

1 **Calibrating the glycerol dialkyl glycerol tetraether temperature signal in**
2 **speleothems**

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26 **Abstract**

27

28 Paleotemperature proxies based on glycerol dialkyl glycerol tetraethers
29 (GDGTs) lipids have been established for marine and lacustrine environments, but
30 there has been relatively little study of their application in speleothems. In this study
31 we analyse the GDGT content of 33 speleothem samples from 16 different sites
32 around the globe, and test whether proxies based on isoprenoid tetraethers (TEX₈₆) or
33 branched tetraethers (MBT/CBT) are correlated with measured surface and cave mean
34 annual air temperature (MAT). The results show that the TEX₈₆ has a strong
35 relationship with measured temperature ($r^2 = 0.78$, standard error of the estimate 2.3
36 °C, when calibrated with surface MAT). Furthermore, the MBT/CBT also showed a
37 significant relationship with temperature ($r^2 = 0.73$, standard error of the estimate 2.7
38 °C, when calibrated with surface MAT). Some issues remain requiring future work,
39 in particular the development of a larger calibration sample set with measured cave
40 temperature data, and the investigation of controls other than temperature on GDGT
41 distribution, but overall the results indicate that GDGT based proxies derived from
42 speleothems may be highly viable new methods for reconstructing continental
43 paleotemperatures.

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46 **Keywords**

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48 Speleothems, stalagmites; paleotemperature; tetraethers; GDGT; MBT/CBT, TEX₈₆

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51 **1. Introduction**

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53 The recovery of both global and regional palaeotemperature records is an issue
54 of vital importance in palaeoclimatic research, being central to both our understanding
55 of past environmental change, and providing the basis for the development of models
56 for future climate. A number of palaeotemperature proxies have been developed, but
57 these have tended to focus on ice-cores and oceanic sediments (e.g. Dansgaard et al.,
58 1993; Müller et al., 1998; Petit et al., 1999; Elderfield & Ganssen, 2000; Johnsen et
59 al., 2001) with less work focused on the development of an independent
60 palaeotemperature measure for continental regions.

61 Stalagmites are ideal archives for terrestrial climate proxies as they form in
62 incremental laminations, are easy to date precisely via uranium series analysis, and
63 collect a wide range of chemical signals transported by the feed drip-water, including
64 stable isotopes, trace elements, and organic matter. To date, attempts to recover
65 temperature proxies from stalagmites have focused on stable isotopes in the calcite
66 matrix (e.g. Gascoyne et al., 1980; Talma & Vogel, 1992; Mangini et al., 2005),
67 clumped isotope thermometry of the calcite (Affek et al., 2008; Meckler et al., 2009),
68 or isotopic or noble gas analysis of fluid inclusions (Schwarcz & Harmon, 1976;
69 Genty et al., 2002; van Breukelen et al., 2008; Scheidegger et al., 2010). Analysis of
70 $\delta^{18}\text{O}$ in stalagmite calcite is now largely recognised as being an unreliable proxy for
71 temperature due to the large number of confounding factors that affect the signal
72 (McDermott, 2004). Clumped isotope analysis shows more promise, with the signal
73 being an internal function of the carbonate mineral independent of the isotopic
74 composition of the water (Affek et al., 2008). However, it was found that there is

75 disequilibrium in the clumped isotopes due to kinetic isotopic fractionation, leading to
76 temperature underestimates (Daëron et al., 2011).

77 The development of an organic temperature proxy in stalagmites would
78 provide a measure of temperature completely independent of the isotopic system. The
79 theory behind organic temperature proxies is that certain organisms synthesise
80 molecules with different structures dependent on the environmental conditions
81 (temperature, pH and so on) to which they are subjected (e.g. Castañeda and
82 Schouten, 2011 and references cited therein). Therefore, if these molecules are
83 preserved in a time-series context, it is possible to reconstruct the past environmental
84 conditions from the relative abundance of different structural types.

