Katsuwonus pelamis</i> - Skipjack tuna ^{1,3,4,5,6,7}	✓	✓	✓	✓	✓	✓	✓	✓	✓
	<i>Thunnus alalunga</i> - Albacore tuna ^{1,3,4,5,6,7}	✓	✓	✓	✓	✓	✓	✓	✓	✓
	<i>Thunnus albacares</i> - Yellowfin tuna ^{1,3,4,5,6,7}	✓	✓	✓	✓	✓	✓	✓	✓	✓
	<i>Thunnus maccoyi</i> - Southern bluefin tuna ^{3,6}	–	✓	–	–	–	–	–	–	✓
	<i>Thunnus obesus</i> - Bigeye tuna ^{1,3,4,5,6,7}	✓	✓	✓	✓	✓	✓	✓	✓	✓
	<i>Thunnus orientalis</i> - Pacific bluefin tuna ^{1,3,6,7}	✓	✓	✓	✓	–	–	–	✓	✓
	Reef	<i>Grammatocygnus bilineatus</i> - Double-lined mackerel ³	✓	✓	✓	✓	–	–	–	–
<i>Gymnosarda unicolor</i> - Dogtooth tuna ^{3,6}		✓	✓	✓	✓	✓	–	–	–	–

References: 1. Bagnis et al., (1984); 2. Collette and Aadland (1996); 3. Collette and Nauen (1983); 4. Randall (1985); 5. Randall and Egana (1984); 6. Roberts et al., (2019); and 7. Tinker (1978).

Region Abbreviations: IS = Island Southeast Asia; SA = Australia, New Guinea and Near Oceania; MI = Micronesia; WP = West Polynesia (including Fiji); FP = French Polynesia; EI = Easter Island; HI = Hawaii; NZ = New Zealand.

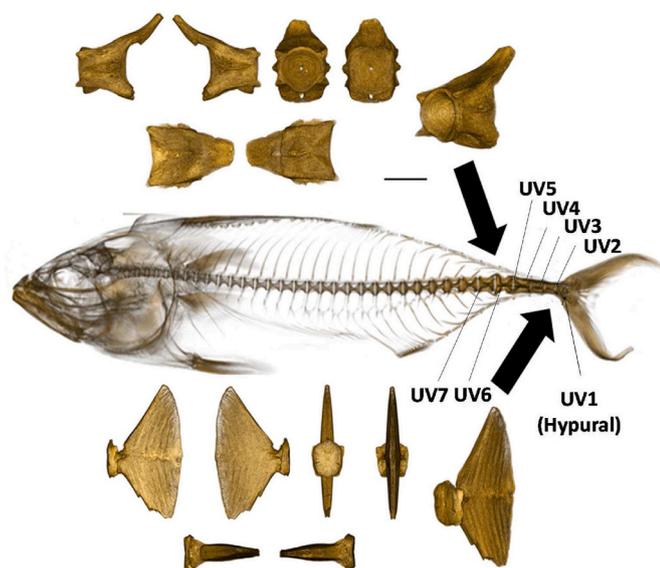


Fig. 1. Example images of vertebrae, showing different angles of a single UV5 vertebra (top set) and a single hypural (bottom set) in relation to skeleton of *Euthynnus* (the latter retrieved from Morphosource, Yale Peabody Museum YPM000827; scanned at 90 μ m resolution); see [Supplementary Figs. S1–S6](#) for images of all scanned archaeological specimens).

their distinctive morphology makes hypurals readily identifiable in archaeological assemblages; and 4) the hypural shows potential for reconstructing fish-length, a useful metric for the analysis of diet and food technologies in the distant past.

