Aminostratigraphical test of the East European Mammal Zonation for the Late Neogene and Quaternary

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Highlights

- Amino acid geochronology of bithyniid opercula independently tests the East European Mammal zonation.

- *Parafossarulus* and *Bithynia* opercula have similar patterns of protein degradation.
- Reworking is evident at some sites.
- Gaps in the regional palaeontological record are identified.
- Anomalously high levels of IcPD from Tizdar may result from local geothermal heating.

Abstract

An aminostratigraphical study was undertaken to provide an independent test of the veracity of the East European Mammal zonation. This important biostratigraphical scheme was originally defined from reference sites in the Azov / Black Sea region of southern Russia, but is now widely used to correlate late Neogene and Quaternary sediments across much of Europe and western Asia. As well as yielding a series of mammal assemblages, these reference sites, which range in age from the late Pliocene (Piacenzian ca. 3.0 Ma) to Late Pleistocene (0.1 Ma), also contain calcitic opercula of two genera (*Bithynia* and *Parafossarulus*) of freshwater gastropod snails that are suitable for amino acid dating. The intra-crystalline protein decomposition (IcPD) of four amino acids (aspartic acid, alanine, valine, and glutamic acid) was analysed from the opercula of these two genera, which showed similar patterns of protein degradation, allowing both to be used for aminostratigraphy. The IcPD data are consistent with the relative ages inferred from the mammal biostratigraphy and also with stratigraphical hiatuses interpreted from the fossil record. The temporal resolution provided by IcPD data from opercula is amino acid dependent, and declines in samples older than ~2 Ma. The high variability of IcPD between opercula samples at some sites suggests reworking. Anomalously high levels of IcPD in samples from the Early Pleistocene site of Tizdar may be due to geothermal heating from local volcanism. This study provides the first large-scale application of IcPD-based aminostratigraphy for the Quaternary of continental Europe, and highlights its importance in testing regional stratigraphic schemes for the Late Pliocene and the Pleistocene.

1. Introduction

Correlation of continental sequences (which are virtually always incomplete and geographically isolated) is a major problem in Quaternary research, especially for those sites beyond the range of radiocarbon dating. Tephrachronology has been useful in linking sequences together, but this cannot be used universally, for example in regions beyond the distal outfall of tephra or for several critical periods when volcanoes were inactive (Lowe *et al*., 2015). The lack of dating methods that can cover the whole of the Quaternary time period has meant that biostratigraphy has been the main technique used for correlation of continental sequences. Pollen analysis has traditionally been the primary biostratigraphical technique, able to discriminate some temperate stages of the Pleistocene, thereby providing differentiation of the intervening cold stages. Pollen analysis underpinned the stratigraphical succession developed for The Netherlands that has since become the standard template for much of NW Europe (Zagwijn, 1985). Other biostratigraphical schemes are based on other biotic groups, especially mammals. During the Quaternary, faunal turnover in mammalian species was relatively high, making them particularly useful for biostratigraphy (Gromov, 1948; Kretzoi, 1987; Fejfar *et al*., 1997; 1998; Mayhew, 2015; and many others). Several zonation schemes have been proposed that are founded on the changing composition of mammalian assemblages in response to climate and on the evolutionary trends within key lineages of arvicoline rodents, such as grass voles (*Allophaiomys/Microtus*), water voles (*Mimomys/Arvicola*) and steppe lemmings (*Borsodia/Prolagurus/Lagurus*). The best known, and most widely used, scheme is the European Land Mammal Ages, now integrated with the Neogene MN zonation (Mein, 1990; Fejfar *et al*., 1998). Combined with regional geological contexts and any possible external age control and calibration points, this has enabled stratigraphical schemes to be constructed for large regions and continents (e.g., Bell *et al*., 2004; Cione and Tonni, 2005; Nomade *et al*., 2014, Flynn and Wu, 2017).

Several independent mammal-based Quaternary chronologies have been developed for western and

central Europe (e.g., Guérin, 1982; Horáček and Ložek, 1988; Rook and Martínez-Navarro, 2010). One of the richest regional records of fossil mammals, including arvicoline rodents, is known in the south of eastern Europe (Alexandrova, 1976; Alexeeva, 1977, 1990; Markova, 1982, 1990, 2007; Topachevsky *et al*., 1987, 1998; Rekovets and Nadachowski, 1995; Bajgusheva *et al*., 2001; Nesin and Nadachowski, 2001; Tesakov, 2004; Titov, 2008; Agajanian, 2009, Markova and Vislobokova, 2016; and many others). The Pliocene and Pleistocene sequences from the Sea of Azov region and neighbouring areas, including margins of the Black Sea, North Caucasus and the lower catchment of the Don River, are particularly important (Fig. 1). Continental deposits of different origin (fluvial, lagoonal, loess-paleosol) can be dated by the evolutionary stage in various lineages including *Archidiskodon/Mammuthus, Equus (Allohippus)/Equus (Equus), Arvernoceros/Megaloceros, Eucladoceros/Praemegaceros, Paracamelus/Camelus, Allophaiomys/Microtus, Mimomys/Arvicola* and *Borsodia/Prolagurus/Lagurus*. The chronological framework for the region in question is currently based on biostratigraphy, lithostratigraphy, and paleomagnetism. One of traditional approaches is the use of small mammal assemblages based on reference faunas and first appearance events (e.g., Markova, 2007; Markova and Vislobokova, 2016). However for the Early Pleistocene this system is insufficiently detailed. An important advance in refining the regional stratigraphy was the establishment of the MQR/MNR mammal zonation for the Plio-Pleistocene of southern East Europe and western Asia. This was based on the consistent application of concurrent range zones for several rapidly-evolving phyletic lineages of arvicoline rodents (Vangengeim *et al*., 2001; Tesakov, 2004; Tesakov *et al*., 2007a). Fourteen zones were defined in the Quaternary (three MNR units for the Gelasian and 11 MQR units for the rest of Quaternary, ca. 2.6 Ma to Recent (Fig. 1). This East European Mammal zonation has since been applied to other regions including the Ponto-Caspian (Krijgsman et al., 2019), the Southern Caucasus (Tesakov, 2016; Tesakov *et al*., 2019a), Lower Volga (Zastrozhnov *et al*., 2018), Urals (Borodin *et al*., 2019), Anatolia (van den Hoek Ostende *et al*., 2015), central Europe (Mayhew, 2012) and as far west as the Netherlands and Britain (Mayhew, 2015, Preece *et al*., 2020).

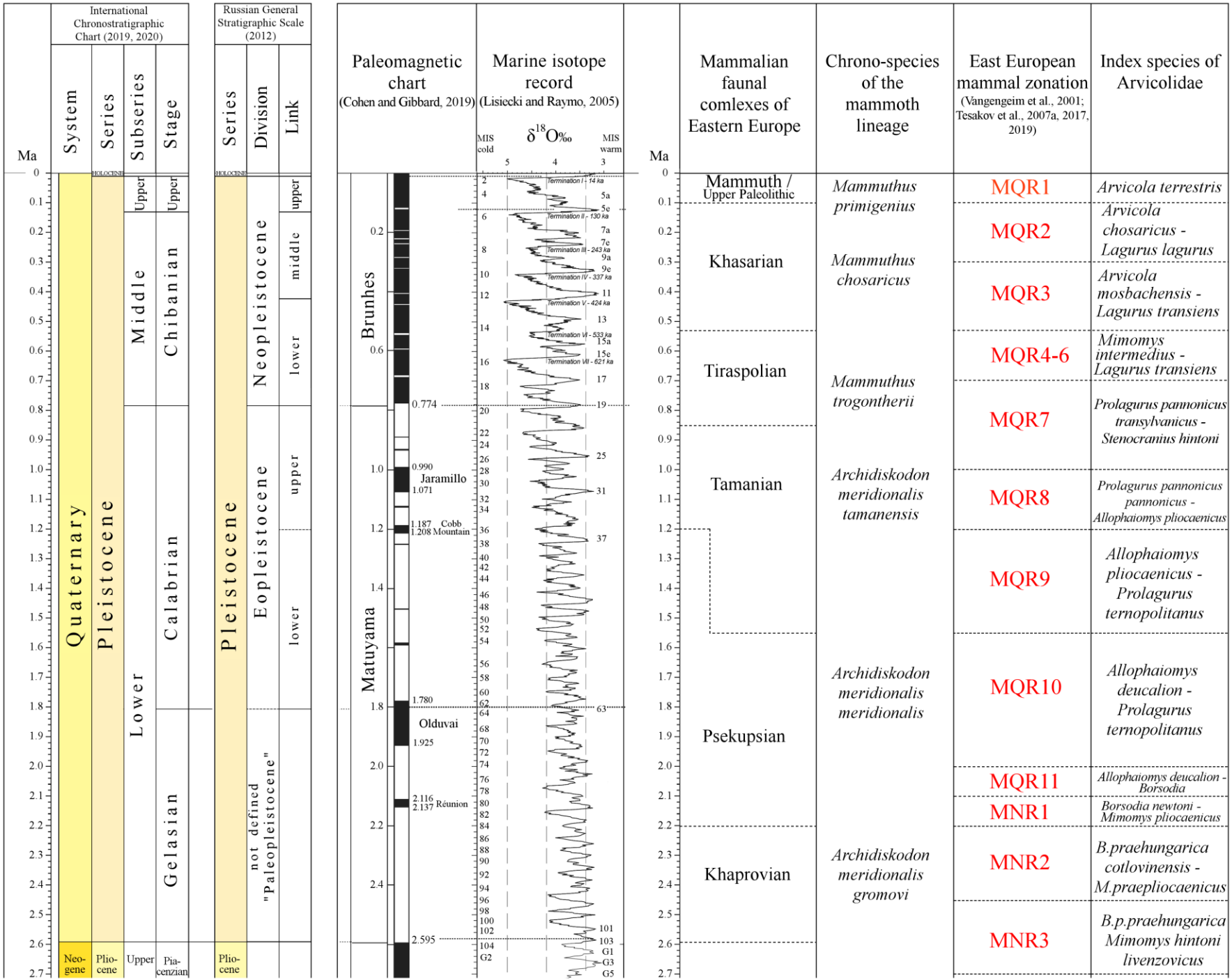

International Chronostratigraphic Chart (2019, 2020)
Russian General Stratigraphic Scale (2012)
Ma
System
Series
Subseries
Stage
Series
Division
Link
Quaternary
Pleistocene
Lower
Middle
Upper
Gelasian
Calabrian
Chibanian
Upper
Pleistocene
Eopleistocene
Neopleistocene
not defined "Paleopleistocene"
lower
upper
middle
lower
upper
Neo-gene
Plio-cene
Upper
Pia-cenzian
Plio-cene
Paleomagnetic chart
(Cohen and Gibbard, 2019)
Brunhes
Matuyama
0.774
0.990
Jaramillo
1.071
1.187
1.208
Cobb Mountain
1.780
Olduvai
1.925
2.116
2.137
Réunion
2.595
Marine isotope record
(Lisiecki and Raymo, 2005)
MIS cold
MIS warm
δ18O‰
Termination I - 14 ka
Termination II - 130 ka
Termination III - 243 ka
Termination IV - 337 ka
Termination V - 424 ka
Termination VI - 533 ka
Termination VII - 621 ka
Mammalian faunal comlexes of Eastern Europe
Mammuth / Upper Paleolithic
Khasarian
Tiraspolian
Tamanian
Psekupsian
Khaprovian
Chrono-species of the mammoth lineage
Mammuthus primigenius
Mammuthus chosaricus
Mammuthus trogontherii
Archidiskodon meridionalis tamanensis
Archidiskodon meridionalis meridionalis
Archidiskodon meridionalis gromovi
East European mammal zonation
(Vangengeim et al., 2001; Tesakov et al., 2007a, 2017, 2019)
MQR1
MQR2
MQR3
MQR4-6
MQR7
MQR8
MQR9
MQR10
MQR11
MNR1
MNR2
MNR3
Index species of Arvicolidae
Arvicola terrestris
Arvicola chosaricus - Lagurus lagurus
Arvicola mosbachensis - Lagurus transiens
Mimomys intermedius - Lagurus transiens
Prolagurus pannonicus transylvanicus - Stenocranius hintoni
Prolagurus pannonicus pannonicus - Allophaiomys pliocaenicus
Allophaiomys pliocaenicus - Prolagurus ternopolitanus
Allophaiomys deucalion - Prolagurus ternopolitanus
Allophaiomys deucalion - Borsodia
Borsodia newtoni - Mimomys pliocaenicus
B.praehungarica cotlovinensis - M.praepliocaenicus
B.p.praehungarica Mimomys hintoni livenzovicus

Figure 1. Biostratigraphical chart of south-eastern Europe based on mammals (adapted from Tesakov *et al*., 2017, 2019b); taxonomy of *Archidiskodon/Mammuthus meridionalis* follows Baygusheva and Titov (2012).

Biostratigraphy has been successful for establishing a regional succession, but correlation between regions can be problematic because migrational events can be diachronous (Walsh, 1998). It is also limited by the ranges and preservation of critical species, which are not always represented in the fragmentary continental fossil record. In Europe, the Early Pleistocene is an important time period for early human evolution, and it is essential that a reliable chronology is developed for this period. A robust chronology needs external dating and cross-checking. Attempts to test this mammalian biostratigraphy using radiometric and luminescence dating are ongoing, but challenging due to temporal constraints of the methods and absence of volcanic deposits throughout the region. In this study we use the developments in amino acid geochronology from the intra-crystalline fraction of molluscan opercula to test the robustness of the East European Mammal Zonation.

