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Holocene temperature and hydrological changes reconstructed by bacterial 3-hydroxy fatty acids in a stalagmite from central China

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16

17 **Abstract**

18 To achieve a sufficient understanding of the spatial dynamics of terrestrial climate

19 variability, new proxies and networks of data that cover thousands of years and run up

20 to the present day are needed. Here we show the first Gram-negative bacterial

21 3-hydroxy fatty acid (3-OH-FA) based temperature and hydrological records from any

22 palaeoclimate archive globally. The data, covering the last 9 ka before present (BP),

23 are generated from an individual stalagmite, collected from Heshang Cave, located on

24 a tributary of the Yangtze River, central China (30°27′N, 110°25′E; 294 m). Our

25 results indicate a clear early-to-middle Holocene Climatic Optimum (8.0-6.0 ka BP)

26 followed by a long-term monotonic cooling and increasing variability over the last 0.9

27 ka BP. The hydrological record shows two relatively long wet periods (8.8-5.9 ka BP

28 and 3.0-0 ka BP) and one relative dry period (5.9-3.0 ka BP) in central China. We

29 show that 3-OH-FA biomarkers hold promise as independent tools for palaeoclimate

30 reconstruction, with the potential to deconvolve temperature and hydrological signals

31 from an individual stalagmite.

32

36

37 **1. Introduction**

38   Nearly half of the Earth's population live within the influence of the modern

39   monsoon and its importance to terrestrial eco-systems, societal wellbeing and the

40   global economy can not be overstated (Webster et al., 1998). Records of past

41   Holocene rainfall and temperature, which extend the relatively short instrumental

42   record, can constrain natural monsoon variability and are particularly important for

43   the Asian monsoon region where prediction of future changes in rainfall using climate

44   models has proven challenging (IPCC, 2014). Such records can also illustrate the

45   influence of the monsoon on prehistoric cultures and settlements (Xie et al., 2013).

46   Stalagmites have become a key archive in Quaternary palaeoclimatic

47   reconstruction due to their ability to yield continuous and undisturbed records, precise

48   and absolute chronologies, and their global terrestrial distribution (Blyth et al., 2016;

49   Fairchild et al., 2006; Fairchild and Baker, 2012; McDermott, 2004; Wong and

50   Breecker, 2015). Oxygen isotopes are effectively the 'master' or standard approach

51   for speleothem analysis, but inherently encode a mix of climatic signals (Lachniet,

52   2009; McDermott, 2004), including, at the regional scale: temperature changes, the

53   isotopic composition of source waters and precipitation amount. In addition, complex

54   site-specific factors must be taken into account, such as drip rate (Dreybrodt and

55   Scholz, 2011) and $CaCO_3$ precipitation (Fairchild and Baker, 2012). Many previous

56   studies have focused on the interpretation of oxygen isotopes in speleothems, but

57   deconvolving independent temperature and precipitation signals from speleothem

58    $CaCO_3$ remains highly challenging, as evidenced by the paucity of such deconvolved

59   records (Hu et al., 2008b; Yuan et al., 2004).

60   Biomarker based proxies are now firmly established in the fields of

61   paleoceanography and paleolimnology (Castañeda and Schouten, 2011; Eglinton and

62   Eglinton, 2008; Schouten et al., 2013). Recently attention has turned to the potential

63   of organic matter and biomarker techniques for speleothem research (Blyth et al.,

64   2008; Blyth et al., 2016). A number of biomarkers with known paleoclimatic utility

65   have been discovered and measured in speleothems, including glycerol dialkyl

66   glycerol tetraethers (GDGTs) (Blyth et al., 2014; Blyth and Schouten, 2013; Yang et

67   al., 2011), plant derived biomarkers (Blyth et al., 2007; Blyth et al., 2011; Blyth et al.,

68   2010; Bosle et al., 2014; Xie et al., 2003), branched fatty acids and hydroxy fatty

69   acids (Blyth et al., 2006; Huang et al., 2008; Wang et al., 2012). Furthermore, Blyth

70   and Schouten (2013) recently proposed a novel GDGT calibration, based on samples

71   derived from 33 globally distributed speleothems from caves with a range of average

72   air temperatures.

73   Biomarkers in stalagmites may originate from the overlying vegetation,

74   overlying soil ecosystem, limestone aquifer and cave fauna (Blyth et al., 2008).