The identification of Scombridae in archaeological faunal assemblages is of particular interest because certain scombrids, especially tuna, live mainly in the open sea (Collette and Nauen, 1983; Froese and Pauly, 2017; Johannes, 1981). As tuna and other pelagic species are usually caught in offshore waters, identifying them in the archaeological record can be significant in implying the regular use of seaworthy watercraft - a basic component of prehistoric maritime cultures and seafaring (e.g. Anderson 2013a; O'Connor et al., 2011; Rolett, 1998). In the Pacific, where men skilled at catching tuna are highly respected, traditional tuna fishing practices are rooted in ancient times (Fraser, 2001). Archaeologically excavated faunal assemblages date Scombridae fishing to more than 3000 years ago in the western Pacific (Butler, 1994). Records of pre-European contact Scombridae fishing exist for the western Pacific, Micronesia and Polynesia. In general, fishing for scombrids was more important on islands without barrier reefs, than on islands with lagoons (e.g. Ono and Intoh, 2011). Archaeological records for the Marquesas, in particular, reveal the highest levels of scombrid fishing known for the Pacific (Allen, 2017; Dye, 1990; Leach et al., 1984, 1997; Rolett, 1998).

Archaeological studies of Scombridae fishing are constrained, however, by the lack of a reliable means to systematically distinguish among various scombrids, including offshore and inshore species, using bone morphology. Without being able to separate inshore from offshore scombrids in archaeological fishbone assemblages, the potential for using evidence of scombrids as a proxy for seafaring remains unfulfilled (Anderson, 2013a, 2013b; Lambrides and Weisler, 2015). Here, we show that this problem can be addressed through the application of collagen peptide mass fingerprinting of archaeofaunal remains, or Zooarchaeology by Mass Spectrometry (ZooMS; Buckley et al., 2009), which allows species-level taxonomic identifications of Scombridae (Rick et al., 2019). To mitigate the impacts on faunal assemblages, researchers are beginning to turn to microCT for the digital preservation of specimens, as this enhances their value for further studies. Here we combine ZooMS and microCT in application to over one hundred scombrid remains from the Hanamiai archaeological site in the Marquesas.

1.2. The Marquesan environmental, cultural and archaeological context

The Marquesas are an archipelago of ten high-volcanic islands in geographic isolation along the eastern margin of French Polynesia. They are remarkable for their steep valleys, rugged terrain and the lack of coastal plains. In comparison with most other tropical Polynesian high-volcanic islands, the marine environments are also distinctive. Most striking is the scarcity of coral reefs. Barrier reefs are completely absent, as are lagoons, so that the only sheltered inshore waters consist of bays situated on the leeward coasts. Another distinctive feature of the marine environments is the presence of underwater terraces surrounding the islands at depths of 60–75 m, and extending up to 4 km from the shore (Brousse et al., 1978). Marquesan waters are rich in fish (Randall, 1985), including the same species of Scombridae present throughout French Polynesia. This profusion of pelagic fishes, especially tunas, is likely caused by upwelling currents linked with an exceptional abundance of phytoplankton (Taquet et al., 2016).

Even today the Marquesan diet is dominated by fish and most of the fishing methods employed can be traced back to the pre-European contact era (Rolett, 1998). Scombridae, especially yellowfin tuna (*kahi*), skipjack tuna (*atu*), and wahoo (*ono*), are highly sought after and captured on a regular basis. Schools of skipjack, associated with smaller numbers of yellowfin tuna, are spotted from a distance by flocks of birds feeding on the same small prey that attracts the larger fish. Skipjack schools are mainly found offshore but according to Native Marquesan fisherman Manuhi Timau they occasionally enter sheltered bays (personal communication to BR, August 3, 2020). Skipjack and wahoo are caught only during the day, by trolling in surface waters using an unbaited composite fishhook (Bagnis et al., 1984:320; Rolett, 1998:125). Yellowfin can also be captured in the same way. Additional observations by Timau are significant for interpreting Scombridae bones recovered from Marquesan archaeological sites. Based on his 50 years of experience fishing in Marquesan waters, Timau estimates that yellowfin caught together with skipjack are about the same size as the skipjack, averaging roughly 50 cm in fork length. The largest skipjack seen by Timau measure no more than 70–80 cm in fork length (personal communication to BR, November 3, 2020; see also Froese and Pauly, 2017). Yellowfin grow up to ca. 150 cm in length (Froese and Pauly, 2017), however Timau notes that the larger ones are caught only at night, by fishing with a baited hand line in deep offshore waters (personal communication, November 20, 2020; see also Bagnis et al., 1984:319; Rolett, 1998:124). Catches from two night fishing sorties targeting yellowfin were recorded on Tahuata by BR in June and July 2017. Both sorties involved hand line fishing in deep offshore *to'a* (“fishing holes”) in front of Hapatoni Bay, near Vaitahu. Altogether, 28 yellowfin were caught. The smallest was around 90 cm long, while the largest reached about 120 cm in length.