The advances in intra-crystalline preparative methods (Penkman *et al*., 2008) and choice of material for analysis (Penkman *et al*., 2007, 2010) have improved the precision and accuracy in amino acid geochronology, providing relative age estimates that extend far beyond the limits of current radiocarbon, U-series and luminescence timescales. The calcite opercula of the bithyniid freshwater snails are an excellent repository for the original protein, providing an intra-crystalline closed system that is better protected from post-depositional environmental contamination other than mineral diagenesis. The measurement of opercula intra-crystalline protein degradation (IcPD) has become an inexpensive and rapid method for establishing a robust relative chronology that can be calibrated against sites of known age (Penkman *et al*., 2011, 2013).

The presence of bithyniid opercula from the same reference sites on which the East European Mammal zonation of the Black Sea and Azov Sea regions was defined provides an opportunity to test the veracity of this scheme. In this paper, we develop an aminostratigraphy for the Sea of Azov region using two genera (*Parafossarulus* and *Bithynia*) of freshwater gastropod snails from the family Bithyniidae that have

calcitic opercula.

2. Materials and methods

Bithyniids are common freshwater gastropods that inhabit a wide variety of habitats. *Parafossarulus* can be separated from *Bithynia* by its larger size, well-developed spiral sculpture and differences in the opercula (Annandale, 1924), which have a concentric form in *Bithynia* but are paucispiral in *Parafossarulus* (Girotti, 1972; Meijer, 1974; Zatravkin *et al*., 1989; Sanko, 2007, see Fig. 2). This generic distinction has recently been supported by molecular phylogenetic data (Wilke *et al*., 2013). In Europe, *Bithynia* is still extant, but *Parafossarulus* became extinct during the Middle Pleistocene, ~ 400 ka (Meijer, 1974; 1986; 1989; Preece, 1990; Gittenberger *et al*., 1998; Sanko, 2007; Lewis *et al*., 2004). *Parafossarulus* still inhabits eastern Asia, where its distribution suggests that it possibly has a greater affinity for warmer environments than *Bithynia*. In most Early and early Middle Pleistocene localities in the Azov region both genera occur. The extinction of *Parafossarulus* in Europe may be related to the specific features of the 100 ka climatic cyclicity period and the more extreme minimum temperatures attained during the glacial periods.

Figure 2. Opercula of *Bithynia* (1), with a concentric form, and *Parafossarulus* (2), which is paucispiral. Scale bar equals 1 mm.

Ninety-five opercula from *Parafossarulus* and *Bithynia* were selected from 26 horizons at 16 sites (Table 1) that have also yielded mammal remains that enable them to be directly linked to the mammal assemblage zones of the regional biostratigraphy (Vangengeim *et al*., 2001; Tesakov *et al*., 2007a). Most of the fossil localities are situated along the shores of the Taganrog Gulf of the Sea of Azov, the lower catchment of the Don River, and the western part of North Caucasus including the Taman Peninsula (Fig. 3). Material from multi-layered Early Pleistocene locality Kryzhanovka (SW Ukraine) came from the lower, Gelasian, part of the section (Tesakov, 2004), not to be confused with the well-known upper bed of Kryzhanovka correlated with the Gelasian-Calabrian transition (Rekovets, Nadachowski, 1995). The Port-Katon 4 locality (Tesakov et al., 2007), of mid Middle Pleistocene age, is much younger than the well-known Early Pleistocene site of Port-Katon (Markova, 1982, 1990). The opercula, collected between 1988 and 2018, were mostly extracted in the field, but some sediment samples needed hot water for disaggregation in the laboratory. Heating increases amino acid D/L values, but the duration of the hot water disaggregation (opercula would only have experienced heating > 40°C for less than 30 minutes) would not have been sufficient to alter the D/L values. Samples were stored at the Geological Institute RAS (Moscow) and Southern Scientific Centre RAS (Rostov-on-Don) before transfer to the University of York for amino acid analysis.

To establish the IcPD behaviour of *Bithynia* and *Parafossarulus*, we also analysed 11 opercula from two additional sites outside the main study region where both genera co-occur. These sites are Korotoyak V7 (=Korotoyak 3a, Uspenka Suite, Early Pleistocene, not to be confused with a slightly younger Early Pleistocene site of Korotoyak 3c, Ostragozh Suite described by Markova, 2005) in the upper catchment of the Don River (Iossifova and Semenov, 1998; Agajanian, 2009) and the Tiglian type-site at Tegelen, the Netherlands (Freudenthal *et al*., 1976; Penkman *et al*., 2013).

173 Table 1: Samples analysed in study (full details in SI).

Locality Number and code	**Site & reference**	**Geographical location**	**Chronostratigraphy**	**MQR/ MNR**	**Age range estimate, Ma**	**Genus**	**n**
1. Krv	Krivsky [Chegis *et al*., 2017]	Lower Don River	Late Pliocene, Piacenzian	MNR5	>2.5	*Parafossarulus*	4
2. Kr2	Kryzhanovka 2 [Tesakov, 2004]	NW Black Sea coast, Ukraine	early Early Pleistocene, Gelasian	MNR3	ca. 2.4	*Parafossarulus*	3
2. Kr3	Kryzhanovka 3 [Tesakov, 2004]	NW Black Sea coast, Ukraine	Early Pleistocene, Gelasian	MNR2	2.2-2.3	*Parafossarulus*	4
3. Psk	Psekups [Tesakov, 2004]	North Caucasus	Early Pleistocene, Gelasian	MNR1	2.1-2.2	*Bithynia*	3
4. Tz1	Tizdar 1 [Tesakov, 2004]	Taman Peninsula	Early Pleistocene, Gelasian-Calabrian transition	MQR11	2.0-2.1	*Bithynia*	4
4. Tz1	Tizdar 1 [Tesakov, 2004]	Taman Peninsula	Early Pleistocene, Gelasian-Calabrian transition	MQR11	2.0-2.1	*Parafossarulus*	4

4. TzK	Tizdar K [Shchelinsky *et al*., 2016]	Taman Peninsula	Early Pleistocene, Gelasian-Calabrian transition	MQR10	1.6-2.0	*Bithynia*	4
4. Tz2	Tizdar 2 [Tesakov, 2004]	Taman Peninsula	Early Pleistocene, Gelasian-Calabrian transition	MQR10	1.6-2.0	*Bithynia*	4
4. Tz2	Tizdar 2 [Tesakov, 2004]	Taman Peninsula	Early Pleistocene, Gelasian-Calabrian transition	MQR10	1.6-2.0	*Parafossarulus*	3
5. Srk	Sarkel [Dodonov *et al*., 2007; Nikolskiy *et al*., 2014]	Lower Don River	Early Pleistocene, Gelasian-Calabrian transition	MQR8	1.0-1.2	*Bithynia*	4
6. MaK	Malyi Kut [Pilipenko *et al*., 2015, and new data]	Taman Peninsula	Calabrian, late Early Pleistocene	MQR7-8	0.9-1.0	*Parafossarulus*	2
7. Se1	Semibalki 1 [Tesakov *et al*., 2007b]	Taganrog Gulf	Calabrian, late Early Pleistocene	MQR7	0.8-1.0	*Bithynia*	4
7. Se1	Semibalki 1 [Tesakov	Taganrog Gulf	Calabrian, late Early	MQR7	0.8-1.0	*Parafossarulus*	4

	et al., 2007b]		Pleistocene				
8. Mg2	Margaritovo 2 [Tesakov *et al*., 2007b]	Taganrog Gulf	Calabrian, late Early Pleistocene	MQR7	0.8-1.0	*Bithynia*	4
8. Mg2	Margaritovo 2 [Tesakov *et al*., 2007b]	Taganrog Gulf	Calabrian, Early Pleistocene	MQR7	0.8-1.0	*Parafossarulus*	4
9. Zel	Zelenyi [new data]	Taganrog Gulf	early Middle Pleistocene	MQR4-6	0.5-0.7	*Bithynia*	4
9. Zel	Zelenyi [new data]	Taganrog Gulf	early Middle Pleistocene	MQR4-6	0.5-0.7	*Parafossarulus*	3
7. Se2	Semibalki 2 [Tesakov *et al*., 2007b]	Taganrog Gulf	early Middle Pleistocene	MQR4-6	0.5-0.7	*Parafossarulus*	4
10. Pla	Platovo [Tesakov *et al*., 2007b]	Taganrog Gulf	early Middle Pleistocene	MQR4-6	0.5-0.7	*Parafossarulus*	3
11. Stf	Stefanidinodar [new data]	Taganrog Gulf	early Middle Pleistocene	MQR4-6	0.5-0.7	*Bithynia*	4

12. Tag	Taganrog [new data]	Taganrog Gulf	early Middle Pleistocene	MQR4-6	0.5-0.7	*Parafossarulus*	4
13. PK4	Port-Katon 4 [Tesakov *et al*., 2007b]	Taganrog Gulf	early Middle Pleistocene	MQR3	0.3-0.5	*Parafossarulus*	4
14. BgA	Beglitsa A core [new data]	Taganrog Gulf	late Middle Pleistocene	MQR2	0.2-0.3	*Bithynia*	2
14. BgB	Beglitsa B [Tesakov *et al*., 2007b]	Taganrog Gulf	late Middle Pleistocene	MQR2	0.2-0.3	*Bithynia*	4
15. Leb	Lebyazhiy (=Veshenskaya) [Baygusheva *et al*., 2014]	Lower Don River	late Middle Pleistocene	MQR2	0.2-0.3	*Bithynia*	4
16. SYr	Siniy Yar ‘ [Tesakov *et al*., 2012]	Lower Don River	Late Pleistocene	MQR1	0-0.1	*Bithynia*	4
17. Ky7	Korotoyak V7 [= Korotoyak 3a in Iossifova and	Upper Don River	late Early Pleistocene	MQR8	1.0-1.2	*Bithynia*	4

	Semenov, 1998; = Korotoyak 3 in Agajanian, 2009]						
17. Ky7	Korotoyak V7 [= Korotoyak 3a in Iossifova and Semenov, 1998; = Korotoyak 3 in Agajanian, 2009]	Upper Don River	late Early Pleistocene	MQR8	1.0-1.2	*Parafossarulus*	2
18. Teg	Tegelen [Freudenthal *et al*., 1976; Penkman *et a*l., 2013]	Netherlands	Early Pleistocene, Gelasian	MNR1	2.1-2.2	*Bithynia*	4
18. Teg	Tegelen [Freudenthal *et a*l., 1976; Penkman *et al*., 2013]	Netherlands	Early Pleistocene, Gelasian	MNR1	2.1-2.2	*Parafossarulus*	4

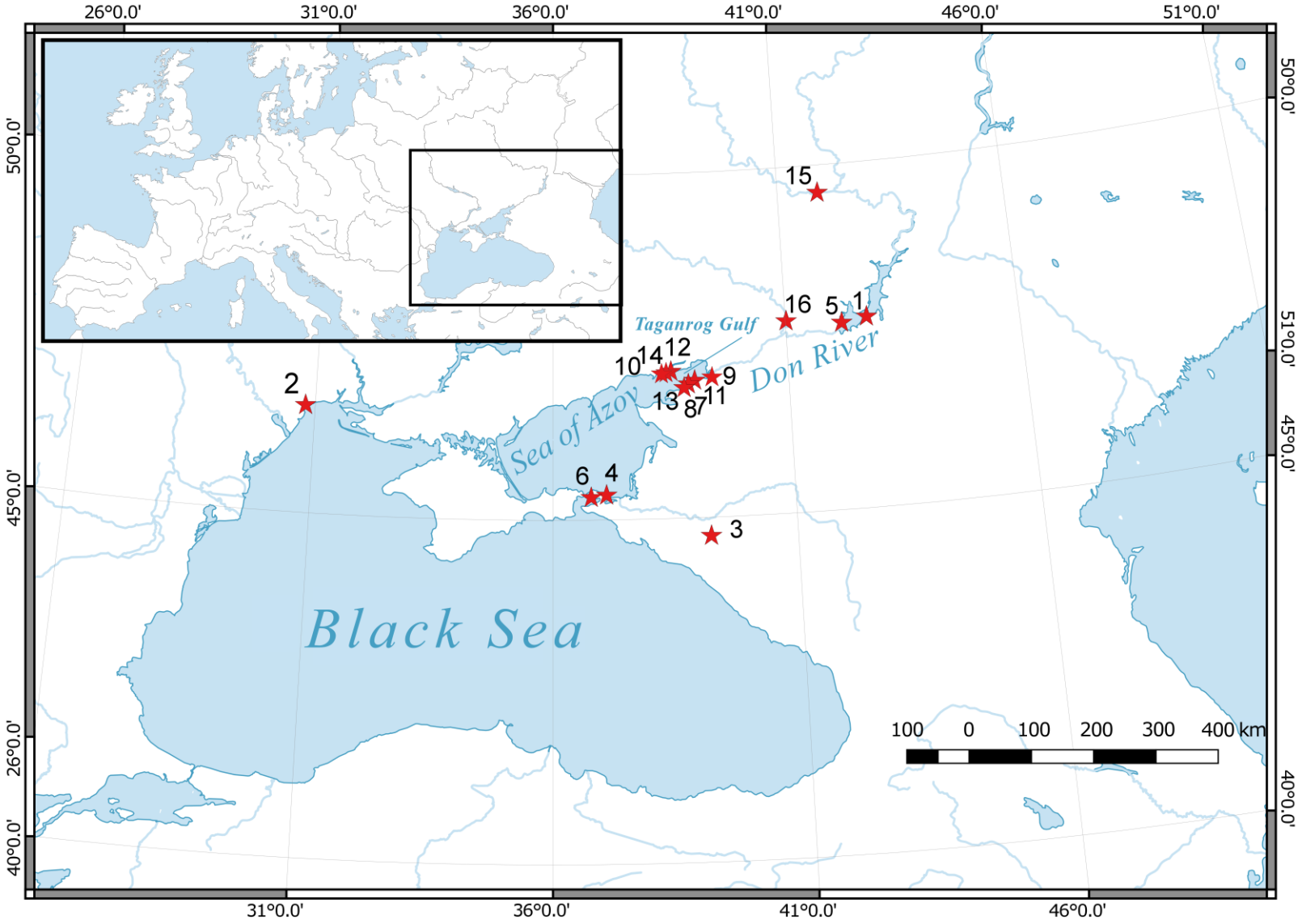


Figure 3. Map of the fossil sites in the Azov region. 1. Krivsky, 2-3. Kryzhanovka 2 and 3, 4.Psekups, 4.Tizdar, 5. Sarkel, 6. Malyi Kut, 7. Semibalki 1 and 2, 8. Margaritovo 2, 9. Zelenyi, 10. Platovo, 11. Stefanidinodar, 12. Taganrog, 13. Port-Katon 4, 14. Beglitsa, 15. Lebyazhiy, 16.