85 Glycerol dialkyl glycerol tetraethers (GDGTs) are lipid molecules derived
86 from microbial membranes that have been shown to vary in structure in relation to
87 temperature and pH. Two main groups have been identified: isoprenoid archaeal
88 GDGTs and branched GDGTs, which are believed to be principally derived from
89 bacteria (Weijers et al., 2006).

90 Archaeal GDGTs have isoprenoid carbon skeletons (Figure 1), with the
91 number of cyclopentane moieties in the structure having been shown to have a
92 positive relationship with temperature (Gliozzi et al., 1983; Schouten et al., 2002,
93 Wuchter et al., 2004). Of particular interest has been the kingdom Crenarchaeota,
94 which initially was thought to be formed of hyperthermophilic organisms, but has
95 now been shown to include ‘cold’ planktonic organisms living in lakes and oceans
96 within a ‘normal’ temperature range (0 – 30 °C) (DeLong, 1992; Fuhrman et al.,
97 1992). Genomic analysis of enriched or isolated ‘cold’ Crenarchaeota have shown
98 that they form a separated phylum called Thaumarchaeota (Brochier-Armanet et al.,
99 2008; Spang et al., 2010), which also include some (hyper-)thermophiles (De La

100 Torre et al., 2008). These Thaumarchaeota synthesise the same broad spectrum of
101 isoprenoid GDGTs as hyperthermophilic Crenarchaeota, but also produce
102 crenarchaeol, a GDGT characterised by possession of a cyclohexane moiety
103 (Schouten et al., 2000; 2002; Sinninghe Damsté et al., 2002). The sensitivity of the
104 molecular composition of thaumarchaeotal membrane lipids to temperature has made
105 them strong candidates for use as organic temperature proxies. This has been
106 exploited in the development of the TEX₈₆ proxy (Schouten et al., 2002), which
107 measures the relative distribution of different isoprenoid GDGTs derived from aquatic
108 Thaumarchaeota. TEX₈₆ has principally been used in marine studies, although studies
109 have also been made in continental aquatic contexts (e.g. Powers et al., 2004; Blaga et
110 al., 2009; Powers et al., 2010). Thaumarchaeota, as well as other Archaea, also occur
111 widely in the terrestrial realm (e.g. Buckley et al., 1998; Ochsenreiter et al., 2003;
112 Leininger et al., 2006; Weijers et al., 2006), and their membrane lipids are detectable
113 in soils, albeit at much lower abundance levels than those derived from bacteria, and
114 at much lower abundances than found in the marine realm (Gattinger et al., 2003;
115 Weijers et al., 2006). However, TEX₈₆ is not generally considered a viable proxy for
116 land or soil based palaeothermometry due to the additional contribution of isoprenoid
117 GDGTs from for example, methanogenic Euryarchaeota (Weijers et al., 2006).

118 Bacterially derived branched GDGTs have a carbon skeleton containing 4 to 6
119 branched methyl groups and up to two cyclopentane moieties (Figure 1). In a global
120 study of GDGTs in soils, Weijers et al. (2007) demonstrated that mean annual air
121 temperature (MAT) and soil pH were major controls on the number of methyl
122 branches in bacterial GDGTs, while the number of cyclopentane rings was primarily
123 related to soil pH alone. This led to the creation of two indices designed to quantify
124 the distribution of the different branched GDGT structures within a sample: MBT

125 reflecting soil pH and MAT, and CBT reflecting soil pH. As both these indices have
126 soil pH as a common factor, it is therefore possible to use the two indices together to
127 derive a calculated MAT. This approach has now been applied mainly to ocean
128 margin sediments (e.g. Weijers et al., 2007; Schouten et al., 2008;).