The kawakawa tuna is occasionally caught in surface waters among schools of skipjack and yellowfin, but in the Marquesas it is more often captured by nighttime inshore hand-line fishing at intermediate depths (Manuhi Timau, personal communication to BR, August 3, 2020). Big-eye and dogtooth tunas are usually caught in deep offshore waters (Bagnis et al., 1984:318; Rolett, 1998:124). Albacore tuna are present but rarely captured in the Marquesas; in the Society Islands they are almost always caught in deep offshore waters (Bagnis et al., 1984:318).

The Hanamiai archaeological site lies on the leeward coast of Tahuata, an island in the southern Marquesas with a surface area of 61 km² and a maximum elevation of 1050 m. The site is a sand dune remnant facing a sheltered bay backed by an amphitheater-shaped valley. Excavations at Hanamiai reveal a sequence of four prehistoric cultural phases with calcareous sand archaeological deposits extending from the surface to depths of around 1.85 m (Rolett, 1998). A revised radiocarbon chronology dates Phase I, marking initial settlement of the site, to around AD 1250 (Rolett and Dye, unpublished data). This situates Hanamiai as one of the oldest human colonization sites in East Polynesia (Rolett, 1998; Kirch, 2017). The prehistoric deposits represent

a series of occupations in a generalized habitation area. Artifacts and faunal remains are well preserved in the sandy deposits and were recovered by sieving the deposits through 1/8" mesh screens. A 20 m² area excavation at Hanamiai North yielded more than 18,000 bones, revealing a broad-spectrum subsistence orientation with a heavy reliance on fishing. Numerous fishhooks, mostly made of pearl shell (*Pinctada margaritifera*), including trolling lure points and shanks, were discovered in direct association with the faunal remains (Rolett, 1998). More recently, additional excavations have been carried out at Hanamiai South, an area lying about 50 m south from the Hanamiai North location.

2. Materials and methods

2.1. Reference specimens

Prior to this study, published Scombridae reference spectra were available for only the albacore (*Thunnus alalunga*) and Atlantic bluefin (*T. thynnus*) tunas (Rick et al., 2019). Modern samples for the six other scombrid species found in French Polynesia were obtained to complete the reference collection (Table 2). A single skeleton was obtained for each species and multiple bones from each specimen were selected for ZooMS analysis.

2.2. Archaeological specimens

From the Hanamiai assemblage of fish remains identified on the basis of morphology, 137 bones were selected for the study (Supplementary Tables S1–S2). These consist of 135 scombrid bones and two others representing neritic coral-feeding fishes, to serve as control samples. Among the bones identified on the basis of morphology as representing scombrids, 111 are hypurals from the 20 m² area excavation at Hanamiai North (Supplementary Table S1). This set comprises a complete sample of all scombrid hypurals from that excavation. Because a single fish skeleton contains only one hypural, this set represents 111 individuals. The hypural assemblage includes specimens from all five cultural phases in the Hanamiai sequence, spanning the time period from ca. AD 1250–1900 (Supplementary Table S1). An additional 26 morphologically-diagnostic scombrid vertebral centra and dentaries were selected from the Hanamiai North and South assemblages (Supplementary Table S2). As these bones are less useful for determining the minimum number of individuals (MNI) represented, they were considered to be less valuable than the hypurals. This set of 24 scombrid bones was used to experiment with minimally-destructive methods for ZooMS analysis.