Siniy Yar. Main map source: modified from Natural Earth maps (2019). Inset map: modified from D-maps (2017)

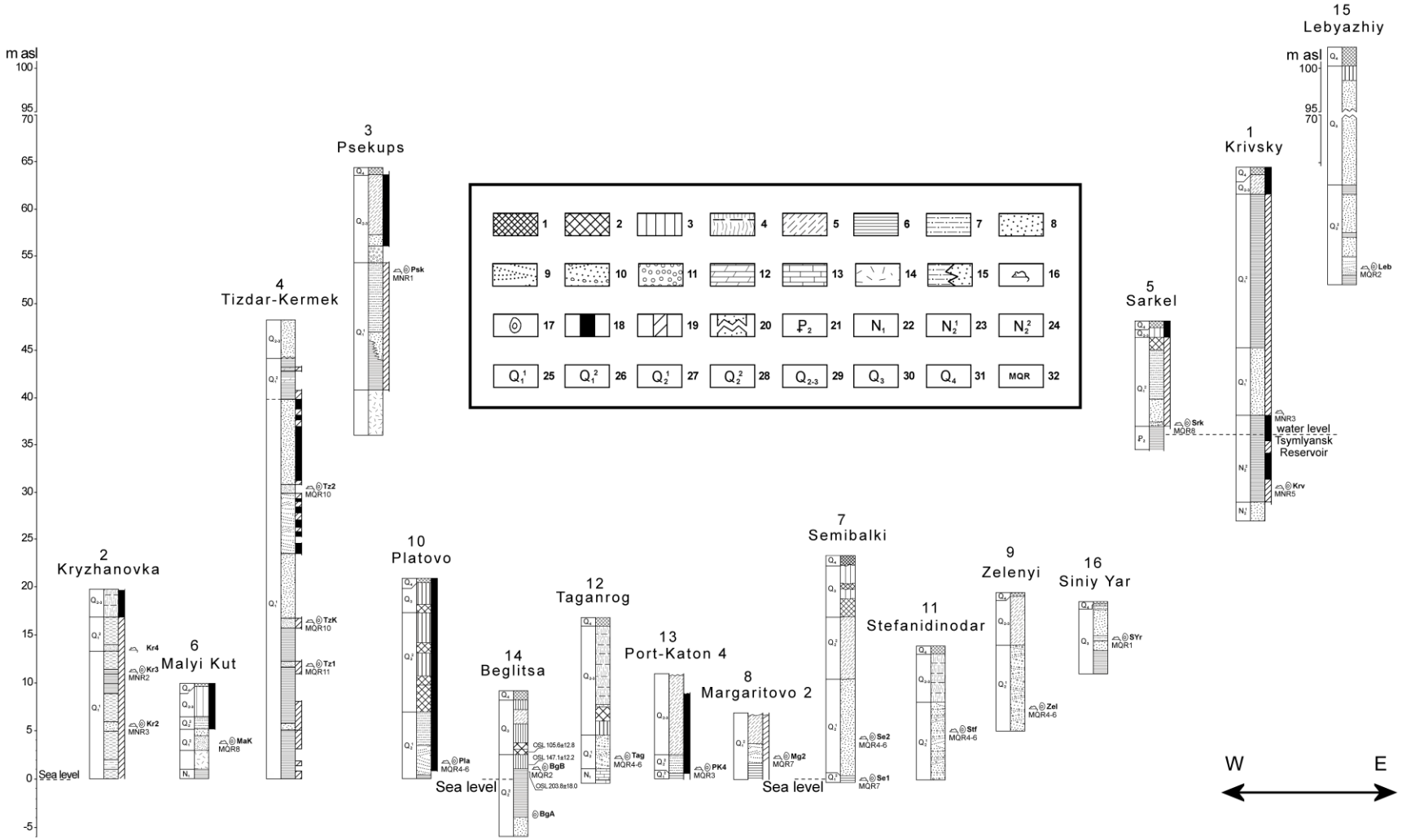


Figure 4. Schematic geological sections of the localities studied with the elevation above sea level (m). 1. Modern soil. 2. Palaeosol. 3. Loess. 4. Loess-palaeosol sequence. 5. Subaerial loam. 6. Clay. 7. Sandy clay. 8. Sand. 9. Cross-bedded sand. 10. Gravel. 11. Pebbles.12. Marl. 13. Limestone. 14. Talus. 15. Facies boundaries. 16. Mammal remains relevant to biostratigraphy. 17. Opercula sampled in this study. 18. Normal palaeomagnetic polarity. 19. Reversed polarity. 20. Thickness not to scale. 21. Eocene. 22. Miocene. 23. Early Pliocene. 24. Late Pliocene. 25. Early Pleistocene (Gelasian). 26. Early Pleistocene (Calabrian). 27. early Middle Pleistocene. 28. late Middle Pleistocene. 29. Middle-Late

Pleistocene. 30. Late Pleistocene. 31. Holocene. 32. Biostratigraphical zones. Data from Baygusheva *et al*., 2014; Chegis *et al*., 2017; Chen *et al*., 2018; Dodonov *et al*., 2007; Pilipenko *et al*., 2015; Shchelinsky *et al*., 2016; Tesakov, 2004; Tesakov *et al*., 2007b, 2012, 2019b. Numbers above section names match site numbers in Figure 3.

All samples were prepared using the procedures of Penkman *et al*. (2008) to isolate the intra-crystalline protein by bleaching. In brief, two subsamples were then taken from each operculum; one fraction was directly demineralised and the free amino acids analysed (referred to as the 'free' amino acids, FAA), and the second was hydrolysed at 110°C for 24 hours to release the peptide-bound amino acids, thus yielding the 'total' amino acid concentration, referred to as the ‘total hydrolysable amino acid fraction (THAA). Samples were analysed in duplicate by RP-HPLC, with standards and blanks run alongside samples. During hydrolysis, both asparagine and glutamine undergo rapid irreversible deamination to aspartic acid and glutamic acid, respectively (Hill, 1965). It is therefore not possible to distinguish between the acidic amino acids and their derivatives and they are reported together as Asx and Glx, respectively.

The D/L values of aspartic acid/asparagine, glutamic acid/glutamine, serine, alanine and valine (D/L Asx, Glx, Ser, Ala, Val) and the concentrations of Ser and Ala ([Ser]/[Ala]) were then assessed to provide an overall estimate of intra-crystalline protein decomposition (IcPD). These amino acids are the best chromatographically resolved enantiomer pairs for opercula (Powell *et al*., 2013), and between them also cover a wide temporal range (Penkman *et al*., 2011). In a closed system, the amino acid ratios of the FAA and the THAA subsamples should be highly correlated, enabling the recognition of compromised samples (e.g., Preece and Penkman, 2005). The D/L of an amino acid will increase with increasing time, whilst the [Ser]/[Ala] value will decrease. Each amino acid racemises at different rates, and therefore is useful over different timescales. The D/L of Ser is less useful as a geochronological tool as its breakdown patterns mean that a single D/L value can represent more than one time-point in samples of this age. However, D/L Ser is reported here as aberrant values are useful indications of contamination (e.g. Williams and Smith, 1977; Kosnik and Kaufman, 2008).

3. Results and Discussion

3.1 Comparison of D/L values in co-occurring Parafossarulus *and* Bithynia

Five Russian sites yielded *Bithynia* and *Parafossarulus* opercula from the same horizons: two from the Taman Peninsula (Tizdar 2, Tizdar 1) and three from the margins of the Taganrog Gulf (Margaritovo 2, Semibalki 1, Zelenyi). IcPD data from these have been compared with data from Korotoyak V7 (Upper Don region) and previously published data from the type-site of the Tiglian (Penkman *et al*., 2011; 2013).

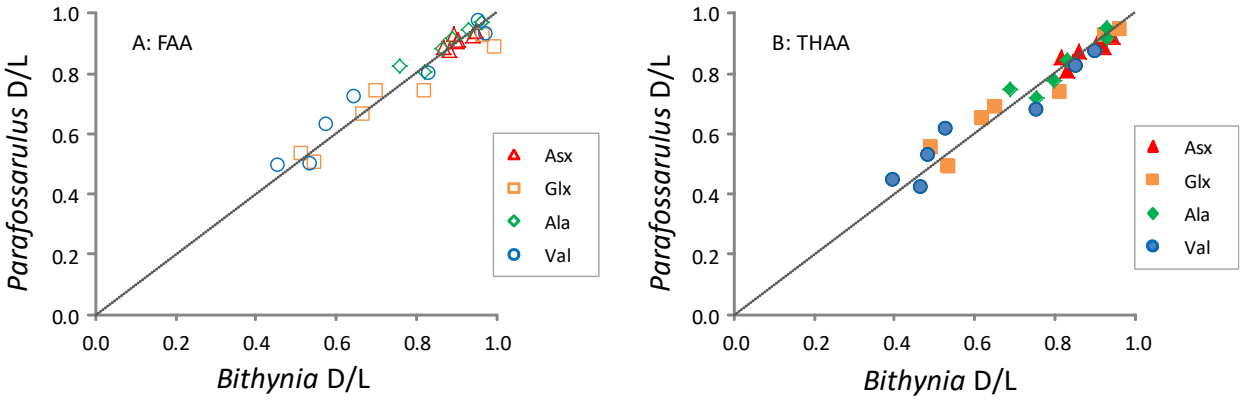


Fig. 5: Comparison of mean D/L values of *Parafossarulus* and *Bithynia* for (A) the free amino acids (FAA) and (B) the total hydrolysable amino acids (THAA) Asx, Glx, Ala and Val from the Azov sites Tizdar 1, Tizdar 2, Margaritovo 2, Semibalki 1 and Zelenyi, the Lower Don site Korotoyak and the Tiglian type-site at Tegelen; the grey line indicates a 1:1 relationship.

There is a strong correlation between the extent of racemisation in the opercula of each genera from the same horizons (Fig. 5). A Student's 2-tailed t-test (or Mann-Whitney for data that was not normal), which assesses the probability that the two samples are derived from the same population, showed that in 23 cases (41%) the amino acids in *Bithynia* were more racemised than in *Parafossarulus* from a site. In 14 cases (25%) amino acids from *Parafossarulus* were more racemised than those from *Bithynia*, and in 19 cases there was no significant difference (34%). There are therefore no consistent differences between the racemisation of these genera from the same stratigraphical horizon (Table 2; Fig.6). Analysis of a greater number of individuals per site (as well as a larger number of sites) will enable any interspecific differences to be better resolved, but we conclude from the existing dataset that it is possible to make

direct comparisons of the IcPD data from the opercula of these two genera, which can therefore be used interchangeably, providing complementary data.