75   Moreover, different biomarker classes may have different sources, for example, Yang

76   et al. (2011) found that the majority of the archaeal isoprenoid and bacterial branched

77   GDGTs measured in stalagmite samples from Heshang cave were likely produced *in*

78   *situ*. Most recently, Blyth et al. (2014) found that GDGTs preserved in stalagmites in

79   the UK and Australia likely originated from the *in situ* microbial communities within

80   cave systems. An artificial irrigation experiment conducted in Cathedral Cave,

81   Australia, found different GDGT distributions among speleothem, soil and drip water

4

82   samples (Baker et al., 2016). In contrast, a 2 year monitoring experiment of drip

83   waters in Heshang Cave found that fatty acids in drip waters were most likely derived

84   from the overlying soil and/or groundwater system via particulate entrainment and

85   deposition (Li et al., 2011). It is noteworthy that the fatty acid ratios (ratios of

86    $nC_{16:1}/nC_{16:0}$ and $nC_{18:1}/nC_{18:0}$; the prefix n indicates normal, the number before the

87   colon specifies the number of C atoms, and the number after the colon gives the

88   number of double bonds) showed a strong negative relationship with the external

89   temperature recorded in Yichang meteoric station (located ca.100 km east of Heshang

90   Cave), whereas the two ratios displayed no relationship with internal cave

91   temperatures recorded at the HS4 site, which suggests that *in situ* cave microbes are

92   probably not the predominant source for C_{16} and C_{18} acids in drip water collected in

93   Heshang Cave. Li et al., (2011) concluded that, based the distributional patterns of the

94   fatty acids, microbes living in the overlying soils and/or groundwater system are the

95   dominant source of fatty acids to the Heshang Cave drip waters. We note that

96   Vaughen et al. (2011) discovered microbia living on speleothem surfaces in Kartchner

97   Caverns, USA. Such consortia of microbes are an inevitable source of *in situ* fatty

98   acids. Thus fatty acids measured in stalagmites may be derived from mixed sources,

99   including overlying soils/ sediments (Li et al., 2011), the ramifying network of

100   conduits and reservoirs in the limestone and *in situ* microbes (Vaughan et al., 2011).

101   However, even though the origin and pathways of inclusion into speleothems of

102   biomarkers may be complex (Blyth et al., 2008; Blyth et al., 2016), it doesn't hinder

103   the utilization of biomarkers in paleaoclimate reconstruction. Site specific

104   interpretation and ground truthing is required, but this is also true for established

105   paleoclimate techniques, as outlined above. In summary lipid biomarkers preserved in

106   speleothems show clear potential for paleoclimate reconstruction. However, very few

107   such biomarker based paleoclimatic reconstructions have been published (Blyth et al.,

108   2011; Huguet et al., 2018; Li et al., 2014; Xie et al., 2003).

109   Gram-negative bacterial 3-hydroxy fatty acids (3-OH-FAs) are abundant in

110   stalagmites (Blyth et al., 2006; Huang et al., 2008; Wang et al., 2016; Wang et al.,

111   2012) and are characteristic compounds of Lipid A, the lipid component of the

112   lipopolysaccharides (LPS) located in the outer membrane of Gram-negative bacteria

113   (Szponar et al., 2003; Szponar et al., 2002; Wollenweber and Rietschel, 1990). Based

114   on strong relationships with environmental pH and temperature from an altitudinal

115   transect of soils on Shennongjia Mountain (Mt.) central China, a number of novel

116   3-OH-FA based proxies have been proposed (Wang et al., 2016). For example, the

117   ratio of *anteiso* to *normal* C_{15} 3-hydroxy fatty acid (RAN_{15}) was propounded to be a

118   novel temperature proxy, and the ratio of the total sum of *iso* and *anteiso* 3-OH-FAs to

119   the total amount of *normal* 3-OH-FAs (Branching Ratio) and RIAN (negative

120   logarithm of Branching Ratio) were propounded to be novel pH proxies (Wang et al.,

121   2016).

122   In this study we present inferred temperature and hydrological records, spanning

123   the last 9 ka BP, based on 3-OH-FA derived proxies from a single stalagmite

124   collected from Heshang Cave, central China (Fig. 1). This work is the first

125   demonstration of the application of 3-OH-FA based proxies for paleoclimatic

126   reconstruction and suggests that such approaches may be used to derive independent

127   quantitative temperature and qualitative hydrological signals from an individual

128   stalagmite.

129

130   **2.   Materials and methods**

131   **2.1 Sampling site and sample information**

132   Heshang Cave is located at 294m above sea level (a.s.l.), on the Qing River, a

133   tributary in the middle reaches of the Yangtze River, central China (30°27′N,

134   110°25′E) (Fig. 1A). Heshang Cave is one of several caves which characterize the

135   regional karst landscape. The overlying dolomite is ca. 400 m thick and is capped

136   with a mature layer of soil (20-40 cm-thick) and reasonably dense vegetation (Fig.