2.3. MicroCT scanning

In order to digitally preserve the archaeological bone specimens prior to sampling for ZooMS analysis, high-throughput digitisation by microCT was carried out. Specimens were placed in individual wells

Table 2
Details of Scombridae reference specimens analysed in duplicate² or triplicate³.

Common name	Species	Fork length	Weight	Place collected
Skipjack tuna ³	<i>Katsuwonus pelamis</i>	48 cm	NA	Tahuata, Marquesas
Yellowfin tuna ³	<i>Thunnus albacares</i>	89 cm	8 kg	Tahuata, Marquesas
Dogtooth tuna ³	<i>Gymnosarda unicolor</i>	56 cm	1.5 kg	Hiva Oa, Marquesas
Bigeye tuna ²	<i>Thunnus obesus</i>	NA	NA	Oahu, Hawai'i
Kawakawa tuna ²	<i>Euthynnus affinis</i>	50 cm	NA	Oahu, Hawai'i
Wahoo ²	<i>Acanthocybium solandri</i>	115 cm	NA	Oahu, Hawai'i

within microtiter plates and subsequently microCT scanned at Manchester X-Ray Imaging Facility (University of Manchester, UK) in a Nikon X TH 225 scanner, at a voxel size of 48 µm. Samples were scanned using a tungsten target and a 0.1 mm copper filter, at a voltage of 120 kV and energy of 110 µA. A total of 5013 projections were collected, at an exposure time of 500 ms. Scans were reconstructed in CTPRO and exported as 32 bit float files for archiving. For the purposes of visualisation, individual specimens were cropped from the dataset in Fiji (Schindelin et al., 2012), converted to 8 bit and volume rendered in Horos (horosproject.org).

2.4. Collagen peptide mass fingerprinting

Initially we tested for collagen from a selection of the ziplock bags that the archaeological bones were individually shipped in (e.g., McGrath et al., 2019) or wipes with fibre lapping film (Fibre Fox, UK), through the addition of 200 µL 50 mM ammonium bicarbonate (ABC), manual mixing and leaving overnight prior to being removed and digested with trypsin at 37 °C overnight. However, following poor results we subsequently submerged all archaeological specimens in 0.3 M hydrochloric acid (HCl; e.g., Buckley et al., 2016) for 4 h and then ultrafiltered the recovered acid using 10 kDa Molecular Weight Cut Off ultrafilters directly into 50 mM ABC and digested with 0.4 µg sequencing grade trypsin at 37 °C overnight. For all samples, 1 µL of the digests was then spotted onto stainless steel target plates for MALDI-ToF analysis as described elsewhere (Buckley et al., 2016).

For each of the newly added reference taxa we carried out LC-MS/MS analyses of the above extracts for sequence interpretation using an UltiMate 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA, USA) coupled to an Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer (120 k resolution, full scan, positive mode, normal mass range 350–1500). Peptides were separated using an Ethylene Bridged Hybrid (BEH) C18 analytical column (75mm × 250 µm i.d., 1.7 µm; Waters) with a gradient from 92% A (0.1% FA in water) and 8% B (0.1% FA in ACN) to 33% B in 44 min at a flow rate of 300 nL min⁻¹. Peptides were automatically selected for fragmentation by data-dependent analysis; six MS/MS scans (Velos ion trap, product ion scans, rapid scan rate, centroid data; scan event: 500 count minimum signal threshold, top 6) were acquired per cycle, with dynamic exclusion employed. One repeat scan (i.e., two MS/MS scans total) was acquired in a 30 s repeat duration with that precursor being excluded for the subsequent 30 s (activation: collision-induced dissociation (CID), 2+ default charge state, 2 m/z isolation width, 35 eV normalized collision energy, 0.25 activation Q, 10.0 ms activation time).

