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Species identification of ancient Lithuanian fish remains using collagen fingerprinting

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ABSTRACT

Morphological identification of ancient bone is often problematic due to heavy fragmentation that generally influences zooarchaeological assemblages. Fish bones are more taphonomically sensitive than those of other vertebrates as they are typically smaller and less biomineralised. Thus, taxonomic identification based on the preservation of morphological features is often extremely limited and can reduce or eliminate the usefulness of an assemblage for inferring taxon information. Currently, one of the most time- and cost-efficient methods of achieving faunal identity from ancient bone is by the collagen fingerprinting technique known as ZooMS (Zooarchaeology by Mass Spectrometry). ZooMS harnesses the potential of preserved collagen, which is the most dominant and time-stable protein in bone. In this research, ZooMS is applied to ancient Baltic region fish assemblages that are between 500 and 6000 years old in order to define species identity and construct assemblage compositions. Alongside inferences into environmental and biological shifts from the Neolithic era to present day in the Baltic region, we demonstrate for the first time the ability to distinguish between recently diverged members of the *Salmo* (salmon) and *Scophthalmus* (turbot) genera. ZooMS analysis highlights 7% of the collagen-containing assemblage as having been morphologically identified incorrectly and has facilitated taxonomic refinement of a further 28% of samples, including some of the morphologically indeterminate bone fragments. This research emphasises the great potential of ZooMS in identifying ichthyoarchaeological bone remains to species-level, and provides a case for the use of collagen fingerprinting in contributing to baseline fisheries and ecological data to inform modern management.

1. Introduction

The Baltic Sea is a young, shallow, semi-enclosed sea that was carved from the last glaciation event. Today the sea covers an area of 415,000 km² (Emeis et al., 2002) and features the coastlines of many countries, including Lithuania. The modern Baltic Sea features a range of salinities extending from low-salinity (near-freshwater) environments on the eastern edge and on the upper surface of the sea, to high salinity environments in the west and at depth that support ecosystems of marine origin (Emeis et al., 2002; Harff et al., 2007; Mohrholtz et al., 2015).

1.1. Brief history of fishing in Lithuania

The history of the Baltic Sea from the last glacial event to the Holocene is rich in evidence of past human activities, particularly near the coasts. In the last 14,000 yr BP (years before present), the sea has

endured vast alterations in environmental conditions (climate, temperature, salinity), which have been accompanied by shifts in biota (Weckström et al., 2017). The Mesolithic communities in Lithuanian territorial waters favoured the complex ecotopes of river mouths and findings of stationary fishing constructions dating to 8505 yr BP (the oldest known of their kind in the Baltic region) demonstrate early exploitation of freshwater fish in these regions (Girininkas and Žulkus, 2017). The Mesolithic people showed preference towards harvesting salmon (*Salmo salar*), carp (*Carassius carassius*), perch (*Perca fluviatilis*) and catfish (*Silurus glanis*) (Sloka, 1986; Zagorska, 1992), and are likely to have used hunting tools such as daggers, spears, fish traps, harpoons and hooks (Enghoff, 1994; Meadows et al., 2014), with the latter tradition being notably common throughout Mesolithic Europe (Gramsch et al., 2013). Fishing with mesh nets in the Baltic region began in the late Mesolithic (7000–5300 yr BP) and continued into the Neolithic and beyond, indicated by a prevalence of smaller fish bones being found alongside larger ones in ancient assemblages (Olson and Walther, 2007;

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Piličiauskas et al., 2015).