137   1B). The regional climate is strongly impacted by the East Asian Monsoon, with a hot

138   and moist summer, but relatively cold and dry winter (An, 2000). Regional average

139   annual precipitation is 1161 mm, based on the recent 65 years (1951-2014) of

140   meteorological data from Yichang station. The seasonal temperature ranges, inside

141   and immediately outside the cave, were constrained by 2-hour resolution logging

142   between 2004 and 2007 using HOBO H8 Pro T loggers (Hu et al., 2008a). The

143   modern temperature immediately outside the cave varies seasonally from 3°C to 30°C,

144   with an annual average of 18°C and is statistically identical to that of the nearest

145   government meteorological station in Changyang county (Hu et al., 2008a). The

146   annual mean temperature inside the cave is identical to the outside measurements.

147   However, the amplitude of the internal temperature range is about one fifth of the

148 external cycle and lags the external temperatures by about 10 days (Hu et al., 2008a).

149 Heshang cave extends a distance of ≈250 m, roughly horizontally from it's opening

150 (see Fig. 1C) and is well decorated with stalagmites, rimstone pools, and less frequent

151 stalactites (including an exquisite 'Lotus Flower' stalactite).

152   The HS4 stalagmite is 2.5 m long, and was actively growing when collected

153 from ca. 150 m within Heshang Cave in 2001 (Fig. 1C). It shows clear annual banding

154 throughout its growth axis, generated by the strong seasonal cycle at this site (Johnson

155 et al., 2006). Highlights of previous work on this stalagmite include a quantitative

156 Holocene Asian monsoon rainfall record (Hu et al., 2008b) and high resolution 8.2 ka

157 event record (Liu et al., 2013; Owen et al., 2016). The HS4 stalagmite was divided

158 longitudinally into 4 sections. Each section was dedicated to a different branch of

159 analyses (e.g. δ^{18}O, trace elements, organic geochemistry etc.). 206 subsamples were

160 taken from the organic geochemistry section along the stalagmite growth axis and

161 73 subsamples were selected at intervals for biomarker analysis. All the outer layers

162 of the subsample were removed during sampling to avoid any potential contamination.

163 Based on annual layering each sample has a resolution of several decades to >100

164 years.

165   In 2013 seventeen cave sediment samples were collected within Heshang cave

166 from the entrance to the deepest accessible part of the cave (Fig. 1C) and nine

167 overlying soil samples were collected from the land-surface immediately above the

168 cave (which slopes upwards from an altitude of 457 m to 489 m) (Fig. 1B). The

169 sediment inside the cave is oligotrophic with < 6 g/kg total organic carbon (Gong et

170   al., 2015).

171 **2.2 Chronology**

172   The chronology of HS-4 was established independently by U-Th dating and layer

173 counting. Twenty-one subsamples were sampled and prepared in a class-1000 clean

174 lab before being analyzed by multi-collector inductively coupled plasma mass

175 spectrometry (MC-ICP-MS) at Oxford University (Nu Instruments), following the

176 techniques of Robinson et al. (2002). Layer counting was used for the uppermost 150

177 years and U-Th dating for the period ca. 378 ± 57 to 9446 ± 146 years BP. The

178 chronology of each sample is based on linear interpolation between the ^{230}Th dates,

179 the average age uncertainty is 67 yrs. Further details on the chronological techniques

180 and model are reported by Hu et al. (2008b).

181 **2.3 pH measurement**

182   The pH of cave surface sediments and overlying soils was measured following

183 the methods of Yang et al. (2014). Samples were mixed with ultrapure water in a ratio

184 of 1:2.5 (g/mL). After standing for 30 min, the pH of the supernatant was measured

185 using a meter with a precision of ± 0.01.

186 **2.4 Lipid extraction and work-up**

187   The stalagmite samples were treated with an optimized acid digestion method

188 following Wang et al. (2012). In brief, 10 grams of stalagmite sample were digested

189 with 3M HCl, then re-fluxed at 130°C for 3 hours with a condenser/ heating mantle

190 assembly. An internal standard (pregn-5-en-3.beta.-ol) was quantitatively added to

191 each sample to quantify the amount of lipids in the stalagmite. After cooling, the

192 residue was extracted by dichloromethane (15mL×4) and the extracts combined.