Tandem mass spectra were then searched against a local database containing selected COL1 sequences (Supplementary Material) retrievable from a protein BLAST search of stickleback (*Gasterosteus aculeatus*) COL1A2 (ENSGACT00000008691.1) as well as the COL1A1a and COL1A1b (also known as COL1A3) southern bluefin tuna (*T. maccoyii*) sequences for error tolerant matches using the Mascot search engine (version 2.5.1; Matrix Science, London, UK). Each Error Tolerant search included the variable modifications for oxidation of lysine and proline to account for common collagen post-translational modifications. Enzyme specificity was trypsin-P (first batch of analyses) with only one missed cleavage allowed; mass tolerances were set at 5 ppm for the precursor ions and 0.5 Da for the fragment ions and all spectra were considered as having either 2+ or 3+ precursors.

3. Results and discussion

3.1. Quality of the digitisation

Although a resolution as low as 5–7 µm could potentially be achieved using lab-based microCT, this would require specimens to be scanned individually. Alternatively, the resolution would also be improved through the use of smaller dimension microtiter plates, although these

are not as readily compatible with subsequent high-throughput ZooMS analyses (Buckley et al., 2016). However, given the size of these specimens, a resolution of ~50 μm was clearly adequate to resolve fine-scale surface features of the specimens (e.g., Fig. 1 & Supplementary Figs. S1–S2).

3.2. Taxonomic resolution of ZooMS for scombrids

The ability to resolve genus-level distinction in these taxa is not surprising given the known evolutionary divergences of these scombrids. For example, *Katsuwonus* and *Euthynnus* are thought to have separated from each other ~32 Ma, which as a group separated from *Thunnus* ~48 Ma (Santini et al., 2013). The divergence from the grouping of *Acanthocybium* and *Gymnosarda* is even deeper having occurred approximately 60 Ma (and the separation between these two at ~58 Ma). Within the *Thunnus* genus, all separations of which occurred within the last ~5 Ma, *T. albacares* is sister to *T. obesus*, both sister to *T. thynnus*, all sister to *T. alalunga*. It is interesting to note although few of the selected biomarkers reflect these phylogenetic clades, with the exception of m/z 2510 for *Thunnus*, m/z 1445 for the Thunnunini (*Thunnus* + *Katsuwonus* + *Euthynnus* of this study), and m/z 2112 for the Sardini (*Gymnosarda*) + Thunnunini clade. The ability to separate to the species level within *Thunnus* is potentially a key advantage in using ZooMS (for example all four species using the 3t47, 2t13 and 1t55/56 markers). However, even separating some of the other more distantly related species by ZooMS is advantageous because doing so using morphology alone can be challenging with particular skeletal elements.

Determination of the sequences for each of these markers by interpretation of tandem mass spectra (Table 3; Supplementary Figs. S10–22) was more challenging for some taxa than for others, largely due to taxonomic distance to any known sequences on which the probability-based matches are based; only sequences for the two of the three collagen (I) chains, the alpha 1 and alpha 3 chains derived from the COL1A and COL1B genes respectively, were retrievable from the bluefin tuna (*T. thynnus*) genome, lacking the more variable alpha 2 (I) sequence. The inability of Error Tolerant searches to achieve confident matches to all markers highlights this but the variation of more than a single amino acid (the limitation of this modified search approach) would be expected given the ~50 Ma divergence in some taxa. However, even for these (e.g., the *Katsuwonus* m/z 1082 marker; Table 3), homology of the markers could still be assumed through partial sequence recognition and lack of observation in the LC-MS/MS data that has assessed thousands of spectra per sample, in addition to lack of nearby peaks in the MALDI reference spectra. This is an important advantage that sequencing analysis has over the more recently introduced

Table 3

Selected collagen peptide biomarkers for scombrids considered within this study (cell shading used to highlight taxon separation); modern reference spectra shown in Fig. 2 & Supplementary Fig. S7 with labels deriving from Harvey et al. (2018). Superscripted numbers relate to tandem mass spectra shown in supplementary material; brackets indicate marker not expected due to tryptic site, *indicates that homology was not confidently confirmed through sequence analysis but speculated based on proximity to known markers to the exclusion of others present across spectra; no numbers indicate homologous markers were not inferred and X indicates marker sequence location was not determined.