129 The use of the MBT/CBT soil derived proxy in lakes has proved problematic,
130 with calculated temperatures consistently coming out colder than measured
131 temperatures (Tierney et al., 2010; Blaga et al., 2010; Pearson et al., 2011). This is
132 hypothesised to be the result of mixed environmental inputs, with branched GDGTs
133 being derived from both transported terrestrial material and in situ production
134 (Tierney & Russell, 2009; Tierney et al., 2010; Pearson et al., 2011). Therefore,
135 recently other statistical techniques have been applied, focusing on regression models
136 and weighted GDGT proxies, which has yielded improved correlations between
137 branched GDGT distribution and lake temperatures (Tierney et al., 2010; Pearson et
138 al., 2011).

139 The analysis of GDGTs preserved in stalagmites has in principle great
140 potential to provide an organic terrestrial palaeothermometer independent of isotopic
141 records. The strong time-series control provided by U series disequilibrium dating
142 provides a robust temporal framework within which the temperature records can be
143 set, while the presence of other chemical proxies for climatic and environmental
144 changes (such as fluctuations in rainfall, or changes in the overlying vegetation)
145 would allow investigation of how changes in temperature relate to other changes in
146 the wider environment. However, investigation of the presence and utility of GDGTs
147 in stalagmites has been limited, with Yang et al. (2011) the only in depth investigation
148 we are aware of. Yang et al. (2011) analysed the GDGT content from palaeo and
149 modern speleothem samples in Heshang Cave, China, along with samples of drip-

150 water, bedrock from within the cave, and overlying soils. They found that the
151 stalagmite GDGTs were dominated by archaeal compounds (particularly
152 crenarchaeol), and that both archaeal and bacterial GDGTs differed markedly in
153 stalagmites compared to those in the overlying soils. They therefore hypothesised that
154 GDGTs in stalagmites are primarily cave or aquifer derived. In terms of proxy utility,
155 Yang et al. (2011) concluded that TEX₈₆ might relate to temperature as it showed
156 some correlation ($r^2 = 0.51$ for TEX₈₆, and 0.43 for TEX_{86'}) with depletion in $\delta^{18}\text{O}$ in
157 the calcite, which is related to monsoon intensity (via surface precipitation), which in
158 turn relates to temperature. However, there are a number of reasons why changes in
159 rainfall might influence GDGT composition, including variation of input from
160 different sources with changes in transport flow, and variation in microbial activity
161 with changes in nutrient input and moisture. It is worth noting that, although not
162 commented on in the study, the MBT/CBT index showed a negative correlation with
163 $\delta^{18}\text{O}$ of the same magnitude ($r^2 = 0.47$). The study noted that the MBT/CBT index in
164 the modern stalagmites was a poor match for modern temperature, showing a much
165 lower value than expected for the region. However, as this is a record from only one
166 site, and the calibration applied was derived from soils, to judge more
167 comprehensively whether GDGT proxies in stalagmites have potential as reliable
168 temperature proxies, it is necessary to undertake a broader multi-site study, and create
169 speleothem specific calibration equations. Here we therefore analysed the GDGT
170 content of a suite of modern and known age samples to create a new calibration to
171 establish the validity of GDGTs in speleothems as a palaeotemperature proxy.

172

173 **2. Material and methods**

174

175 2.1 Stalagmite sampling and comparative data

176

177 Thirty-three samples from recent stalagmites, straw stalactites, and flowstones
178 from sixteen sites around the world were analysed (Table 1; Fig. 2). These cover a
179 MAT range of 7 – 26 °C, and were chosen to encompass a range of different climatic
180 zones. Comparative modern surface air temperatures were obtained for 15 sites (data
181 for Nettlebed cave, New Zealand was not available) from measurements published or
182 determined by the original speleothem workers at the sites, and otherwise from the
183 records of the nearest weather station (Table 1). Due to the nature of the temperature
184 data, full information on collection method and errors is not available. This imposes a
185 limitation on our data set (discussed in section 3.8), but does not invalidate it. Where
186 possible (8 sites), internal cave temperature data were also obtained from the original
187 speleothem studies. Both cave and surface temperatures are necessary to test the
188 calibrations because cave temperature will be important if GDGTs derive from in situ
189 cave organisms, while surface temperature will be important if GDGTs derive from
190 soil organisms.