193 Solvents were removed by rotary evaporation (Buchi R210) under reduced pressure.

194 Soil samples and cave surface sediments underwent the same work-up protocol as the

195 stalagmite samples. The condensed lipids were further derivatized by BF_3-methanol

196 (14% BF_3/methanol, Sigma) and BSTFA (N, O-bis(trimethylsilyl) trifluoroacetamide,

197 Supelco) before undergoing gas chromatography-mass spectrometry (GC-MS). In

198 order to minimize contamination, all glassware was soaked in a decontamination

199 solution, rinsed with ultra purified water, and heated for 6 h at 500 °C. The HCl was

200 pre-extracted with dichloromethane (DCM, ×4), and all other reagents were tested for

201 background contaminants.

202 **2.5 Instrumental analysis**

203 All the samples were analyzed using GC-MS with a Hewlett Packard 6890 gas

204 chromatograph coupled to a Hewlett Packard 5973 mass selective detector. Separation

205 was performed on a ZB-5MS fused silica capillary column (60 m×0.25 mm id.; 0.25

206 μm film thickness). The GC oven temperature was programmed from 70°C to 200°C

207 at 10°C per min, then from 200°C to 300°C at 2°C per min, and finally held at 300°C

208 for 27 min. The carrier gas was He (1 mL/min). The spectrometers were operated in

209 electron-impact (EI) mode, the ionization energy was set at 70 eV and the scan range

210 was from 50 to 550 aum.

211 **2.6 Proxy calculation**

212 The RAN_{15} and RIAN in the HS4 stalagmite samples were calculated using the

213 relative abundances of the 3-OH-FAs with carbon numbers from C_{10} to $C_{18,}$ which are

214   derived from Gram-negative bacteria. Standard deviations for the RAN_{15}-MAAT and

215   RIAN were calculated by a duplicate extraction and analyses on 9 randomly selected

216   samples.

217

218   **3.   Results and Discussion**

219   **3.1 Distribution and source of 3-OH-FAs**

220   Below we discuss the distributional characteristics of 3-OH-FAs in Heshang

221   cave sediments and overlying soils and, with consideration of recent bacterial

222   monitoring of the cave environment and drip waters (Liu et al., 2010; Yun et al.,

223   2016b), constrain their possible sources and pathways.

224   The average distributions of 3-OH-FAs in the overlying soils, cave surface

225   sediments and the HS4 stalagmite samples are illustrated in Figure 2. There is an

226   overall similarity in the distribution patterns of the three sample sets (with some

227   differences discussed below), with the C_{10}, C_{12}, C_{14}, C_{16} and C_{18} homologues being

228   typically most abundant. The carbon number of the detectable 3-OH-FA homologues

229   varies from C_8 to C_{30} (Fig. 2), however only the overlying soil samples contain the

230   lowest carbon numbers of the 3-OH-FAs (C_8, i-C_9, a-C_9, C_9) (Fig. 2A). The

231   distribution of 3-OH-FAs in the HS-4 stalagmite reported here agrees with previous

232   studies of the HS4 stalagmite from Heshang cave (Huang et al., 2008; Wang et al.,

233   2012). The only other reports of 3-OH-FAs in speleothem samples comes from an

234   Ethiopian stalagmite (Blyth et al., 2006) and a British stalagmite (Blyth et al., 2011).

235   The molecular distributions reported by Blyth et al. (2006) and (2011) are similar

236 (maxima at C_{12}, C_{14}, C_{16} etc.) to the HS4 distributions, but the higher molecular

237 weight 3-OH-FAs ($>C_{20}$) we detected were not previously reported.

238 Normal and branched 3-OH-FAs homologues of C_{10} to C_{18} chain length are

239 abundant constituents of Lipid A, a constituent of Lipopolysaccharide (LPS), the main

240 component of the outer membrane of Gram-negative bacteria (Lee et al., 2004;

241 Szponar et al., 2003). 3-OH-FAs have been used to quantify and characterize the

242 Gram-negative bacterial community in atmospheric aerosols (Lee et al., 2004), marine

243 dissolved organic matter (DOM) (Wakeham et al., 2003) and snow samples (Tyagi et

244 al., 2016; Tyagi et al., 2015), and have recently been utilized to define a number of

245 novel terrestrial paleoclimate proxies (Wang et al., 2016). The fractional abundance of

246 the individual 3-OH-FA homologues varies from 0 to 30% in the studied samples. The

247 3-OH-FAs generally show a strong even/odd predominance (Fig. 2). In the HS4

248 stalagmite, this general even/ odd predominance is accentuated for the homologues in

249 the range from C_{11} to C_{15}, but is reversed for the i-C_{17} 3-OH-FA which is notably

250 higher than the n-C_{16} 3-OH-FAs (Fig. 2C). Furthermore, the n-C_{12} 3-OH-FAs account

251 for ca.18% in the stalagmite samples, but only ca.11% in both the overlying soils and

252 cave surface sediments. The higher proportion of n-C_{12} and i-C_{17} homologues in the

253 stalagmite may derive from *in situ* bacterial production or possibly better preservation

254 of these compounds in stalagmites.