	3t47	3t62	1t62	2t62	X*	2t61	1t55/56*
Dogtooth (<i>Gymnosarda</i>)	1040 ¹⁰	1455 ¹²	1443	1572 ¹³		2552 ¹⁸	2857 ²⁴
Wahoo (<i>Acanthocybium</i>)	1052 ¹¹	1455	1443	1545 ¹⁵		2540 ¹⁹	2895
Skipjack (<i>Katsuwonus</i>)	1082 ¹²	1445 ¹⁴	1443	1557 ¹⁷		2484 ²⁰	
Kawakawa (<i>Euthynnus</i>)		1445	1443	1572		2482 ²¹	2885
Albacore (<i>T. alalunga</i>)	1038	1445	1443	1572	1620		2867
Bigeye (<i>T. obesus</i>)	1052	1445	1443	1572	1620		2867
Bluefin (<i>T. thynnus</i>)	1052	1445	1443	1572	1620		2897
Yellowfin (<i>T. albacares</i>)	1052	1445	1443	1572	1604		2867

approaches using machine learning of training set spectra (e.g., Gu and Buckley 2018).

Taxonomic identifications for the experimental subset of 24 bones show that the majority can be attributed to the skipjack tuna, with only one to yellowfin tuna (Supplementary Fig. S8). Our collagen fingerprinting of modern reference specimens representing additional, previously unpublished species, confirms the ability to obtain species-level taxonomic resolution within the *Thunnus* genus. Two deliberately distinct ‘unknowns’ (319 and 347; Supplementary Fig. S9) representing near-shore coral-feeding fish were included (by BR) as control samples.

3.3. ZooMS collagen fingerprinting of the 111 hypurals

This is the first study using a combination of ZooMS and microCT to investigate ancient scombrid fishing in the Pacific Ocean. The 111 hypural specimens comprise the total assemblage of scombrid hypurals recovered during the Hanamiai North areal excavation. These are fairly evenly distributed among the early (Phases I, II) and late (Phases III, IV) prehistoric cultural phases, with only five found in the Phase V historic era deposits (Table 4). Because an individual scombrid skeleton contains only one hypural, the assemblage represents 111 fish. Among these, we confidently matched 71 of the 73 retrieved collagen fingerprints to skipjack tuna (Table 4), one to *Thunnus* (with species resolution not achieved due to the poor quality of this spectrum which, along with several others, would have been considered a ‘fail’ in terms of screening for radiocarbon dating due to lack of high m/z peaks (Harvey et al. 2016)), and one that did not match anything in our reference collection despite being a good quality spectrum (Fig. 3). As with ZooMS analysis of other taxa such as bear (e.g., Mychajliw et al., 2020), some identifications can be more readily made using low m/z values that are present in poor collagen fingerprints, in this case that of the skipjack tuna which dominates this assemblage (although all four of the markers for this taxon were identified in all archaeological spectra in this study; Supplementary Tables S1–S2).

Although collagen fingerprints were not retrieved from one third of the assemblage ($n = 38$), the overwhelming dominance of skipjack (71 of the 73 spectra that yielded collagenous peptides) allows us to consider one of the key questions regarding the kinds of tuna captured - whether or not there is change, or continuity, over time. Despite the fact that other lines of faunal evidence, as well as the artifact assemblages, suggest significant cultural changes over the period from initial settlement

Table 4

Number of specimens of fish remains from the Hanamiai excavation identified by ZooMS. ¹One non-skipjack hypural yielded a fingerprint showing only low m/z collagen peptides ruling out *T. alalunga* but not able to distinguish the other *Thunnus* taxa); ²One spectrum yielded was of high quality, with well-resolved high m/z peaks, but did not match any reference spectrum; ³All other (non-hypural) bones were identified as skipjack except for one⁴ identified as yellowfin tuna.