191 Multiple samples are included from single caves (Uamh an Tartair; Cathedral
192 Cave, Wellington), and from separate caves in a single area (Cathedral and Gaydon
193 Cave, Wellington, the Bats Ridge Cave system) in order to test the consistency of the
194 signal at individual sites. Sample choice was dictated by availability and the
195 constraints of cave conservation. However, although these samples are from single
196 areas, they should not be considered true replicates in the way multiple samples from
197 a single speleothem would be, as caves are heterogeneous environments, with
198 variations in microenvironment due to position in the cave, and variations in external
199 input between drip pathways. This is illustrated by the two samples from Nettlebed

200 cave in New Zealand, which have different measured cave temperatures for their
201 collection sites (Table 1).

202 Calcite samples of 5 – 20 g were sectioned from stalagmites and flowstones
203 using a 2.5 cm diamond tipped circular saw attached to a hand drill. Where samples
204 were taken from stalagmites which had been actively depositing at collection, the
205 subsample was taken from the outer (youngest) layers, in order to ensure that the most
206 recent growth was sampled. Straw stalactites were processed intact.

207

208 **2.2 Extraction method**

209

210 Prior to processing, each sample was cleaned by immersion in 3 M pre-
211 cleaned hydrochloric acid (HCl) to remove the outer 2 mm surface layer, rinsed in
212 cleaned MilliQ water, and sonicated in dichloromethane (DCM) to remove any
213 remaining organic surface contamination. Samples were individually digested in 3 M
214 HCl, and boiled under reflux at 100 °C for two hours, which serves to liberate all the
215 GDGTs present, including those from intact polar lipids (Schouten et al., 2013). After
216 cooling, each sample was subject to manual liquid – liquid extraction in DCM,
217 following Blyth et al. (2006). The extracts were separated over an activated Al₂O₃
218 column and eluted sequentially with DCM and DCM / methanol (1:1) following
219 Weijers et al. (2007). The polar (DCM / methanol) fraction was dried under nitrogen,
220 ultrasonically dissolved in hexane / propanol (99:1), and filtered through a 0.45 µm
221 PTFE filter (ø 4 mm) prior to analysis.

222

223 **2.3 Analytical method**

224

225 Polar fractions were analysed for GDGTs using high performance liquid
 226 chromatography/ atmospheric pressure positive ion chemical ionization mass
 227 spectrometry (HPLC/ APCI-MS) following Schouten et al. (2007). HPLC/APCI-MS
 228 analyses were using an Agilent 1100 series LC/MSD SL and separation and a Prevail
 229 Cyano column (2.1 x 150 mm, 3 mm; Alltech), maintained at 30°C. The glycerol
 230 dialkyl glycerol tetraethers (GDGTs) were eluted using a changing mixture of hexane
 231 and propanol as follows: 99% hexane: 1% propanol for 5 minutes, then a linear
 232 gradient to 1.8% propanol in 45 minutes. Flow rate was 0.2 ml per minute. Single ion
 233 monitoring was set to scan the [M+H]⁺ ions of the GDGTs with a dwell time of 237
 234 ms for each ion. Only peak areas above limit of quantitation were considered.

235 The following equations were used to calculate the proxy values

236

$$237 \text{TEX}_{86} = (\text{GDGT 2} + \text{GDGT 3} + \text{Cren isomer}) / (\text{GDGT 1} + \text{GDGT 2} + \text{GDGT 3} +$$

$$238 \text{Cren isomer}) \quad [\text{Eq. 1}]$$

239

$$240 \text{BIT} = (\text{GDGT III} + \text{GDGT II} + \text{GDGT I}) / (\text{Crenarchaeol} + \text{GDGT III} + \text{GDGT II} +$$

$$241 \text{GDGT I}) \quad [\text{Eq. 2}]$$

242

$$243 \text{MBT} = (\text{GDGT I} + \text{GDGT Ib} + \text{GDGT Ic}) / ((\text{GDGT I} + \text{GDGT Ib} + \text{GDGT Ic}) +$$

$$244 (\text{GDGT II} + \text{GDGT IIb} + \text{GDGT IIc}) + (\text{GDGT III} + \text{GDGT IIIb} + \text{GDGT IIIc}))$$

$$245 [\text{Eq. 3}]$$

246

$$247 \text{CBT} = -\text{LOG}((\text{GDGT Ib} + \text{GDGT IIb}) / (\text{GDGT I} + \text{GDGT II})) \quad [\text{Eq. 4}]$$