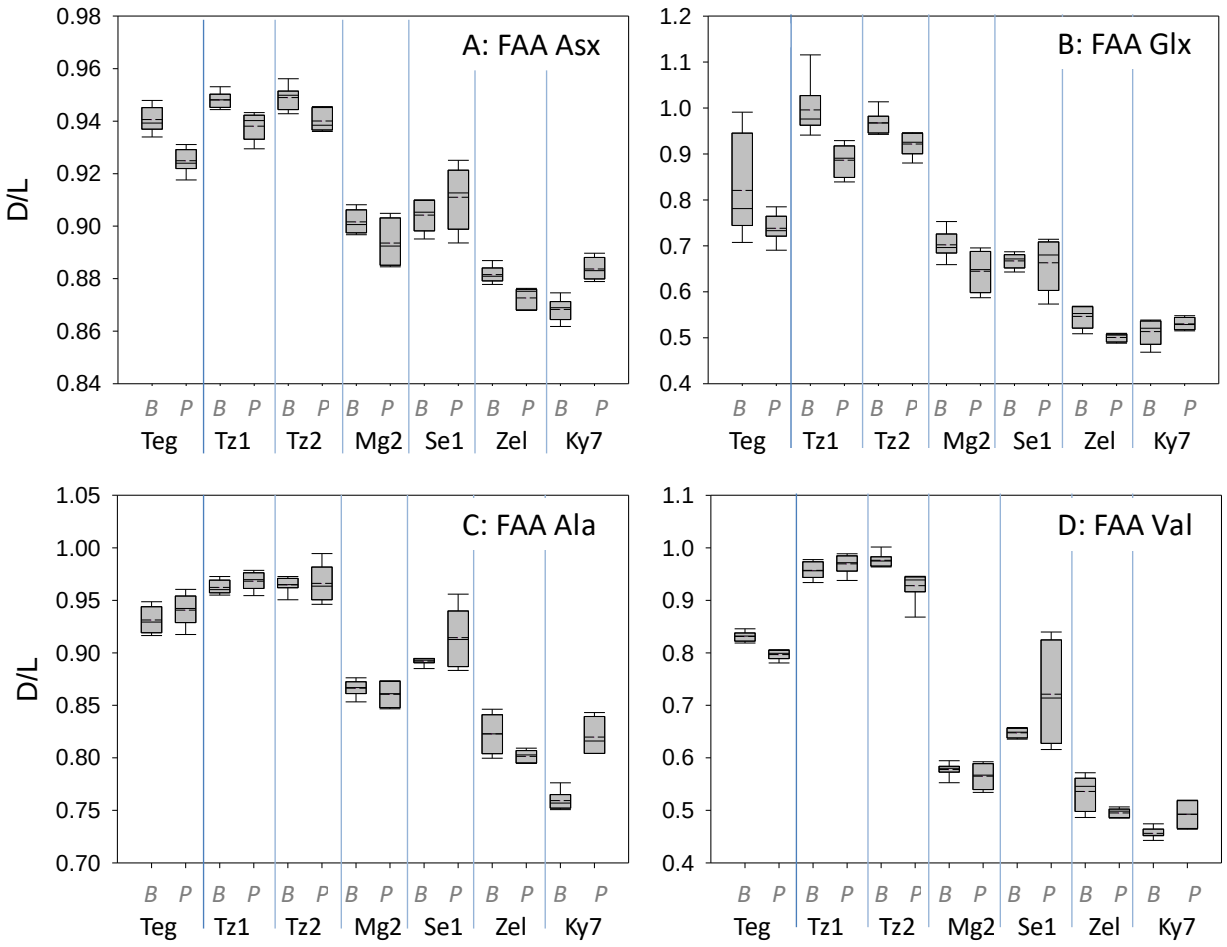
239

	FAA								THAA								Which genus is higher?		
Site	Asx		Glx		Ala		Val		Asx		Glx		Ala		Val		*B* > *P* (n)	*P* > *B* (n)	no sig. diff.
Teg	**0.000**	*B*	*0.050*		0.184		**0.000**	*B*	***0.003***	*B*	***0.013***	*B*	*0.108*		**0.001**	*P*	4	1	3
Tz1	**0.001**	*B*	**0.001**	*B*	0.186		0.201		**0.035**	*B*	**0.009**	*B*	**0.000**	*P*	**0.000**	*P*	4	2	2
Tz2	**0.002**	*B*	**0.005**	*B*	0.847		***0.002***	*B*	0.993		0.852		**0.032**	*B*	***0.003***	*P*	4	1	3
Mg2	0.068		**0.024**	*B*	0.364		0.267		***0.004***	*B*	0.115		**0.001**	*B*	**0.000**	*P*	3	1	4
Se1	0.166		0.846		**0.049**	*P*	0.054		*0.161*		0.272		0.423		**0.029**	*P*	0	2	6
Zel	**0.001**	*B*	**0.002**	*B*	**0.028**	*B*	**0.024**	*B*	**0.011**	*B*	**0.008**	*B*	**0.001**	*B*	**0.000**	*B*	8	0	0
Ky7	**0.000**	*P*	0.285		**0.000**	*P*	**0.009**	*P*	***0.004***	*P*	***0.004***	*P*	**0.000**	*P*	**0.000**	*P*	0	7	0
B > *P* (n)	4		4		1		3		*4*		3		3		1		23		
P > *B* (n)	1		0		2		1		*1*		1		2		6			14	
no sig. diff.	2		3		4		3		*2*		3		2		0				19
%																	41.1%	25.0%	33.9%

240

241 Table 2: p-values for the Student's 2-tailed t-test (for normally distributed data) and Mann-Whitney tests (for non-normal data, p-value is
242 italicised) for Asx, Glx, Ala and Val D/L in both FAA & THAA for *Parafossarulus* (*P*) and *Bithynia* (*B*) at each site: Teg = Tegelen; Tz1 = Tizdar
243 1; Tz2 = Tizdar 2; Mg2 = Margaritovo; Se1 = Semibalki 1; Zel = Zelenyi; Ky7 = Korotoyak. Bold values show that the extent of racemisation is
244 statistically different between the two genera at that site at the 95% confidence level; the genus that is higher is signified by its initial. No
245 consistent offset between the two genera is observable.

246


A: FAA Asx
B: FAA Glx
C: FAA Ala
D: FAA Val
D/L
B P
Teg
Tz1
Tz2
Mg2
Se1
Zel
Ky7

247

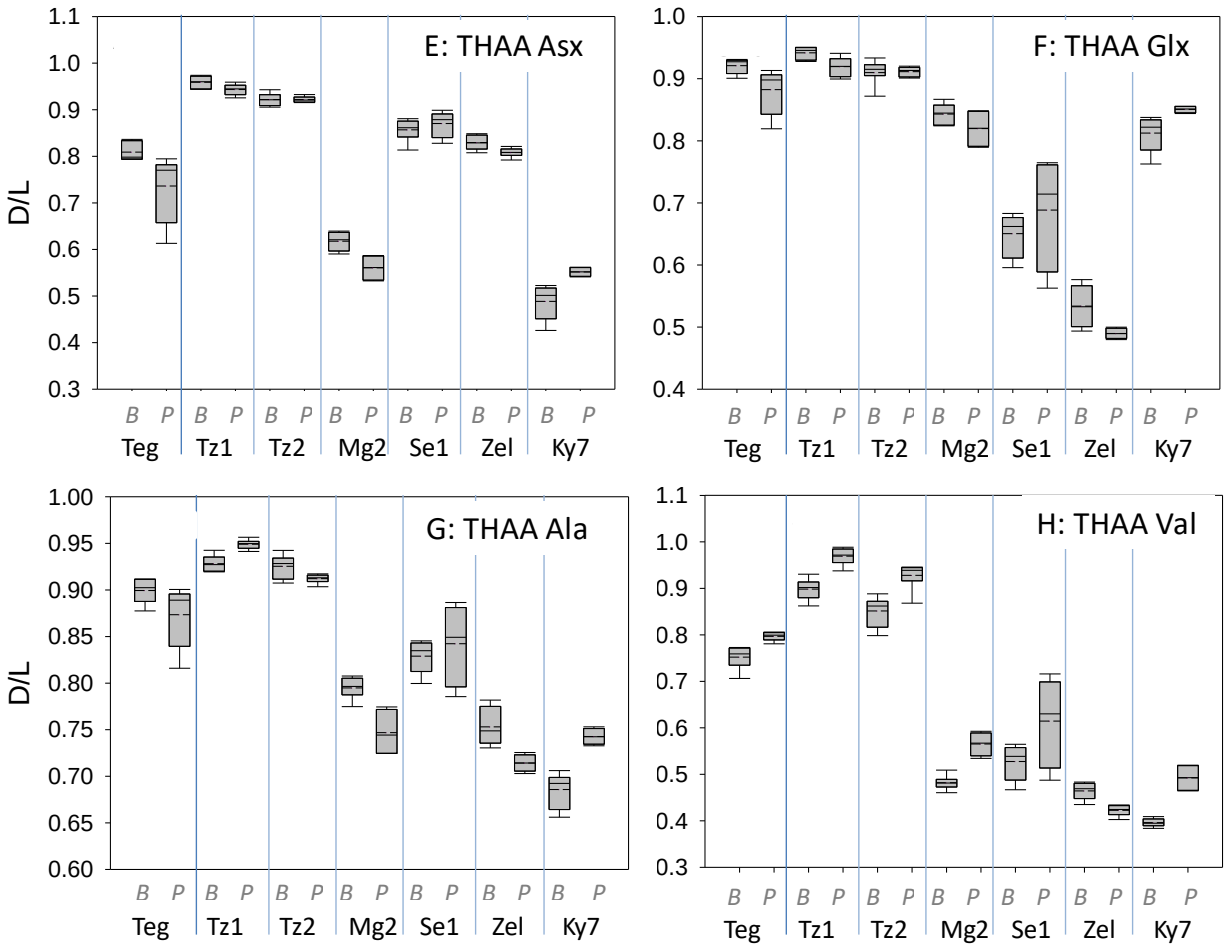


Figure 6: D/L of free amino acid (FAA: A, B, C & D) and total hydrolysable amino acid (THAA: E, F, G & H) intra-crystalline fractions of *Bithynia* (*B*) and *Parafossarulus* (*P*) opercula from the same horizons for the 7 sites. Site abbreviations are as in Table 2. For each sample, the box encloses the 25th and 75th percentiles. Within the box, the solid line indicates the median and the dashed line shows the mean. Where enough data points are available, the 10th and 90th percentiles can be calculated (shown by lines below and above the boxes respectively). The results of each duplicate analysis are included in order to provide a statistically significant sample size. No consistent difference between the D/L values of *Bithynia* and *Parafossarulus* is apparent in this dataset.

3.2. D/L vs mammal zones

The Quaternary biostratigraphical chronology established in southeastern Europe on the basis of stages of mammalian evolution can be compared with the opercula IcPD (D/L values) for the four amino acids reported: alanine (Ala), aspartic acid (Asx), glutamic acid (Glx), and valine (Val). The D/L values plotted against the MQR zonation show a generally consistent pattern of increasing racemisation in successively older samples (Fig. 7).

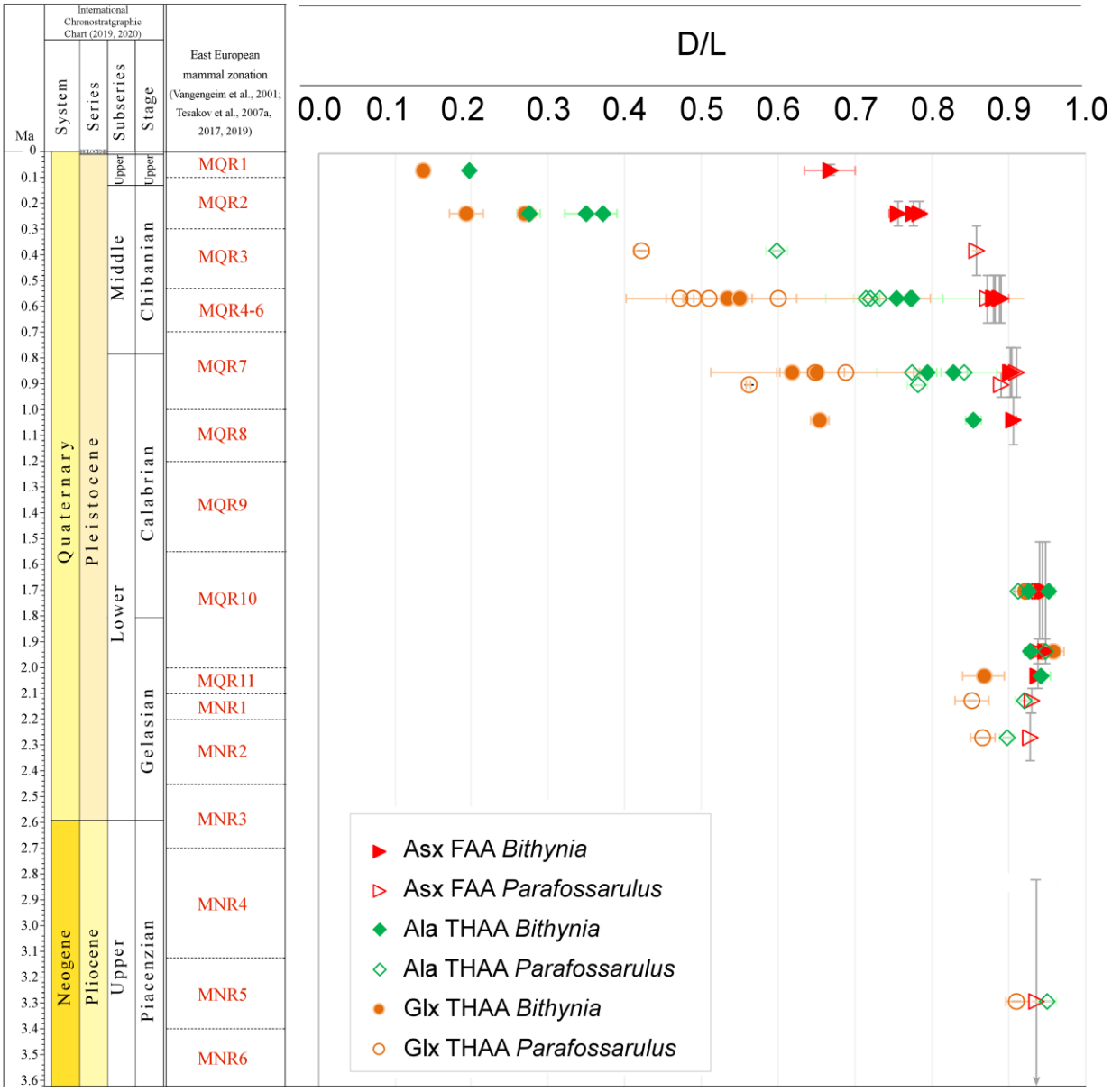
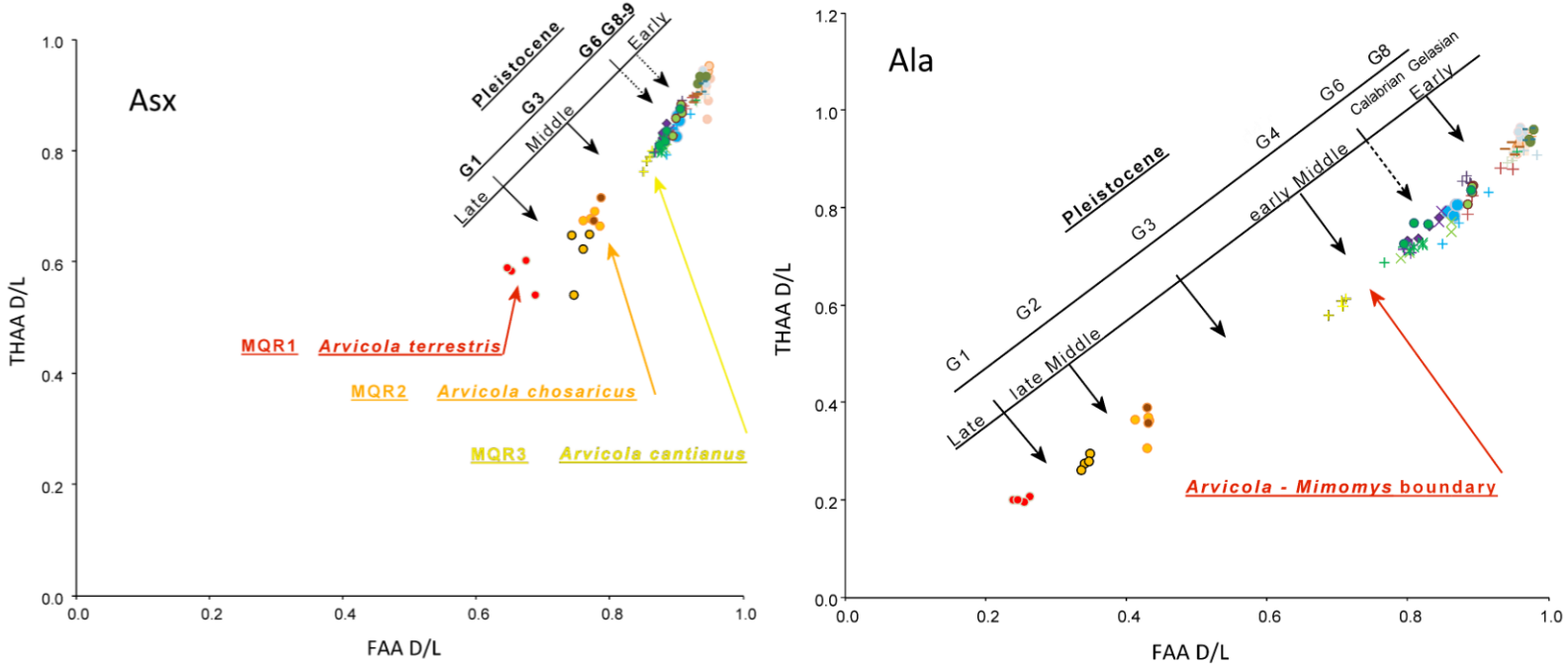