Cultural chronology	Early prehistoric		Late prehistoric		Historic	Total
	Phase I	II	III	IV		
Hanamiai North: Complete assemblage of hypurals						
Skipjack	8	14	12	33	4	71
<i>Thunnus</i> spp. ¹			1			1
Unknown ²			1			1
Insufficient Collagen	17	12	4	4	1	38
Other bones from Hanamiai North and South³						
UV2			1 ⁴		2	3
UV3				1	2	3
UV4				1	2	3
UV5			1	1	1	3
UV6				2	1 ³	3
UV7		1		1	1	3
UV8	1				2	3
Dentary				1		1

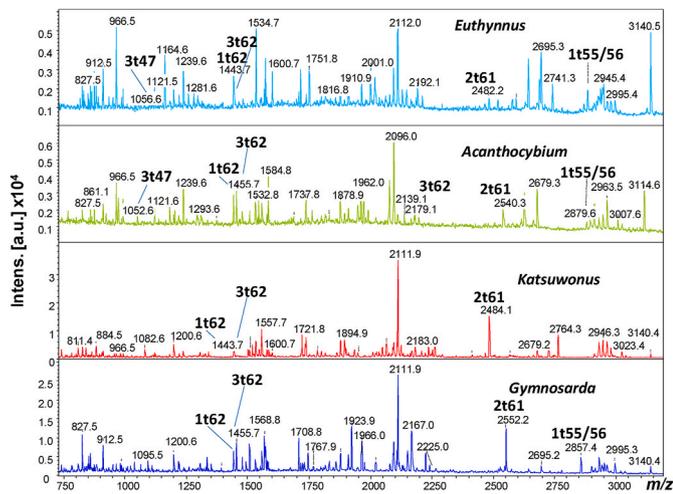


Fig. 2. Peptide mass fingerprint spectra of collagen extracted from four scombrid genera.

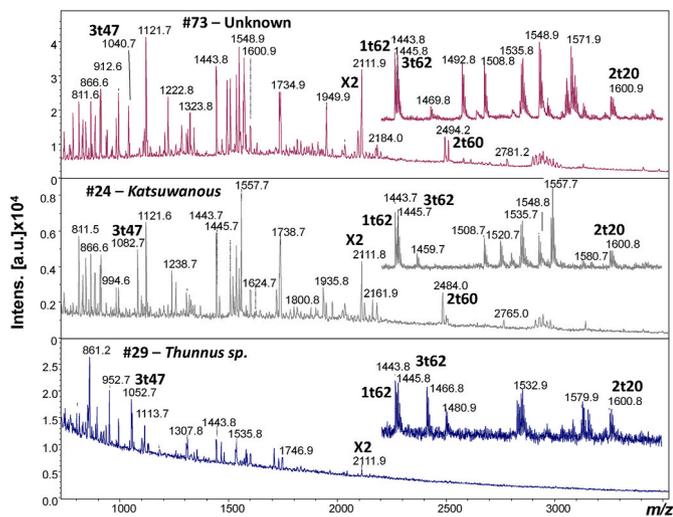


Fig. 3. Example collagen peptide mass fingerprint spectra of the three distinct groups identified amongst the archaeological hypurals.

around AD 1250 until the time of European contact (Rolett, 1998), we do not see this reflected in the evidence for scombrid fishing (Table 4). Our study demonstrates the usefulness of ZooMS results for understanding big picture developments in Marquesan culture, with implications for other Polynesian cultures as well.

3.4. Archaeological significance

Skipjack tuna are caught only during the day and their schools are usually found offshore. While they occasionally enter sheltered bays, such as the one facing the Hanamiai site, the traditional fishing strategy involved canoes suitable for navigating the open sea. At the time of European contact, Marquesan canoes varied in size, including single-hull outrigger canoes and the double-hull type generally associated with long distance voyaging. Both kinds were fitted with sails but were also well suited for paddling (Fig. 4). The dominance of skipjack tuna in the Hanamiai scombrid assemblages suggests that fishing strategies involving sailing canoes, as documented during the early historic period (Coulter, 1845:156–160), were present from the time of initial settlement.