255 Culturable bacteria in drip waters from Heshang Cave are dominated by

256 Gram-negative heterotrophs, derived from Proteobacteria with the dominance of

257 Gamma proteobacteria (Liu et al., 2010). A two-year drip water monitoring

258   experiment in Heshang Cave likely demonstrates a pathway for transporting

259   3-OH-FAs from the overlying soil microbial community to cave sediments and

260   speleothem surfaces (Li et al., 2011). Meanwhile, a recent molecular survey of

261   bacterial communities in Heshang cave drip waters, over the period of 2008 to 2013,

262   confirms a diverse Gram-negative bacterial community and reveals a seasonal control

263   on Proteobacteria, whereby Beta-proteobacteria are supplied in the summer and

264   Gamma-proteobacteria in the winter (Yun et al., 2016b). A latest investigation shows

265   that Proteobacteria are both abundant in Heshang cave overlying soils and drip waters

266   (Yun et al., 2016a). This demonstrates that the seasonal signal of changes in the

267   Gram-negative bacterial community is transmitted readily through the Heshang cave

268   system to drip waters and to the cave and speleothems. This seasonal cycle is positive

269   from a paleoclimate perspective, suggesting minimal attenuation of bacterial based

270   climate signals transmitted from the overlying soils to the HS4 stalagmite, at least

271   sufficient for centennial to millennial scale paleoclimate studies.

272   As noted above, a distinctive feature of the 3-OH-FA distribution in the HS4

273   samples, is the greater relative abundance of the n-C_{12} and i-C_{17} 3-OH-FAs compared

274   to both overlying soils and cave sediments (Fig. 2). This may suggest an additional

275   contribution of 3-OH-FAs, derived from microbes from the cave drip water, which are

276   sequestered and trapped in the calcite matrix (Supplementary Information). We note

277   that Pacton et al. (2013) report that microbial activity can initiate calcite deposition in

278   the aphotic zone of caves before inorganic precipitation of carbonates. We also note

279   the low abundances of long chain 3-OH-FAs (C_{20}-C_{26}), which might originate from

280 fungi, and/or Gram-positive actinomycetes (Keinänen et al., 2003). However, we

281 suggest that the broad similarity of 3-OH-FA distributions in the overlying soils and

282 stalagmites, supported by the site-specific analyses of bacterial diversity and transport

283 pathways (Liu et al., 2010; Yun et al., 2016b), supports a major contribution of

284 3-OH-FAs from Gram-negative bacteria dwelling in the overlying soils to the HS4

285 stalagmite samples. This is consistent with previous findings that lipids preserved in

286 speleothems are principally derived from the overlying soil ecosystem and vegetation,

287 having been transported from the surface by percolating groundwater, although a

288 proportion may be derived from the cave ecosystem (Blyth et al., 2014; Xie et al.,

289 2005; Xie et al., 2003; Yang et al., 2011).

290

291 **3.2 Holocene hydrological reconstruction**

292   Recent work has demonstrated that pH is a key environmental parameter in

293 controlling soil bacterial community structure and diversity (Bååth and Anderson,

294 2003; Griffiths et al., 2011; Lauber et al., 2009; Shen et al., 2013; Zhang et al., 2015).

295 Notably, Giotis et al. (2007) found that a strain of Gram-negative bacterium

296 increased/decreased the proportion of branched-chain fatty acids in higher pH/lower

297 pH conditions. Recently, a novel pH proxy RIAN which is based on Gram-negative

298 bacterial derived 3-OH-FAs in soils from Shennongjia Mountain was proposed by

299 Wang et al. (2016), with a low RIAN value when pH is high. Based on our finding

300 that 3-OH-FAs in the HS4 stalagmite are mainly derived from the overlying soils,

301 here we interpret the HS4 RIAN record as reflecting local changes of pH (Fig. 3A).