Figure 7. Selected mean D/L values (only FAA Asx, THAA Ala and Glx D/Ls shown for clarity) from all bithyniid opercula from each horizon plotted against the standard global MIS timescale and East European Mammal zonation. For simplicity, the x-error bars, indicating the current uncertainty on the age of the deposits, are shown for the Asx data. Y-error bars indicate two standard deviations about the mean for each site. *Bithynia* opercula are represented by closed symbols; *Parafossarulus* opercula by open symbols.

The extent of IcPD (Fig. 7) increases with increasing age and is stratigraphically consistent with the mammal zonation. Amino acids with faster rates of racemisation (e.g., Asx) enable greater temporal resolution in progressively younger sites, whereas the slower racemisers (e.g., Glx) provide temporal information in this region over at least 2 Ma. In a few cases the D/L values have a larger range than expected, which may result from non-closed system behaviour (Sec. 3.3), an effective diagenetic temperature difference across the region, sites of different ages within a discrete mammal zone (Sec 3.4), or reworking (Sec. 3.5).

3.3. IcPD and closed system behaviour

The extent of intra-crystalline protein decomposition in both the FAA and THAA fractions increases with time, with the temperature dependence of the reactions resulting in increased levels of protein breakdown occurring during warm stages and decreased degradation during cold stages (e.g. Miller *et al*., 1999). Sites within a small geographical area, such as those in the Azov region under discussion, can be assumed to have experienced similar thermal histories, allowing the construction of a regional aminostratigraphic framework on the basis that contemporary sites have similar IcPD values. Plotting FAA D/L values against THAA D/L values shows a high correlation between fractions for each amino acid for the Azov dataset (Figs. 8a-8d). As compromised samples would fall away from the trend (Preece and Penkman, 2005), this indicates that the IcPD in opercula from this region exhibit the expected closed system behaviour. The high variability observed at some sites is therefore unlikely to be due to non closed system behaviour in these samples.


Asx
Pleistocene
G1
G3
G6 G8-9
Late
Middle
Early
MQR1
Arvicola terrestris
MQR2
Arvicola chosaricus
MQR3
Arvicola cantianus
THAA D/L
FAA D/L
0.0
0.2
0.4
0.6
0.8
1.0
Ala
Pleistocene
G1
G2
G3
G4
G6
G8
Calabrian
Gelasian
Late
late Middle
early Middle
Early
Arvicola - Mimomys boundary
THAA D/L
FAA D/L
0.0
0.2
0.4
0.6
0.8
1.0
1.2

294

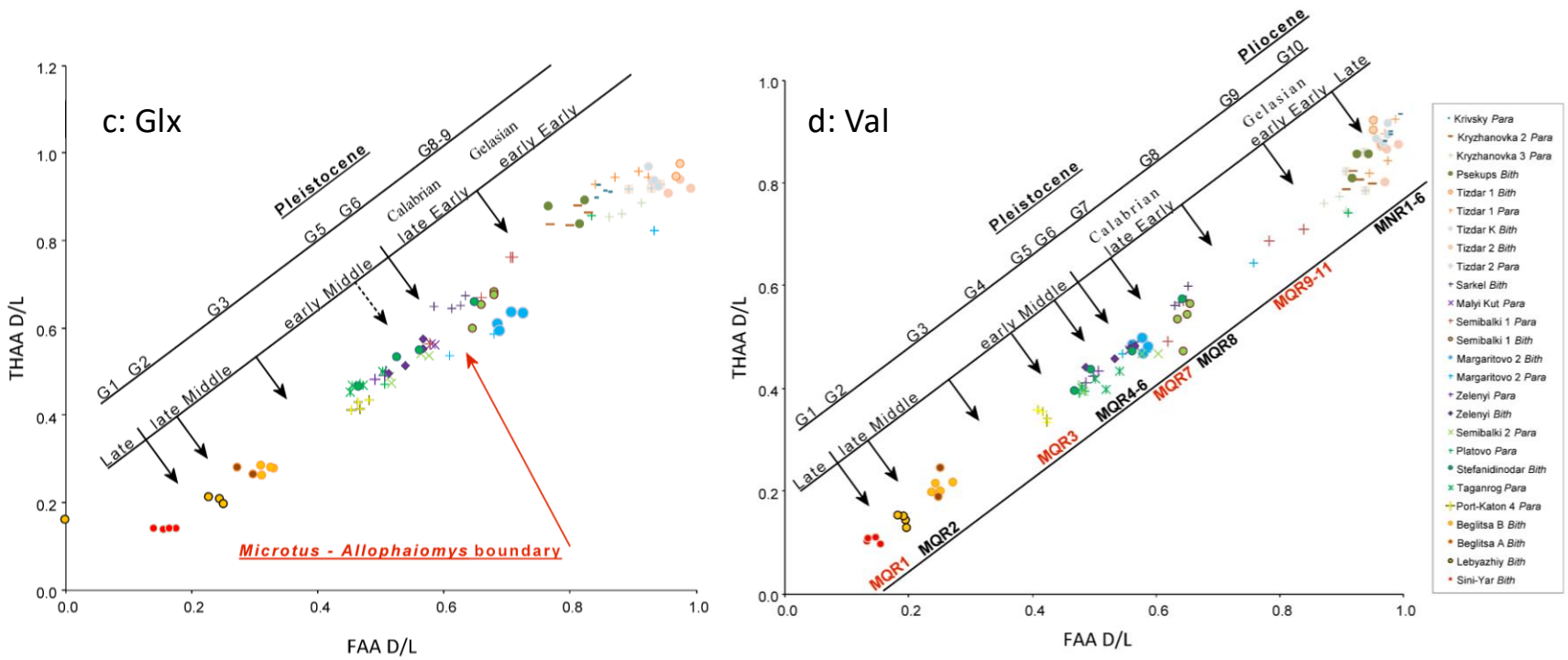


Figure 8. FAA vs THAA D/Ls for (a) Asx, (b) Ala, (c) Glx and (d) Val for the intra-crystalline fraction of individual bithyniid opercula from the Azov region. High correlation between the FAA and THAA fractions indicates closed-system behaviour, providing the foundation for a robust aminostratigraphy. Diagonal axes show how the amino acid data relate to the divisions of the Pleistocene and the East European Mammal zones (MQR/MNR zonation). G1-G10 highlight potential gaps in the range of D/L values that correspond to breaks in the regional fossil record (see section 3.7). Coloured arrows indicate important biostratigraphical events.

3.4 Aminostratigraphic resolution within a mammal l assemblage zone

Fossiliferous levels from Lebyazhiy and Beglitsa are both attributed to MQR2 (late Middle Pleistocene) on the basis of mammalian biostratigraphy and supported by lithostratigraphy. The horizons analysed from Beglitsa are situated below a MIS 5 palaeosol complex Eemian or Mezin paleosol complex of the regional soil stratigraphy (Tesakov *et al*., 2007b)) and the Lebyazhiy fossil bed (Baygusheva *et al*., 2014) are at the base of the third, 40-45 m terrace of the Don River terrace sequence correlated with the late Middle Pleistocene (Krasnenkov and Kazantseva, 1993; Kholmovoi, 200). Both faunas contain water voles with generally undifferentiated enamel (*Arvicola chosaricus*) and an early form of *Mammuthus primigenius* (Tesakov *et a*l., 2007b; Baygusheva *et al*., 2014). The amino acids from opercula analysed from both horizons at Beglitsa (Fig. 4) are consistently more racemised than those from Lebyazhiy (Fig. 8), which either indicates that the Beglitsa opercula are older than those from Lebyazhiy, or that there are significant temperature differences between the sites, which are ~300 km apart, Lebyazhiy being the more northerly. Recent mean annual temperatures in the period of 2000-2019 recorded in the vicinities of both sites differ by about 2°C: with 11.2°C for Beglitsa (meteostation of Taganrog) and 9.1°C (meteostation of Serafimovich) according to the weather archive of the web project “Pogoda i klimat” (accessed May, 2020). The lower temperatures at Lebyazhiy might therefore produce lower IcPD values than contemporary samples from warmer sites, such as Beglitsa. However the IcPD differences might possibly reflect a true difference in age, with Beglitsa being slightly older than Lebyazhiy.

In Beglitsa, the upper horizon with opercula (Beglitsa B) occurs in fluviatile to brackish water estuarine deposits several metres below the Eemian (basal Late Pleistocene) soil complex. It had been assumed to have been deposited relatively close in time to the Eemian, but the duration of the gap between the estuarine deposits with mammals and the Eemian soil was unknown. Recent OSL dating of the Beglitsa section suggests that the age of the lower horizon of the Eemian palaeosol complex (correlated with the Last Interglacial, MIS 5e) is 105.6±12.8 ka, the underlying sandy loess is 147.1 ± 12.2 ka, whereas the basal lagoonal deposits yielding *Bithynia* opercula (Beglitsa B) is 203.8±18.0 ka (Chen et al., 2018), i.e. in

the lower part of MQR2 unit. The lower sample, Beglitsa A, comes from a borehole, 5-6 m below the upper level (Beglitsa B), and shows no significant differences in IcPD from the Beglitsa B sample, implying a similar age for the lagoonal deposits. Although the precise position of the fauna of Lebyazhiy in the time range of MQR2 is unknown, it cannot be excluded that the two sites (Lebyazhiy and Beglitsa) belong to different parts of mammal biozone MQR2 as suggested by IcPD values.

3.5. Evidence of reworking

Bithyniid opercula from discrete horizons at Platovo (MQR 4-6), Margaritovo 2 (MQR7), Semibalki (MQR 4-6 and 7) and Stefanidinodar (MQR 4-6) show a large range of IcPD values between samples, with at least one outlier exhibiting much higher levels of protein breakdown. Reworking of opercula is always a potential issue, because they are composed of calcite that is more stable and robust than shells composed of aragonite, which are more susceptible to diagenetic loss. Moreover, their flat morphology and higher density increases the likelihood of hydrodynamic sorting; indeed, the ratio of bithyniid shell to opercula has been used to identify potential winnowing (Horton *et al*., 1992; Hammarlund and Keen 1994). Both Platovo and Margaritovo are the basal beds of alluvial sequences, where reworking from underlying deposits may be expected. At Platovo, the reworking of the opercula is supported by the reworked small mammals, with redeposited early Calabrian small mammal material present in this horizon (Tesakov *et al*., 2007b).

The sediments from Semibalki 2 are correlated with the early Middle Pleistocene Cromerian complex (MQR4-6). At this site the Cromerian deposits lie immediately on top of latest Calabrian estuarine deposits (‘Tamanian clays’), so there is potential for the opercula with higher D/L values to have been reworked from these underlying deposits. The opercula with the highest D/Ls from Semibalki 2 yielded values similar to the lowest from Semibalki 1 (Fig. 9), a horizon which is correlated with the latest Early Pleistocene and MQR 7. This is therefore consistent with the interpretation that the high D/L value

opercula at Semibalki 2 are reworked from this lower layer. However there is considerable variability in the opercula D/L values at Semibalki 1, indicating significant reworking of the opercula through the sequence at this site (Fig. 9). Although there is no evidence of redeposition from the small mammal fauna, the reworking of the opercula is not inconsistent with the general site histor. The Semibalki 2 deposits form part of the large scale palaeo-Don river, with underlying lagoonal-estuarine deposits of the same large river, so reworking from older deposits due to river erosion is plausible.