Unexpected in our results for the hypurals is the absence of yellowfin tuna, apart from one specimen in the experimental set. Other specimens



Fig. 4. Resolution Bay in the Marquesas. Engraving by B. T. Pouncy after a sketch made by William Hodges in 1774 on Captain James Cook's second voyage to the Pacific. Collection of B. Rolett. Resolution Bay faces Vaitahu and Hanamiai Valleys on Tahuata. The Hanamiai archaeological site lies on the coast. Two Marquesan outrigger canoes of the type used for seafaring and skipjack fishing are shown in the foreground.

in our study for which collagen fingerprints were not retrieved could also be yellowfin. However, sizes of the unidentified specimens (as represented by hypural widths recorded in Table S1) fall into the same range as sizes for the identified skipjack. Yellowfin in this size range are around 50 cm in length on average based on the ecological data and observations by Marquesan fishermen presented above. Because yellowfin this size are captured only during the day among schools of skipjack, regardless of whether the unidentified specimens represent skipjack or yellowfin, our results infer an emphasis on diurnal fishing strategies involving sailing canoes.

Although other fish (e.g. large Serranidae) represented in the Hanamiai North faunal assemblage indicate daytime fishing with a baited hand line in deep offshore waters (Rolett, 1998:118–145), our study reveals no evidence for night fishing using a similar strategy. In sum, the Hanamiai evidence points to a focus on daytime fishing for skipjack rather than night fishing for yellowfin, kawakawa and dogtooth tuna. Future research on scombrid assemblages from other archaeological sites in the Marquesas and elsewhere in the tropical Pacific can test the extent to which these findings display regional patterns.

Marquesan marine environments are unusual in comparison with those for most other tropical Pacific archipelagoes, because there are no lagoons in the Marquesas. Nevertheless, the analytical methods reported here can be used to distinguish inshore from offshore scombrids in other Pacific Ocean settings. They may also help resolve the debate over fishing strategies used to catch scombrids in ca. 40,000 year old Indonesian archaeological sites (Anderson 2013a; Anderson 2013b; O'Connor et al., 2011). However, the main limitation of the ZooMS technique in this regard relates to the survival of collagen. Our study indicates a clear age-related impact on the preservation of collagen; increasing archaeological/stratigraphic age correlates with nearly constant increases in the number of specimens with insufficient collagen. This emphasizes the continued need to combine ZooMS analyses with traditional morphological analyses, the latter of which do not have such limitations other than those related to the survival of morphologically diagnostic features. The combined use of ZooMS with microCT scanning readily allows for both types of analysis to be carried out.

4. Conclusions

Our analysis of over one hundred scombrid hypurals from the Marquesan Hanamiai archaeological site suggests striking continuity over time in scombrid fishing practices from initial Polynesian settlement until the time of European contact, with a strong focus on skipjack tuna and an emphasis on fishing strategies involving sailing canoes. We

demonstrate the potential for combining collagen peptide mass fingerprinting and microCT for the complementary archiving and analysis of archaeofaunal scombrid remains. By scanning in microtiter plate format we are able to reduce the impacts of destructive sampling on important faunal assemblages, rendering them amenable to further morphological study; by using the acid-soluble collagen component of archaeological bones they remain viable for radiocarbon dating.

There are clear advantages of ZooMS over species identification by ancient DNA (aDNA) analyses, where the latter in application to fish bones do not go without problems, including issues relating to contamination and amplification. Sample preservation is one of the main hindrances to the analysis of fish remains, which are relatively brittle and porous compared with most other taxa. The aquatic environment that exposes fish to chemicals causing acidification and oxidation is also likely to have a substantial impact on the survival of aDNA (Oosting et al., 2019). The move into genome sequencing has improved aDNA methodologies, but with much smaller sample numbers being analysed (e.g., Star et al., 2017). As the ZooMS protocols are progressively refined, either methodologically, through sequencing as has been done here, or through machine learning approaches, such high-throughput proteomic approaches could offer a more efficient, cheaper and faster alternative to aDNA. Overall, our results further highlight the utility of ZooMS for identifying archaeological fish bone. In particular, this study shows that ZooMS can be successful for investigating fish remains recovered from deeply stratified dune sites in tropical environments. More broadly, it illustrates the value of ZooMS and microCT as complementary components of multidisciplinary research exploring the human past.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jas.2021.105475>.

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