302   We note that the HS4 RIAN record is, for the period of mutual overlap, consistent

303   with hydrological records based on Hopanoids from the Dajiuhu peatland (Xie et al.,

304   2013) (Fig. 3B; Supplementary Information), situated 130 km to the NW of Heshang

305   Cave and the proportion of soil-derived magnetic minerals ($IRM_{soft-flux}$) incorporated

306   into the HS4 stalagmite, which Zhu et al. (2017) interpret as a proxy for rainfall

307   amount and intensity, with sensitivity to extreme precipitation events (Fig. 3C). These

308   consistent lines of evidence demonstrate RIAN could be used as qualitative

309   hydrological proxy in stalagmites. We argue below that, for the HS4 record, when

310   effective precipitation was higher in the past, the RIAN value is lower and likely

311   indicates leaching of soils and the influence of higher groundwater pH on the

312   3-OH-FA producing Gram-negative bacteria.

313   In soil environments pH reflects the balance between precipitation and

314   evaporation (e.g. hydrologically effective precipitation) and water movement through

315   the soil. Rainwater is naturally acidic due to the reaction with CO_2 in the atmosphere

316   to form carbonic acid. Excess rainfall leaches base cations increasing the relative

317   percentage of H^+ and Al^{3+} ions (and thus acidity) in water. Soil temperatures, pH and

318   aeration conditions also affect soil microbial activity and diversity which determines

319   bacterial respiration of CO_2 (and the formation of carbonic acid), further influencing

320   the pH of soil water and the degree of leaching (Fairchild and Baker, 2012). Thus we

321   argue that in the soils above Heshang cave, during the Holocene, pH was lower with

322   higher effective precipitation rates (and vice versa). This is consistent with the

323   generally observed relationship between effective precipitation and pH in global soils

324 (Slessarev et al., 2016; Yang et al., 2014) and wider evidence of the substantial

325 influence of pH on bacterial communities at both local and continental scales (Lauber

326 et al., 2009; Rousk et al., 2010). However, on the contrary, changes of pH in

327 groundwater systems may display in an opposite trend to that of the overlying soils in

328 response to increased effective precipitation. In a well-drained karst landscape

329 increases in precipitation will also be associated with the increased movement of

330 material (including organic matter and biomarkers), in dissolved or colloidal form to

331 the groundwater system, which may ultimately percolate or flush into cave systems

332 (Blyth et al., 2008). The pH of the groundwater is greatly affected by soil-derived

333 colloids and fine sands flushed into the groundwater system (Fairchild and Baker,

334 2012). The soil processes outlined above influence the latter source of material and

335 thus contribute to the pH of the groundwater system. More importantly, increased

336 rainfall can result in a fall in the total cation content which will lead to an increase of

337 pH in the groundwater system (Fairchild and Baker, 2012). Thus increased effective

338 precipitation may lead to antiphased pH variations between the overlying soils and

339 underlying groundwater systems. Here we interpret Holocene changes in RIAN in the

340 HS4 stalagmite record as primarily reflecting local changes in precipitation regime

341 which control the pH of the soil and groundwater systems. Specifically, we suggest

342 that RIAN records increases in groundwater pH in response to increased rainfall,

343 whereby microbes either initially derived from the overlying soils or already present

344 in the groundwater system modified their membrane lipids to adapt to higher pH in

345 the groundwater system resulting from increased rainfall and soil leaching.

346   From the above, hydrological changes during the last 9 ka BP were qualitatively

347   reconstructed from the HS4 stalagmite using the RIAN proxy (Fig. 3), which we

348   interpret as reflecting local changes in pH of the groundwater, originally driven by

349   changes in precipitation. The RIAN record from HS4 reveals two (relatively long)

350   wetter periods in central China, between 8.8-5.9 ka BP and 3.0-0 ka BP and one

351   relative dryer period from 5.9-3.0 ka BP (Fig. 3A). Both the HS4 and the Dajiuhu

352   peatland records reconstruct a dry middle Holocene with decreased precipitation

353   centered on ca. 5.5, 4.8 and 3.5 ka BP in central China (Fig. 3B, C) and overlap for

354   two long wet periods between > 9 ka to 6 ka BP and 3 to 0.6 ka BP. These drying

355   events occur simultaneously with colder RAN_{15}-MAATs and heavier $\delta^{18}O$ values in

356   HS4 (Fig. 4) and coeval cold/dry events recognized in a number of global NH and

357   regional paleoclimate records (Liu et al., 2013; Ljungqvist, 2010; Mayewski et al.,

358   2004; Owen et al., 2016; Rohling and Palike, 2005). These proxy-inferred

359   hydrological reconstructions re-enforce the conclusion of Xie et al. (2013) that the

360   overturn of distinctive cultures in the Neolithic Period to Iron Age in central China (as

361   observed in the distributions of >1600 prehistoric settlement sites) correlates with wet

362   or flood episodes.