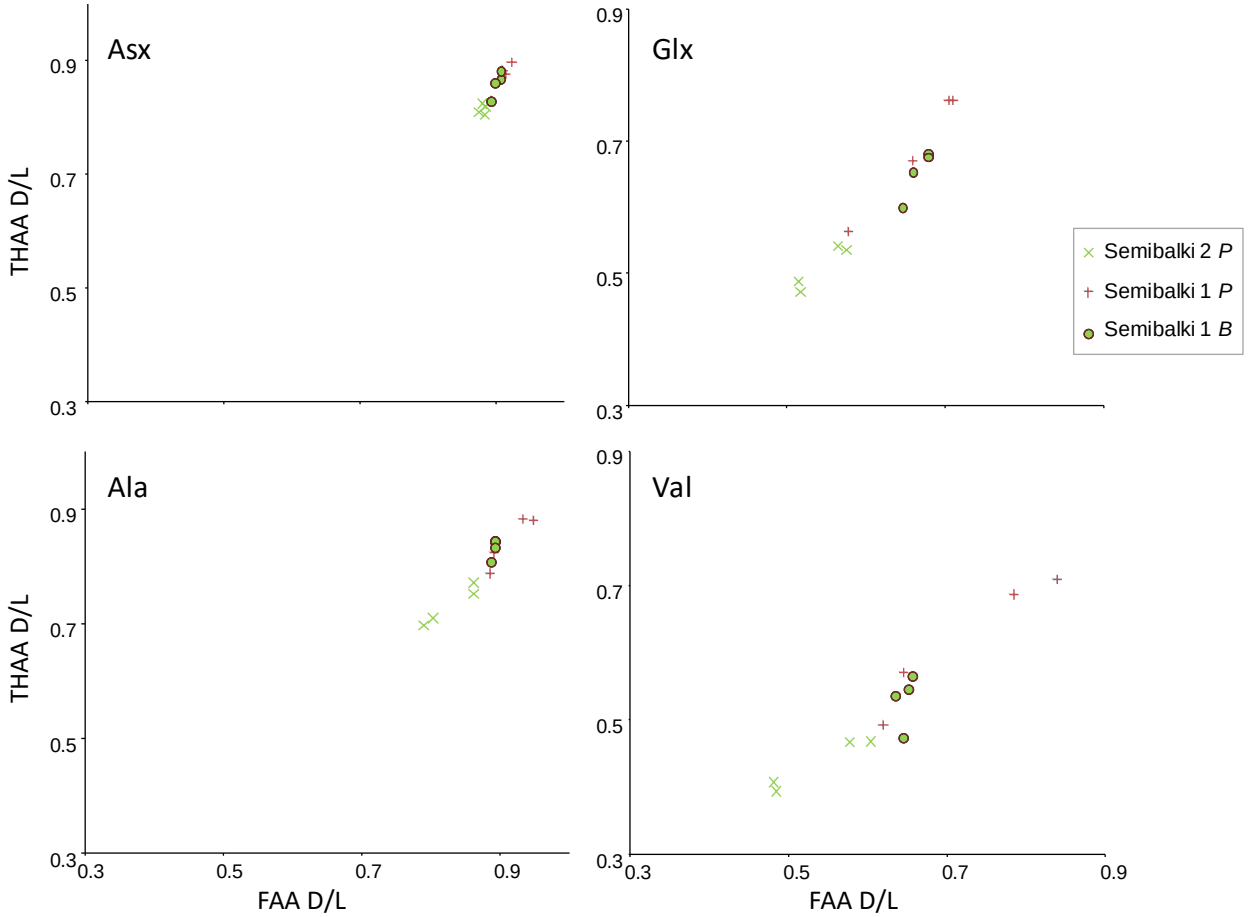


Figure 9. FAA vs THAA D/Ls for Asx, Ala, Glx and Val for the intra-crystalline fraction of bithyniid opercula from Semibalki 1 and 2. N.B. axes do not start at 0.

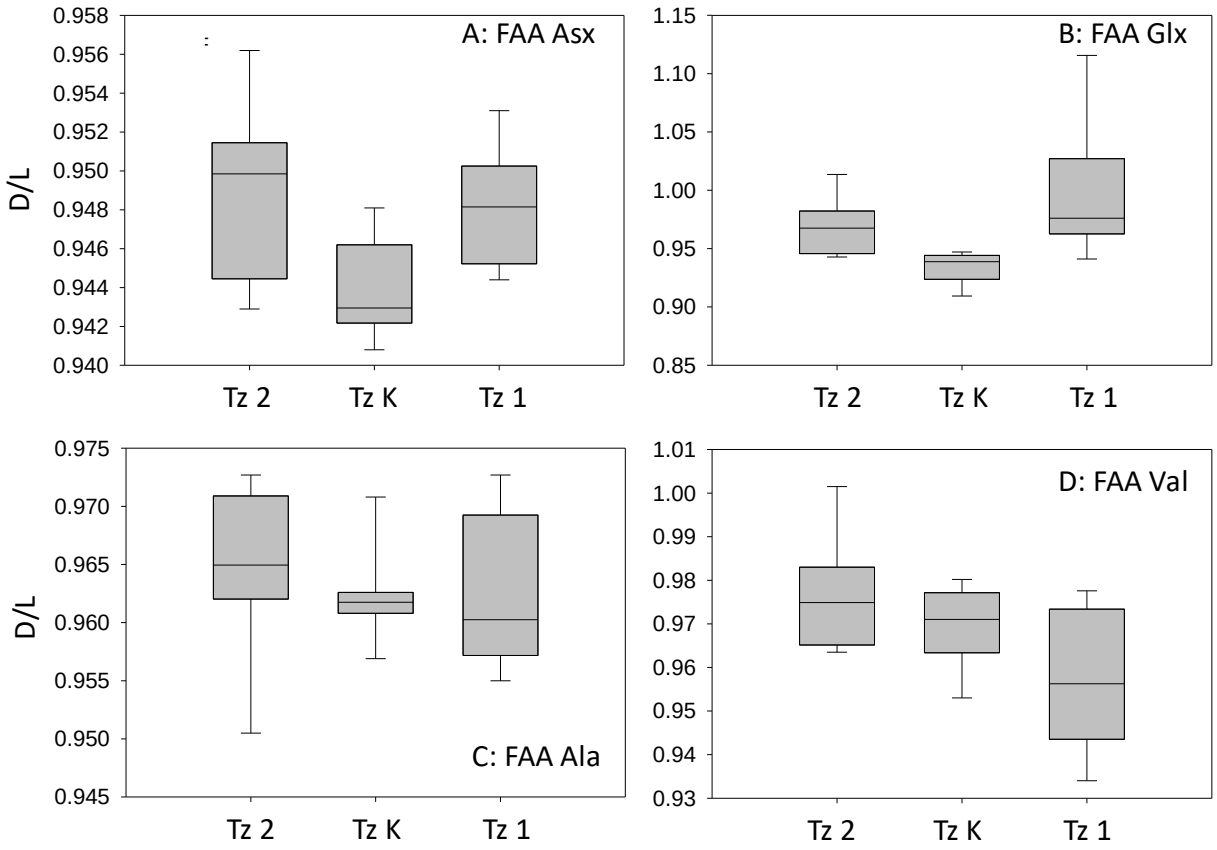
A thick (~20 m) sequence at Stefanidinodar has yielded an early Middle Pleistocene small mammal assemblage attributed to MQR4-6. The IcPD data from three of the bithyniid opercula are consistent with

this age attribution despite exhibiting a larger than expected range of values, but one falls within the cluster of late Early Pleistocene material (Fig. 8). This suggests reworking, although this is otherwise not evident from other palaeontological and lithological data.

The potentially higher potential for reworking of opercula versus other fauna is therefore important to consider when interpreting age based on this method. However IcPD analyses on individual opercula from single horizons could therefore provide a very useful indicator for assessing the integrity of the geological history of fluviatile formations.

3.6 Evidence of geothermal impact on IcPD at Tizdar

Samples have been analysed from three stratigraphically superimposed horizons at Tizdar dating from the Gelasian – Calabrian transition (~ 2 Ma). Tizdar 2, attributed to MQR10 overlies Tizdar K / Kermek (MQR 10), which in turn overlies Tizdar 1 (MQR11). Intra-crystalline protein is clearly still present (Figs. 8a-d) but using FAA alone, the three horizons cannot be resolved (Fig. 10). The THAA for Asx, Glx and Val do show increasing levels of racemisation through the sequence (Fig. 10), which indicates that there is potential age discrimination, although the amino acids are all approaching equilibrium which may preclude any differentiation. Application of the ultra-high pressure liquid chromatography (UHPLC) method (Crisp, 2013), which is able to elute the slower racemising amino acids with baseline resolution, is likely to improve the dating of samples of this age.


A: FAA Asx
B: FAA Glx
C: FAA Ala
D: FAA Val
D/L
D/L
0.958
0.956
0.954
0.952
0.950
0.948
0.946
0.944
0.942
0.940
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.975
0.970
0.965
0.960
0.955
0.950
0.945
1.01
1.00
0.99
0.98
0.97
0.96
0.95
0.94
0.93
Tz 2
Tz K
Tz 1

388

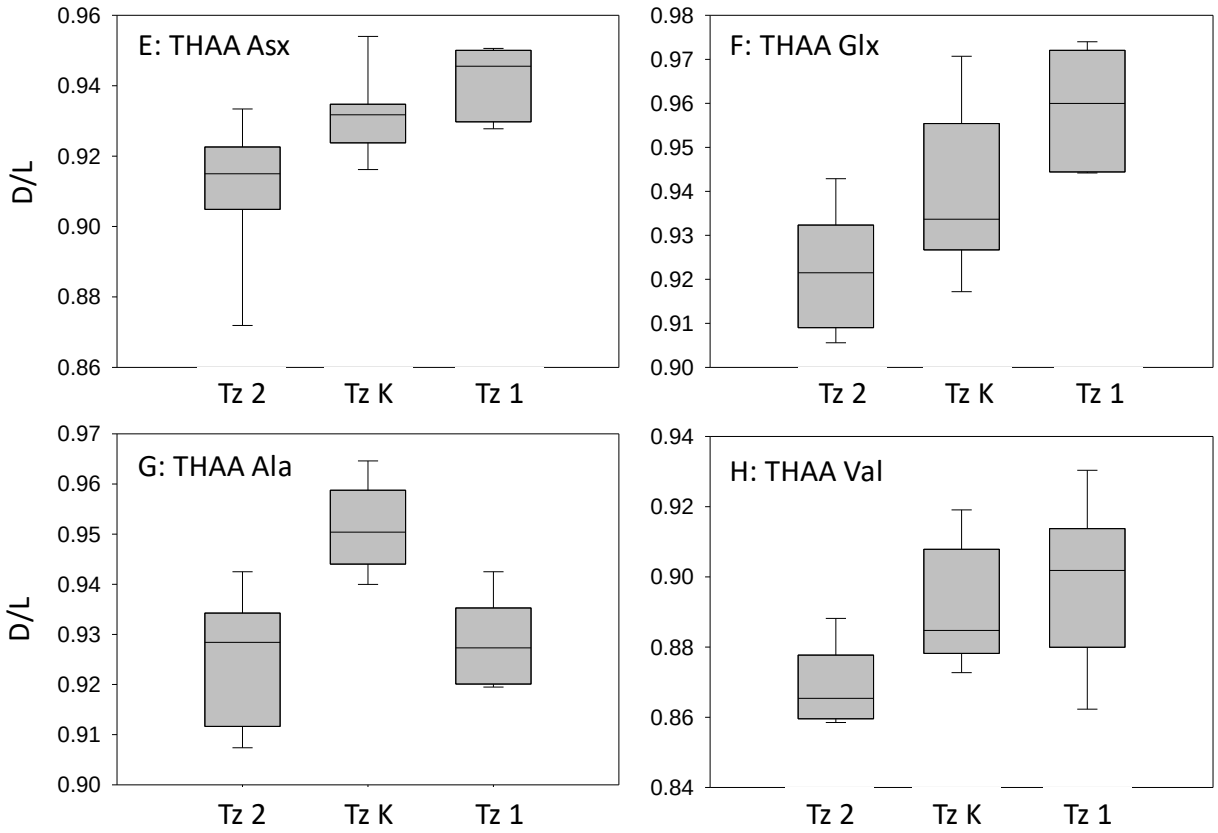
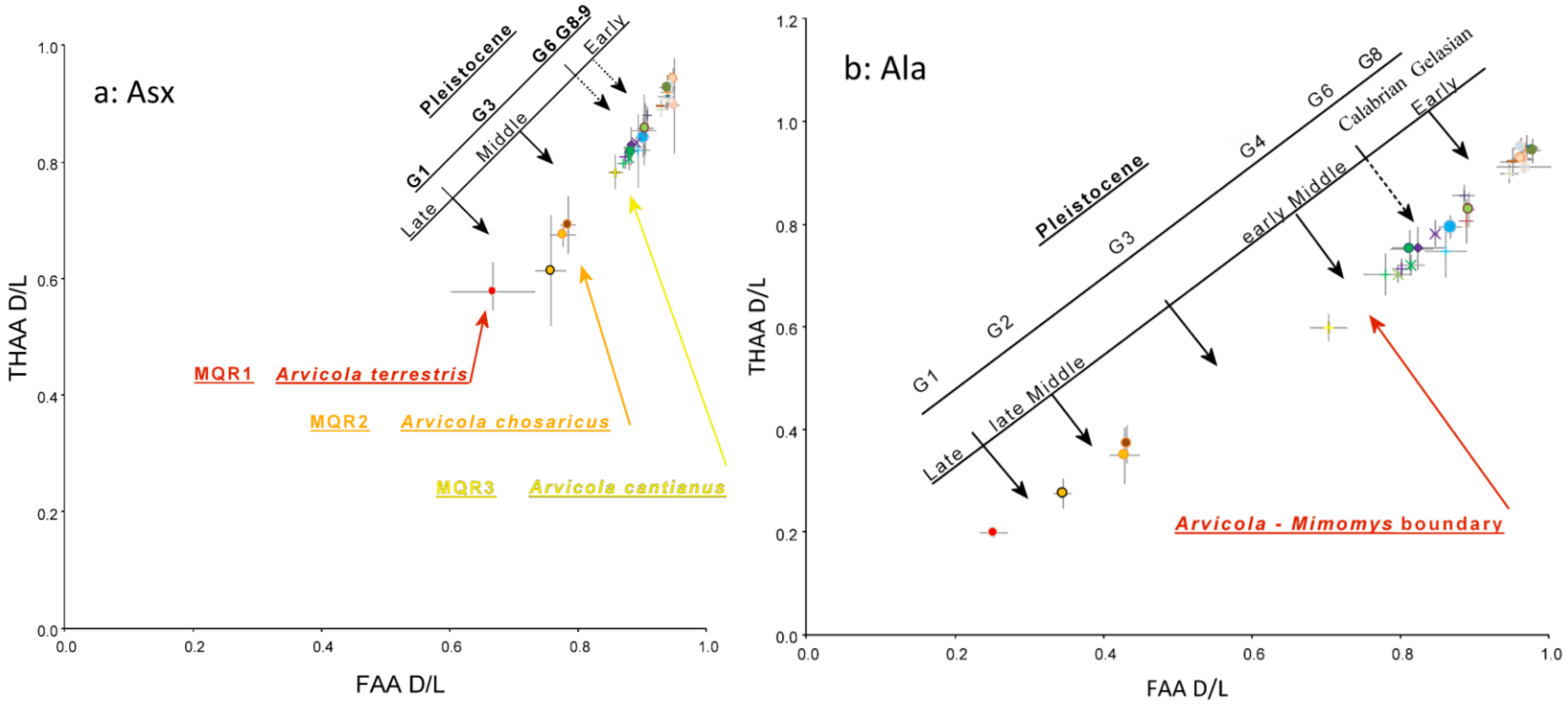