The transition to farming brought about the end of the Mesolithic and the beginning of the Neolithic period, approximately 5500 yr BP in Lithuania (Piličiauskas et al., 2012). In these times, although agricultural practices and animal husbandry were slowly being implemented, hunting and fishing were still dominant and stable methods of acquiring sustenance (Rimantienė, 1992a; Daugnora and Girininkas, 1996; Piličiauskas et al., 2012; Girininkas and Daugnora, 2015). The fishermen of Neolithic Lithuania were proficient, typically using nets as their primary catchment method and targeting large predatory fish such as the northern pike (*Esox lucius*) (Rimantienė, 1992a, 2005; Stančikaitė et al., 2009). Neolithic archaeological collections, such as that of the Šventoji region of north-west Lithuania and lake settlements of the south-east, are famed for their exceptionally preserved artifacts (Rimantienė, 1992b; Daugnora and Girininkas, 1996; Piličiauskas et al., 2012; Girininkas and Daugnora, 2015). Radiocarbon dates of such artifacts demonstrate the regions were occupied from at least 6000–2800 yr BP, a period following the maximal Littorina Sea transgression at ~7500 yr BP (Daugnora and Girininkas, 2004; Rimantienė, 2005; Damušytė, 2011; Piličiauskas et al., 2012)—one of the four recognised stages in the post-glacial progression of the Baltic basin (Andrén et al., 2011). This marine phase, the first connection between the Baltic lake system and the North Sea, established favourable conditions for the exploitation of aquatic resources by early settlers (Jöns, 2011; Girininkas and Daugnora, 2015). The marine bay was then separated from the North Sea by a sandy bar, forcing a semi-open lagoon dated ~7200–3500 yr BP, which favoured a freshwater environment interspersed with further intrusions of brackish water as a consequence of weather extremes and further regression events (Stančikaitė et al., 2009; Andrén et al., 2011; Piličiauskas et al., 2012). These environmental shifts can be evidenced through sediment analyses (Andrén et al., 2000), and through the study of ancient fish bone deposits to identify species that are halophilic (marine), such as brill (*Scophthalmus rhombus*) or halophobic (freshwater), such as pike (*E. lucius*) (Daugnora and Girininkas, 2004; Stančikaitė et al., 2009; Girininkas and Daugnora, 2015). In the early Neolithic period, settlements became more pronounced at the junctions between rivers and seaside lagoons (Lōugas, 1997), and these lagoons were becoming increasingly more saline due to influences from the North Sea (Andrén et al., 2011; Piličiauskas et al., 2012). By the middle and late Neolithic, the diversity of freshwater, diadromous and seawater species being caught was increasing, with greater proportions of marine species typically being targeted as the Neolithic era progressed (Enghoff, 1994, 1999; Lōugas, 1997; Makowiecki, 2003; Daugnora and Girininkas, 2004).

A high dependency on aquatic food resources continued into medieval Lithuania whereby an expansion in fish trade and commercial exchange ensured the retention of fishing practices throughout the middle ages (Zvelebil, 2006; Piličiauskas et al., 2012). Here, catch preference steered again towards more marine-based methods of harvesting, in part due to demand intensification but also as a result of a reduction in freshwater fish stocks (Barrett et al., 2011; Lotze et al., 2014). For example, during the archaeological investigation at the Medieval site of Klaipėda Castle (north-west Lithuanian coast; 14–17th century), a 5–6 m thick cultural layer was unearthed containing over 3000 artifacts, of which 800 were fishhooks and fish bones (Žulkus, 2002). In the late 14th century the main species of interest for the castle's inhabitants included bream (*Abramis brama*), twait shad (*Alosa fallax*), pike-perch (*Sander lucioperca*) and pike (*E. lucius*). However, into the 15th century the menu incorporated increasingly brackish/marine or diadromous species, including cod (*G. morhua*), Atlantic salmon (*S. salar*) and brown sea trout (*Salmo trutta trutta*), alongside artificially grown carp (*Cyprinus carpio*) (Žulkus and Daugnora, 2009).