363

364   **3.3 Holocene temperature reconstruction**

365   Temperature changes during the last 9 ka BP were reconstructed from the HS4

366   stalagmite using the RAN_{15} index (Wang et al., 2016). RAN_{15} is a novel temperature

367   proxy based on 3-OH-FA distributions measured in the soils from an altitudinal

368   transect on Shennongjia Mountain located 120 km to the NW of Heshang Cave (Wang

369   et al., 2016). Higher/lower RAN_{15} values (higher/lower ratio of the *anteiso* to *normal*

370   C_{15} 3-OH-FAs) are obtained in soils with cooler/warmer MAATs. The quantitative

371   correlation between MAAT and RAN_{15} is expressed in the following equation (Wang

372   et al., 2016):

373   MAAT = 23.03 – 3.03×RAN_{15} (R^2 = 0.51, p<0.001, RMSE = 2.6°C)   (3)

374   RAN_{15} in the HS4 stalagmite varies from 0.79 to 2.14 during the last 9 ka BP,

375   with the lowest value at ca. 7.4 ka BP and highest value at ca. 0.5 ka BP (Fig. 4A). By

376   applying equation (3) to the HS4 samples, we obtain RAN_{15}-MAAT reconstructions

377   over the last 9 ka BP (Fig. 4A). The average RAN_{15}-MAAT of 18.4°C over the most

378   recent part of the record (<0.8 ka BP) overlaps with the range of MAATs, ca. 16.2 to

379   18.7 (av. 17.5°C) measured since 1952 at the nearest meteorological station (Yichang,

380   located ca. 100 km away) and is very close to the av. MAAT of 18°C measured

381   directly outside the cave by a temperature logger between 2004 and 2007 (Hu et al.,

382   2008a). This agreement between reconstructed temperatures and instrumental

383   measurements increases our confidence in the potential of the RAN_{15} proxy.

384   RAN_{15}-MAATs in HS4 vary from 16.5 to 20.6°C (av. 19°C), during the last 9 ka BP,

385   and broadly follow a long-term trend of declining temperatures in line with declining

386   solar insolation at 30°N in July (Laskar et al., 2004) (Fig. 4B). The temperature

387   variation (4.1°C) in our record is larger than the calibration error of the RAN_{15} proxy

388   (RMSE = 2.6℃; Wang et al., 2016). The Holocene Climate Optimum (HCO) shown

389   in the RAN_{15}-MAAT record is from 8 to 6 ka BP, with the highest temperature at ca.

390 7.0 ka BP (Fig. 4A). Superimposed on the orbital-scale Holocene trend are centennial

391 to millennial scale climate fluctuations of ca. 1 to 2°C (Fig. 4A). Interestingly, the

392 most recent 0.9 ka BP is distinguished by greater variability with the highest (20.5°C)

393 and lowest (16.5°C) RAN_{15}-MAATs occurring consecutively at 0.6 ka BP and 0.5 ka

394 BP.

395   Our reconstructed RAN_{15}-MAAT follows a similar trend to the $\delta^{18}O$ record (Hu

396 et al., 2008b) from the HS4 stalagmite (Fig. 4C). The high resolution HS4 $\delta^{18}O$

397 record encodes a mixture of temperature and hydrological signals and clearly defines

398 a series of centennial scale episodes of heavier $\delta^{18}O$ (dry/cool events) superimposed

399 on the longer term Holocene trend (Hu et al., 2008b). Although the novel biomarker

400 based proxy has a relatively low resolution it's worth noting that a number of cooler

401 episodes observed in the HS4 RAN_{15}-MAAT record, centered on ca. 8.2 ka, 3.4 ka

402 and 0.5 ka BP (little ice age, LIA) occur simultaneously with heavier values in the

403 high resolution $\delta^{18}O$ record in HS4. Coeval cooling events are broadly recognized in a

404 number of global NH and regional (monsoonal) paleoclimate records (Ljungqvist,

405 2010; Mayewski et al., 2004; Rohling and Palike, 2005). Notably our RAN_{15}-MAAT

406 record is consistent with globally distal $\delta^{18}O$ ice-core record from Greenland (Johnsen

407 et al., 2001) (Fig. 4D) and the Northern Hemisphere Holocene stacked temperature

408 anomalies record (30° to 90°N) (Marcott et al., 2013) (Fig. 4E). Although our

409 sampling resolution is necessarily low due to biomarker sampling requirements (at

410 this stage of analytical development), the HS4 age model is well constrained and we

411 note that these RAN_{15}-MAAT maxima and minima at 0.6 ka BP and 0.5 ka BP

coincide with Northern Hemisphere (NH) scale warm and cold episodes during the late medieval warm periods (MWP) and LIA respectively (Ljungqvist, 2010; Mann et al., 2008; Moberg et al., 2005).