Figure 10. D/L values of amino acids from bithyniid opercula from three horizons at Tizdar: Tizdar 2 (Tz2) overlies Tizdar Kermek (Tz K), which overlies Tizdar 1 (Tz 1). All amino acids are approaching equilibrium so the correct age order is only seen for three amino acids (THAA Asx, Glx and Val). For each sample, the box brackets the 25th and 75th percentiles. Within the box, the solid line indicates the median and the dashed line shows the mean, whereas the 10th and 90th percentiles are shown by lines below and above the boxes respectively. The results of each duplicate analysis are included in order to provide a statistically significant sample size

The extent of the IcPD in the Tizdar samples is, however, higher than expected for their respective mammal zones (Figs. 7, 8a-d), showing greater racemisation than samples from the older Gelasian and Piacenzian sites. Reworking cannot be excluded, but it is less likely at Tizdar as it overlies predominantly marine deposits. However a possible explanation for this unusual situation may be related to the peculiar geological setting of this locality. Unlike all the other sites studied, the Tizdar sequence occurs in an area affected by active tectonics and mud volcanism, as demonstrated by tectonic tilting of the section and the occurrence of volcanic mud breccias that underlie and overlie the fossiliferous beds in this sequence. Occasional high-energy eruptions of mud volcanoes are a common feature of the Taman Peninsula

406 (Sobissevich *et al*., 2008; Ovsyuchenko *et al*., 2017). These eruptions reflect increased geothermal
407 activity, resulting in local explosions and burning of volcanic methane that may potentially involve
408 heating of the local sediments (Mukhtarov, 2003; Ershov *et al*., 2015). These catastrophic eruptions
409 transport to the surface large volumes of hot mud. Thus, given the emplacement of the mud volcanic
410 sediment within this section (Fig. 4), the unexpectedly high IcPD levels could result from such post-
411 depositional heating. Little work has been undertaken on high temperature heating of opercula, but the
412 data obtained on ostrich eggshell (Crisp, 2013) show that heated eggshell tends to show characteristic
413 patterns of lower Glx D/L than expected for a given Asx D/L in the THAA fraction; heating experiments
414 on opercula may provide useful insights into this.
415
416 **3.7: IcPD-based aminostratigraphy of the Azov region**
417
418 The terrestrial mammalian record for south-east Europe is patchy and includes numerous isolated fossil
419 localities unevenly scattered throughout this large region. The current study is based on 20 analysed levels
420 from 16 reference sections (Fig. 4) from which the mammalian assemblages have previously been
421 attributed to the regional biozones (Vangengeim *et al*., 2001; Tesakov, 2004). Due to the natural gaps in
422 the completeness of the geological record, these particular assemblages are distributed unevenly in time,
423 with some intervals over- or underrepresented. We therefore reviewed the aminostratigraphic sequence
424 (Figs. 8 and 11) paying attention to any breaks in its continuity, and compared this pattern to the known or
425 presumed gaps in the local biostratigraphical record. We designated any likely gaps in the
426 aminostratigraphic record as G1-G10; due to the differing levels of temporal resolution between the
427 amino acids, not all gaps are evident in all amino acids, but there is a consistent pattern in the dataset.


a: Asx
Pleistocene
G1
G3
G6 G8-9
Late
Middle
Early
MQR1
Arvicola terrestris
MQR2
Arvicola chosaricus
MQR3
Arvicola cantianus
THAA D/L
FAA D/L
b: Ala
Pleistocene
G1
G2
G3
G4
G6
G8
Late
late Middle
early Middle
Calabrian
Gelasian
Early
Arvicola - Mimomys boundary
THAA D/L
FAA D/L

428

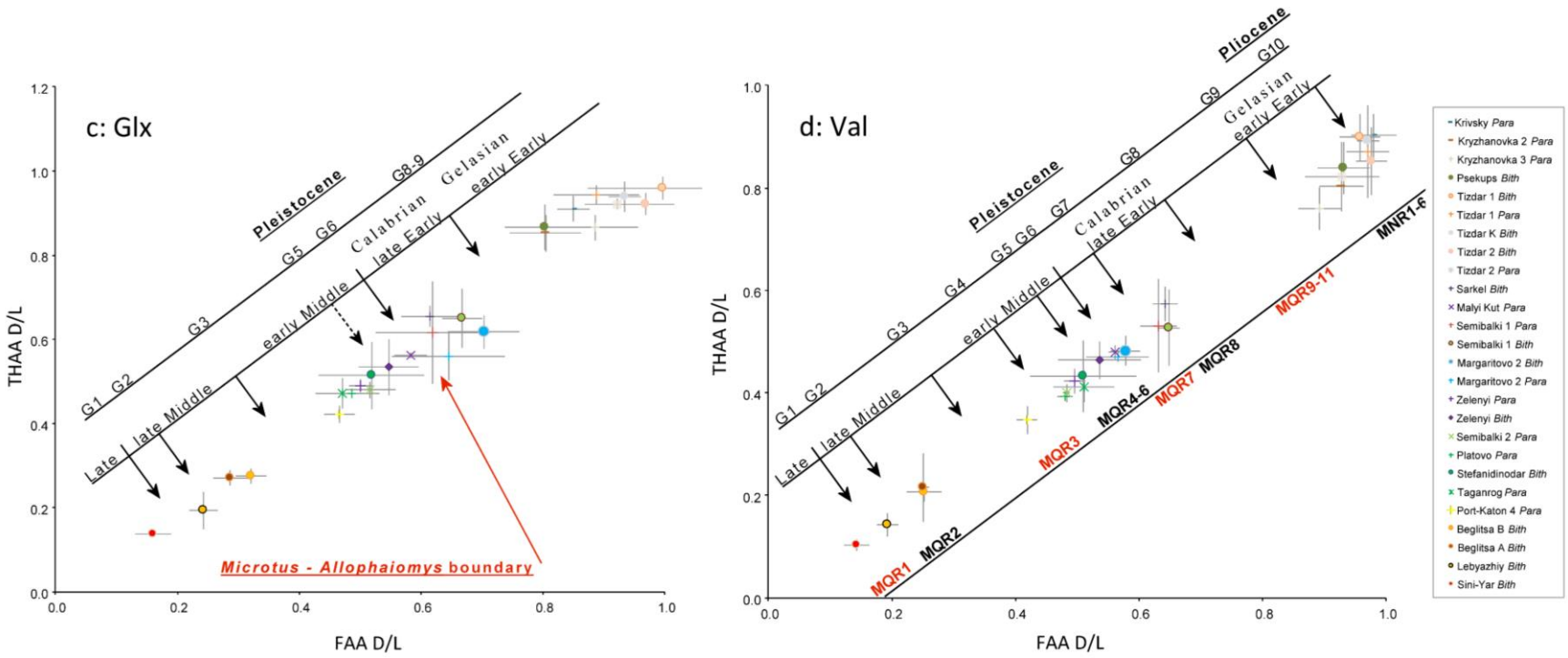


Figure 11. Site mean FAA vs THAA D/Ls for (a) Asx, (b) Ala, (c) Glx and (d) Val for the intra-crystalline fraction of bithyniid opercula from the Azov region, excluding samples identified as reworked. Error bars show two standard deviations about the mean for each site. Diagonal axes show how the amino acid data relate to the divisions of the Pleistocene and the East European Mammal zones (MQR/MNR zonation). G1-G10 highlight likely gaps in the range of D/L values that correspond to breaks in the regional fossil record. Coloured arrows indicate important biostratigraphical events.

Aspartic acid / asparagine (Asx) is the fastest racemiser in our set and shows good closed-system behaviour (Fig. 8a). Asx clearly resolves the Late Pleistocene, post-Eemian, fauna of Siniy Yar that includes *Arvicola terrestris* (MQR1) from late Middle Pleistocene, pre-Eemian faunas that include less advanced *Arvicola chosaricus* (MQR2), but as expected the temporal resolution is poor for older deposits/samples including those of early Middle Pleistocene age (Fig. 11). Two apparent gaps in Asx D/L data correspond to the early Late Pleistocene (G1) and mid-Middle Pleistocene (G3) time intervals not represented in the Azov faunal sequence. Gaps between Middle and Early (G6) and within the Early Pleistocene, i.e. Calabrian and Gelasian (G8-9) are less conspicuous. The largest gap in this record is observed between the youngest studied site of Siniy Yar (MQR1, post-Eemian, possibly MIS 3, Tesakov *et al*., 2012) and modern samples (Penkman *et al*., 2011). This would imply high potential resolution for discriminating terminal Late Pleistocene and Holocene opercula.

Alanine (Ala) is a medium-rate racemiser and in our dataset enables good resolution back to the early Middle Pleistocene, correlated with the Cromerian of NW Europe (Fig 11b). It is also able to separate out the Calabrian from Gelasian - Calabrian transition. The most obvious gap is between late Middle Pleistocene and the late Cromerian faunas (G3), suggesting potentially high resolution of the Ala amino-chronometer for the mid Middle Pleistocene faunas in the region. Also conspicuous is the break within early Middle Pleistocene cloud (G4) encompassing the *Arvicola/Mimomys* transition, a major biostratigraphic boundary in continental Eurasia in the Middle Pleistocene (von Koenigswald and van Kolfschoten, 1996; Maul *et al*., 2000; Maul and Parfitt, 2010).

Glutamic acid / glutamine (Glx) is a slower racemiser providing better differentiation between the older sites (Fig. 11c) than either Asx or Ala. More obvious gaps between clusters are apparent in the Glx D/L values, which clearly highlights a hiatus between the Middle and Early Pleistocene, which corresponds to the *Allophaiomys/Microtus* transition (MQR7). The higher Glx D/L values from Margaritovo 2 are

consistent with a late Calabrian age, with the site probably dating from the interval between the Brunhes-Matuyama and the normally-magnetised event interpreted as the Jamarillo (Tesakov *et al*., 2007b). Sarkel (MQR8) has yielded a mid-Calabrian fauna, and is therefore older than Margaritovo 2 (MQR7), consistent with its higher THAA Glx D/L values. An extended gap (G8-9) between the mid Calabrian and Gelasian implies better resolution in this time interval. Kryzhanovka 2, with *Borsodia paehungarica paehungarica*, is correlated with the earliest Gelasian and has higher FAA Glx D/L than the stratigraphically younger Kryzhanovka 3, with the more advanced *B. p. cotlovinensis*, correlated with the mid-Gelasian. However Glx D/L values are nearing equilibrium by this point, limiting its time-range beyond 2 Ma.

The latest Cromerian site Port-Katon 4 (MQR3) with *Arvicola* has a FAA Glx D/L similar to Cromerian sites (MQR4-6) yielding the ancestral progenitor *Mimomys*. Margaritovo 2 (terminal Calabrian, MQR7) has similar FAA D/L values to older, mid Calabrian clusters of Semibalki 1 and Sarkel (MQR8). The relative difficulty of measuring FAA Glx results from the formation of the pyroglutamic acid lactam (Wilson and Cannan, 1937). This cannot be derivitized and reduces the apparent concentration, which has impacts on the accuracy of the FAA Glx D/L, as shown here by the FAA D/L Glx of Port-Katon 4 and Margaritovo 2. However discrimination between these samples is possible using THAA Glx D/L, and when comparing D/L Ala, Val, and even Asx. The clustering of the sites is therefore consistent with biostratigraphical evidence. It can thus be concluded that in cases where anomalously high values of FAA are seen compared to THAA in Glx, THAA D/L Glx provide the more accurate relative ages.

Valine (Val) is also a relatively slow racemiser, allowing better resolution for the older samples compared to Asx, Ala, and Glx (Fig. 11d). The resolution of Val is consistently high in all parts of the chronological sequence and it reveals the greatest numbers of discrete temporal breaks than any of the other amino acids analysed here. As expected, Sarkel (MQR8) has higher FAA and THAA Val D/L than Margaritovo 2 (MQR7). The discontinuity in the Val D/L record between the Gelasian/Calabrian transition and the mid Calabrian is reflected by faunal differences, because no assemblages attributed to MQR 9 are known from

the region. Tantalisingly the reworked samples from Margaritovo (see Section 3.5) fall within the MQR 9-11 zone (Fig. 8d), indicating that a deposit of this age occurs in the vicinity.