248

249 It was not possible to calculate experimental errors for these samples, due to
250 the limited calcite made available, and the low compound abundance preventing
251 replicate runs. However, laboratory and instrument analytical error is known to be
252 typically better than 0.5 °C, based on previous studies (Kim et al., 2010, Weijers et
253 al., 2007).

254

255 **3. Results and discussion**

256

257 **3.1 GDGT distribution**

258

259 The results show that GDGTs are commonly present in and extractable from
260 stalagmite samples of 5 - 20 g calcite at measurable levels. This is a large calcite
261 sample size compared to some stalagmite techniques, but compares favourably with
262 other organic studies in this context (e.g. Xie et al., 2003; Blyth et al., 2011). Not all
263 samples contained all GDGTs in concentrations above the limit of detection or
264 quantitation, with the crenarchaeol regio-isomer and GDGTs Ic, IIc, IIIc, and IIIb
265 being the least common. Figure 3 shows a ternary plot of the proportional
266 relationships between GDGT 0, crenarchaeol, and the sum of branched GDGTs I, II,
267 & III. This demonstrates a dominance of crenarchaeol in the majority of samples,
268 although also shows that in a handful of samples, branched GDGTs do predominate.
269 Interestingly, for the multiple samples from Wellington, Lower Balls Mine and to a
270 lesser extent, Tartair, the proportion of GDGT-0 is consistent within each site, but
271 there is variation of 10 – 20% between the samples in the proportions of crenarchaeol
272 and branched GDGTs. At Bats Ridge there is a similar distribution of GDGTs in
273 three of the four samples, with the remaining sample showing a lower proportion of

274 crenaracheol and higher proportion of GDGT-0. This within site variability suggests
275 variation in either cave micro-environment or drip source input.

276

277 **3.2 BIT index**

278

279 The dominance of isoprenoid GDGTs versus branched GDGTs can be
280 expressed in the so-called BIT (Branched and Isoprenoid Tetraether) index, a proxy
281 used in marine environments for the relative input of soil organic matter (Hopmans et
282 al., 2004). The BIT index compares the relative amounts of crenarchaeol, mostly
283 aquatic derived, and soil derived non-isoprenoidal branched GDGTs (Hopmans et al.,
284 2004; Weijers et al., 2006). In open marine environments BIT values tend to be <0.1
285 (Kim et al., 2010) while in soils most BIT values are >0.9 (e.g. Weijers et al., 2006).
286 As stalagmites contain distinct signals from soil organic matter (Xie et al., 2003;
287 Blyth et al., 2007, 2010, 2011), it might be expected that they would show a high BIT
288 value due to significant soil derived input. However, the results clearly show that this
289 is not the case, with twenty-eight of the thirty-three samples (from 12 out of 16 sites)
290 showing a BIT index below 0.5 (Fig. 4). This is in line with the results of Yang et al.
291 (2011), who found BIT values ranging from 0.21 - 0.58 in the stalagmite samples
292 from Heshang cave. Our results show that even lower values are common, with
293 twelve of the samples (from 6 sites) having a BIT of 0.1 or below. The BIT values
294 are not consistent between samples from the same site, except at Bats Ridge where all
295 the samples have a BIT of below 0.1. This reflects the within site variation of
296 crenarchaeol and the branched GDGTs seen in the ternary plot (Fig. 3).