4. Conclusion

Hydrological and temperature changes in the middle reaches of the Yangtze River during the last 9 ka BP were reconstructed using Gram-negative membrane lipids extracted from the HS4 stalagmite from Heshang Cave, central China. RAN_{15} is a temperature proxy while RIAN is interpreted as qualitative hydrological proxy. Temperatures varied from 16.5 to 20.6°C during the last 9 ka BP, with a relatively warm period in the early to middle Holocene (8.0-6.0 ka BP), and then a relative cool period in the late Holocene. The hydrological record shows two relatively long wet periods and one relative dry period in central China, 8.8-5.9 ka BP, 3.0-0 ka BP and 5.9-3.0 ka BP respectively. The HS4 Holocene Climatic Optimum (HCO) between 8.0-6.0 ka BP is warmer and wetter than any other period in the Holocene and supports a conclusion of the seminal review of Monsoon palaeoclimate by An et al. (2000) that an early Holocene Optimum in Monsoon strength occurred in the middle and lower reaches of the Yangtze River, China, centered on ca. 6 ka BP. Moreover, this agrees with an ensemble of 18 different model simulations (Joussaume et al., 1999) for 6 ka, which all indicate enhanced low level convergence into the monsoon low over Eurasia, with the summer monsoon flow extending further inland. The present study demonstrates both the first paleoclimate application of 3-OH-FA based

434 proxies and how such biomarker tools can record independent hydrological and

435 temperature signals in speleothems.

436

449

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648

649   **FIGURE CAPTIONS**

650   **Figure 1** The location of Heshang Cave and sample sites. (A) Schematic map

651   showing the main regional surface drainage, revised after Hu et al. (2008a). The red

652   star shows the location of Heshang Cave. (B) The view of Heshang Cave entrance

653   from the opposite site of the Qing River. (C) Sampling locations of HS4 stalagmite

654   and cave surface sediments. Black solid triangle denotes the location of HS4 which

655   was collected in 2001 (Hu et al., 2008a). Black solid circles denote the sampling sites

656   of stream surface sediments; hollow squares denote the sampling sites of cave surface

657   sediment.

658

659   **Figure 2** Distribution and fractional abundance of hydroxy fatty acid homologues in

660   (A) cave overlying soils, (B) cave sediments, and (C) HS4 stalagmites.

661

662   **Figure 3** Comparison of the HS4 RIAN record with other local and regional

663   palaeo-hydrological records. A) Heshang Cave hydrological record inferred from the

664   RIAN record during the last 9 ka BP. U−Th dating errors (Hu et al., 2008b) are shown

665   on the top of the RIAN curve as red line segments. B) Hydrological record based on

666   hopanoids biomarkers from the Dajiuhu peatland (Xie et al., 2013), raw data are

667   shown as triangle symbols, the black line is a locally weighted scatter plot smoothing

668   with a quadratic polynomial (lowess) using a span of 5%. C) $IRM_{soft\text{-}flux}$ in stalagmite

669   HS4. Peaks in $IRM_{soft\text{-}flux}$ indicate intervals with increased precipitation events (Zhu et

670   al., 2017).

671

672   **Figure 4** Comparison of the HS4 stalagmite RAN_{15}-MAAT record with other

673   time-series and proxy records. A) RAN_{15} and RAN_{15}-MAAT record reconstructed

674   from the HS4 stalagmite. The standard deviation of the RAN_{15}-MAAT record is ±

675   0.1°C. The green star represents the mean annual air temperature (MAAT)

676   immediately outside the cave, as by a temperature logger between 2004 and 2007 (Hu

677   et al., 2008a). U−Th dating errors (Hu et al., 2008b) are shown on the bottom of the

678   RAN_{15}-MAAT curve as red line segments. B) Solar insolation changes at 30°N in July

679   during the last 9 ka BP (Laskar et al., 2004). C) The $CaCO_3$ oxygen isotope record

680   from the HS4 stalagmite (Hu et al., 2008b). D) Ice core $\delta^{18}O$ record from North GRIP

681   (Johnsen et al., 2001). E) The Northern Hemisphere stacked temperature anomalies

682   (30° to 90°N) for the 5° × 5° area-weighted mean calculation with its 1 σ

683   uncertainty (Marcott et al., 2013).

684