In Val, the D/L data for the Gelasian sites Kryzhanovka 2 and 3 (MNR3 and MNR2) are very similar which, although consistent with biostratigraphy, indicates a loss of resolution by this time. The biostratigraphically younger site of Psekups (MNR1) also appears to be strongly racemised. It is therefore possible to recognise that all these sites are significantly old, but discrimination between them presents a challenge. The oldest site within the group, Krivsky (Piacenzian, between 3 - 2.5 Ma), also generally has the highest D/L values, although within the range of other Gelasian sites (ca. 2.5 - 2 Ma). As the data from Tizdar show anomalously high D/L values that appear to result from geothermal heating, they cannot be used in the construction of the regional aminostratigraphy (see Section 3.6).

In summary, there are ten potential breaks (G1 –G10) between clusters of D/L values (Figs. 8 & 11): G1 (MQR1/2) occurs between the Late and Middle Pleistocene; G2 (within MQR2), between two late Middle Pleistocene distributions; G3 (MQR2/MQR3), between late Middle Pleistocene and youngest Cromerian record; G4 (MQR3/MQR4-6), between the younger and older Cromerian; G5 (MQR4-6/MQR7), between late and earlier Cromerian levels; G6 (in-MQR7, only really evident in Glx), between early Middle and late Early Pleistocene levels; G7 (MQR7/MQR8), between late Early and mid Early Pleistocene faunal levels; G8 and G9 (MQR8/MQR9-11), between mid Early Pleistocene and the earliest Pleistocene (Calabrian/Gelasian) distributions; and potentially G10 (MNR3/MNR5), between Late Pliocene and Early Pleistocene (Piacenzian/Gelasian), although the Tizdar dataset is complex. Integrating this aminostratigraphic dataset with the biostratigraphy of this well-studied region, we propose that these breaks correspond to actual gaps (lack of deposits/localities) in the regional sequence, as a consequence of local breaks in the continuity of the fossil record (non-deposition, erosion, etc.).

In summary, the increase in opercula IcPD is stratigraphically consistent with the East European Mammal

zonation. As the protein decomposition rate in a closed system depends on time and temperature, the regional pattern of IcPD through time is controlled by the thermal history of that region (Miller *et al*., 1999; Wehmiller *et al*., 2000). Compared to the British aminostratigraphic sequence (Fig. 14 in Penkman *et al*., 2013), the IcPD values from bithyniid opercula in southern Eastern Europe are systematically higher for a given time period, which is logical given the more southerly latitude.

3.8: Comments on the regional biostratigraphy of southern Eastern Europe.

Taking into account the different resolutions in different parts of the chronological sequence, the most important feature of this aminostratigraphy is the reproducibility of the results using four amino acids, and the striking fidelity of the aminostratigraphy in relation to the mammalian biostratigraphy. This enables us to use the aminostratigraphic dataset (Fig. 11) to provide valuable insights into the nature of the regional biostratigraphy.

The substantial gap between the early Middle Pleistocene (Cromerian) and late Middle Pleistocene D/L values (Fig. 11) reflects a substantial stratigraphical hiatus from the interval spanning MIS 9-11, which corresponds to the MQR3-MQR2 transition. Alluvial sites of this age are scarce in the region and not represented in the analyses. Samples from the early Middle Pleistocene (Cromerian), yielding mammalian faunas of the Tiraspol faunal complex (MQR 4-6), are well represented in the data set (7 out of total 20). All belong to the palaeo-Don River Semibalki fluvial formation. Some samples show high variability of D/L values within a single horizon, interpreted as the co-occurrence of both *in situ* specimens (tight clusters) and a tail of reworked outliers (see Section 3.5). Both subclusters are divided by G5, best seen in Glx and Val data (Fig. 8 c,d). The group forming the tail probably reflects a period close to the Brunhes-Matuyama palaeomagnetic reversal that is not represented in the sections studied, and it is likely that opercula from deposits of this period have been reworked by later fluvial activity.

The site of Malyi Kut, poorly characterised by a small mammal fauna, had been tentatively attributed to the Calabrian mammal zones (MQR9-7). The aminostratigraphical evidence (Fig.8 b-d) suggests a

younger age in this range, possibly the early part of MQR7. Data from the Semibalki 1 site plot in the same chronological position, although some of the specimens from here seem to be reworked from two different Calabrian levels. The only reliable mid Calabrian level (MQR8) in our material is represented by the homogeneous cluster of D/L values from Sarkel. Additional specimens that plot next to Sarkel represent reworked material from latest Calabrian (MQR7) and Cromerian sites (MQR4-6).

The large gap in the early Calabrian (G8-9) would be occupied by mammal biozones MQR9-11. It is remarkable that data from reworked bithyniid opercula from the neighbouring sites of Semibalki 1 and Margaritovo 2 (both MQR7) plot in the gap (Fig. 8c-d). This indicates the former presence of early Calabrian deposits on the southern shore of the Taganrog Gulf. The materials from Tizdar (MQR10-11) that would have been expected to plot in G9 may be misplaced as a consequence of geothermal heating (see Section 3.6), and imply that in geothermally active regions the aminostratigraphic technique should be applied with caution.

The temporal resolution using IcPD in bithyniid opercula is poor for sites of Gelasian age. The data for three Gelasian sites (Psekups, Kryzhanovka 3, and Kryzanovka 2) representing three mammal zones during this interval (MNR1-2-3) show similar levels of protein breakdown, precluding finer temporal resolution. The oldest site of Krivsky dating back to the Late Pliocene (mid Piacenzian, > 3 Ma, MNR5) furnished opercula tending to show the highest racemization for Glx and Val, consistent with its greater age. However these values fell within the higher end of the range for other Gelasian sites, and as all of the amino acids reported in this study are nearing equilibrium at this point, so no further resolution is currently possible using IcPD in opercula for this time interval.

4. Implications for Quaternary chronology and biostratigraphy of Europe.

This study provides an example of how this synthetic regional chronology can be developed; combining all available data and cross-testing the sequence, materials and regional geological history. The regional mammal zones were defined from widely scattered sequences in the Azov/Black Sea region (Tesakov,

2004, Tesakov *et al*., 2007), where only rarely do sediments yielding mammals attributable to biostratigraphic zones occur in direct stratigraphical superposition. This is why the independent testing of this system by opercula IcPD provides valuable age constraints. From the aminostratigraphical point of view, following the first regional IcPD framework which was developed for Britain (Penkman *et al*., 2011, 2013), this is the second large-scale application of opercula IcPD that has been undertaken, and is the first in continental Europe. The high fidelity of the independently-constructed chronological sequences of these fossil localities shows the important role aminostratigraphy can play in testing and providing additional support to the standard integrated stratigraphy, and enables the stratigraphic extension to sites where faunas are incomplete (Fig. 11 & 12). This provides a basis to develop regional chronologies for a cross-European aminostratigraphy, enabling us to track IcPD behaviours across latitudes and temperature zones. The Azov region was never covered by glacial ice, and periglacial conditions would only have been present during the glacial stages of the Middle and Late Pleistocene (Velichko *et al*., 2011), so more data will illuminate the impacts of these differing depositional histories on the integrated effective temperatures experienced by the opercula samples.

The reliable temporal range of the opercula IcPD in southern Eastern Europe extends to ~1.5-2 Ma (Fig. 12). For the Early Pleistocene / Late Pliocene, the resolution within current well-chromatographically resolved amino acids is reaching its limit. However there is potential to extend this to other amino acids using UHPLC technology (Crisp, 2013), which improves the chromatographic resolution of certain amino acids as well as resolving addition amino acid D/L pairs. In addition, using other biominerals (such as the hydroxyapatite of tooth enamel) where the racemisation of amino acids is slower (e.g. Dickinson *et al*., 2019) will enable better resolution of Late Neogene samples from this region.

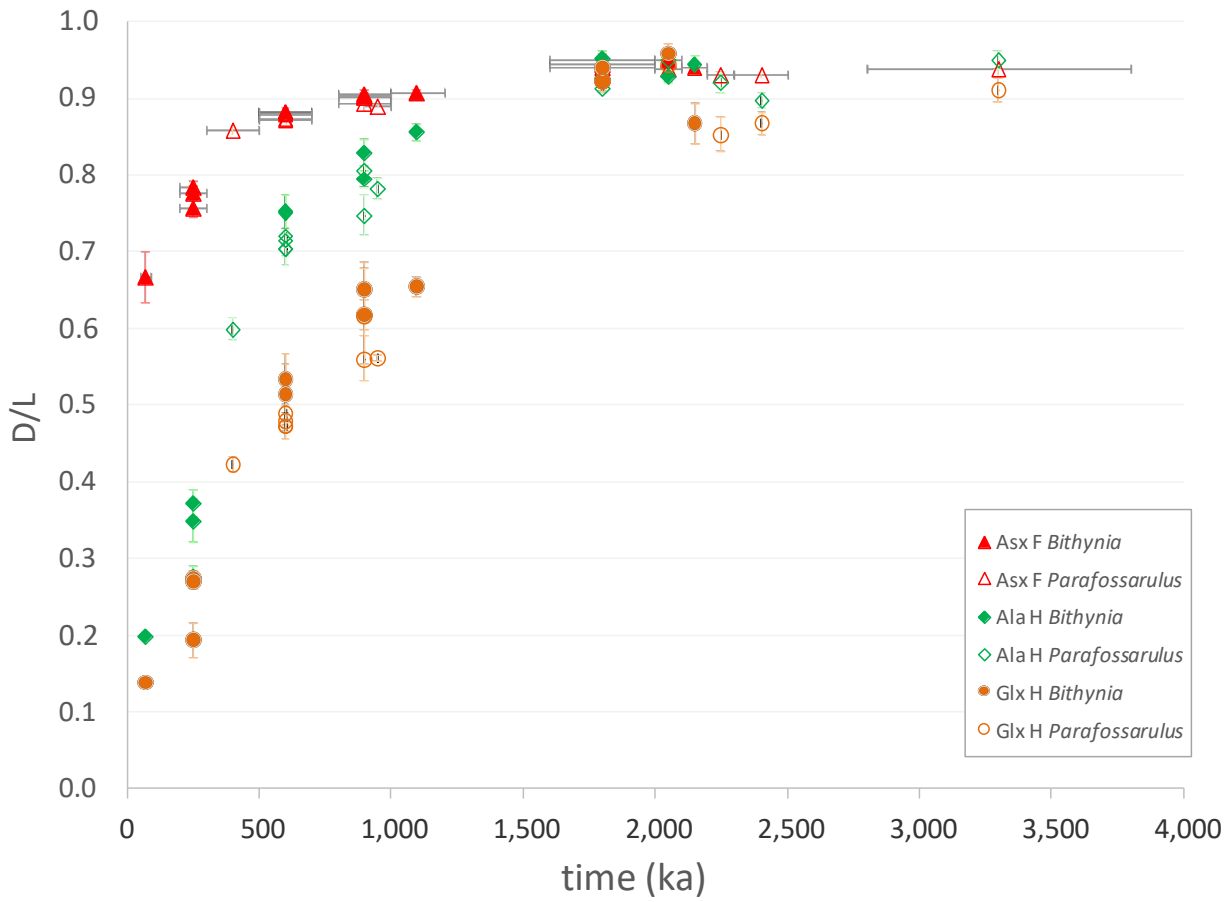


Figure 12. Mean D/L values (Asx, Ala and Glx) for each site, excluding samples identified as reworked, from bithyniid opercula plotted against independent evidence of age. For simplicity, the x-error bars, indicating the current uncertainty on the age of the deposits, are shown for the Asx data. Y-error bars indicate two standard deviations about the mean for each site. *Bithynia* opercula are represented by closed symbols; *Parafossarulus* opercula by open symbols.

Although the East European Mammal zonation was originally based on assemblages of small mammals from reference sites in the Azov Sea and Black Sea regions, it has subsequently been used to correlate Plio-Pleistocene sequences across much of Europe (Gromov, 1948; Rekovets and Nadachowski, 1995; Mayhew, 2015) and western Asia (Borodin *et al*., 2019; van den Hoek Ostende *et al*., 2015). It has been particularly important in dating critical sites in the early Paleolithic, and in estimating the magnitude of regional breaks in paleontological record (such as the late Calabrian '*Allophaiomy*s gap' in England; e.g. Preece *et al*., 2020). The water vole lineage, incorporating the important *Mimomys/Arvicola* transition in the early Middle Pleistocene, has now been shown to exhibit exactly the same sequence of events and

relative chronology as seen in Western and Central Europe (Maul *et al*. 2000). The same is true for the *Mammuthus* lineage, for which our opercula-based data are entirely consistent with the consecutive occurrence of each named chrono-species within the Quaternary. This ground-truthing in the Azov region therefore provides a useful mechanism for testing the assumptions that this East European Mammal zonation scheme is applicable throughout Europe and western Asia (Mayhew, 2015). This new Azov region aminostratigraphic dataset, combined with the British aminostratigraphy, therefore provides the second foundation stone for development of a European-wide chronology.

Data Availability: All amino acid data from this study will be made available through the NOAA repository upon publication: ftp://ftp.ncdc.noaa.gov/pub/data/paleo/aar/.

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Author contributions

Roles of the authors: KP and AT led this study. AT coordinated the synthesis of the stratigraphic and biochronological information on the paleontological record from the studied region; KP coordinated the aminostratigraphic study. PF and VT provided data on the molluscan and large mammal fossil record in southern Russia. TM provided materials on fossil molluscs from the Netherlands. RP, SP and MD

contributed to discussions on the correlations of the Russian and British fossil records. All authors were involved in writing the manuscript.

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