In the Baltic region, the species prevalent in archaeological fish deposits through the ages generally represent the local fish fauna of the region, rather than species that have been brought in through trade (Enghoff, 1999; Žulkus and Daugnora, 2009), although a handful of

archaeological analyses have suggested small-scale commercialization and fisheries trade began to take hold from the 11th–14th centuries (Žulkus and Daugnora, 2009; Orton et al., 2011), with larger and often international enterprises gaining significance well into the 16th century (Holm, 2016). Such harvesting for personal consumption or trade has been changing the Baltic aquatic systems for thousands of years, and anthropogenic pressures have since culminated in a maximal impact during the last 100 years (Rimantienė, 1998; Limburg et al., 2008). Consequently, the Baltic Sea is now recognised as one of the world's most polluted sea areas and is further threatened by overfishing, invasive species introduction, eutrophication and climate change (Weckström et al., 2017; HELCOM, 2017).

1.2. Faunal identification using proteins

The analysis of historical records (e.g. Gaumiga et al., 2007; MacKenzie et al., 2007) and zooarchaeological material (e.g. Holm, 2016; Yurtseva et al., 2015) can enable ecological reconstructions and provide a greater understanding of aquatic baseline fisheries data prior to the major anthropogenic interferences of the modern era. Such analyses are of key importance in the Baltic Sea, which currently relies on [often underreported] fisheries data from the 1950's onwards to assess biological depletion (Zeller et al., 2011). Where past studies assessing fisheries through zooarchaeological analyses have typically utilised morphological methods of bone identification, other tools are now available for zooarchaeologists. For example, preserved proteins such as collagen type I ('collagen (I)') can be harnessed for faunal identification in ancient bone fragments that may otherwise be left unidentified. In this process, termed 'ZooMS' (Zooarchaeology by Mass Spectrometry), collagen (I) is extracted from the bone, enzymatically digested and then the peptide mixture analysed using soft-ionisation mass spectrometry (Buckley et al., 2009). The resulting mass spectrum is called a peptide mass fingerprint (PMF), or more generally a 'collagen fingerprint' as it allows visualisation of the collagen peptides in relation to their mass-to-charge ratios (m/z). Peptide peaks that can be seen as specific to a particular taxonomic level are referred to as biomarkers and it is a combination of these biomarkers that allows the refinement of taxonomic identification to family level (Buckley et al., 2011), genus level (e.g. Buckley et al., 2017), and occasionally to species level (e.g. Buckley et al., 2016). Liquid chromatography tandem mass spectrometry (LC-MS/MS) can also be employed as a supportive technique to assist in the sequencing of peptide biomarkers using probability-based matching. In LC-MS/MS, thousands of peptides from complex proteolytic mixtures can be separated in the first stage of mass spectrometry, and then sequenced in the second stage via collision-induced dissociation (CID). This process generates product ion spectra that detail the m/z of the resulting peptide fragments. When presented with a database of collated protein sequences, probability-based matching allows the inference of specific peptide sequence information that can help interpret homologous biomarkers between taxa (Buckley et al., 2009), or facilitate the building of sequences for phylogenetic analysis (Buckley, 2013).

The collagen (I) molecule is present as a triple helical heterotrimer, with one or more of the three left-handed polypeptide subunits, termed alpha (α) chains, differing in amino acid sequence (Piez, 1965). Collagen (I) in the majority of vertebrates is formed from two identical $\alpha 1$ chains and one chemically dissimilar and quicker evolving $\alpha 2$ chain, each of approximately 1000 amino acids in length (Buckley and Collins, 2011). This formation is termed an '($\alpha 1$)₂ $\alpha 2$ ' arrangement. The $\alpha 2$ chain is less restricted in its requirement for the amino acid proline (Pro), promoting a greater variability in sequence between different taxonomic groups (Buckley et al., 2009), whilst still adhering to the obligatory (Gly-Xaa-Yaa)_n repeating sequence that typifies the collagen (I) α -strands (Kadler et al., 1996). Interestingly however, certain fish species exhibit a more complex heterotrimeric arrangement whereby each of the three α chains differ in sequence to form an ' $\alpha 1_1\alpha 2_1\alpha 3_1$ '

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