297 The BIT results indicate that a strong *in situ* cave / aquatic signal dominated
298 by crenarchaeol is present, and that this is a consistent pattern across a wide

299 geographical range of samples. Secondly, the results suggest that cave microbes are
300 not simply immigrant soil organisms, but distinct cave communities. This hypothesis
301 of the presence of crenarchaeol producing organisms in cave microbial communities
302 is supported by microbiological work showing that thaumarchaeotal 16S rDNA
303 sequences have been found in cave deposits (Gonzalez et al., 2006).

304

305 **3.3 TEX₈₆**

306

307 Given the high crenarchaeol abundance observed, suggesting an active
308 Thaumarchaeotal community in the cave deposits, it would be reasonable to
309 hypothesise that TEX₈₆ might be a suitable proxy to apply. We performed two sets of
310 correlations, plotting TEX₈₆ against MAT derived from local or regional surface air
311 temperature measurements (n = 30, from 14 sites – one sample / site LAB-S2 was not
312 included due to too low an abundance of certain GDGTs), and against measured cave
313 air temperature (n = 19, from 8 sites). In both cases, TEX₈₆ shows a significant
314 correlation ($r^2 = 0.78$, $p < 0.001$ for surface MAT, $r^2 = 0.68$, $p < 0.001$ for cave MAT)
315 (Fig. 5; table 2). This indicates that a temperature dependent signal is being recorded.

316 One method to improve TEX₈₆ correlations is to exclude samples with high
317 BIT values, therefore excluding samples with significant external soil input which
318 may blur the autochthonous signal. This approach has been used in marine sediments
319 whereby BIT values >0.3 are thought to signal a potential bias in TEX₈₆ values.
320 (Weijers et al., 2006). This criterion has also been applied in lakes, and shown to
321 improve calibrations, but in that context has been complicated by the small sample set
322 remaining (Blaga et al., 2009; Powers et al., 2010; Bechtel et al., 2010; Pearson et al.,
323 2011). Due to the persistently low BIT seen in this study, it is possible to exclude

324 samples with a BIT of ≥ 0.5 , and still maintain a reasonable sample set of $n = 27$
325 (from 11 sites) for surface MAT calibration and $n = 16$ (from 6 sites) for the cave
326 MAT calibration. However, trimming the data set in this way does not make a
327 substantial difference to the calibrations (Fig. 6; table 2), with the calibration with
328 surface temperature having an r^2 of 0.77, and with cave temperature an r^2 of 0.70.
329 The latter is a slight improvement, but as can be seen from figure 6a, the correlation is
330 mainly driven by only two samples with high measured MAT. There is no reason to
331 treat these points as abnormal outliers and remove them from the data set, but until
332 additional samples with cave temperature measurements from the mid and high
333 temperature ranges can be obtained, this calibration is currently not very robust. In
334 both the surface and cave datasets, exclusion on BIT > 0.5 improves the standard error
335 of the estimate by 0.1°C only. As the changes to the calibration equation are therefore
336 not substantial, and do not reduce the sample set, at present we do not consider it
337 advisable to exclude data points by BIT in this context, although it may become
338 appropriate during future work.

339 For TEX_{86} , measured in the full sample set, we therefore have calibration
340 equations of:

341

342 Surface MAT = $-7.4 + (33.3 * \text{TEX}_{86})$

343 ($r^2 = 0.78$; $p < 0.001$; standard error of the estimate = 2.3°C) [eq. 5]

344

345 Cave MAT = $-7.3 + (32.2 * \text{TEX}_{86})$

346 ($r^2 = 0.68$; $p < 0.001$; standard error of the estimate = 2°C) [eq.6]

347

348 The standard errors of the regression in these calibrations compare favourably
349 to previous studies in other contexts. In marine sediments Kim et al. (2010) reported
350 standard errors of 2.5 °C for the $\text{TEX}_{86}^{\text{H}}$ excluding polar oceans. In lakes, Powers et
351 al. (2010) found an estimated temperature error of 3.6 °C in a data set with high BIT
352 samples removed. As can be seen from Fig. 5, the calibration line is quite different to
353 those from Kim et al. (2008) or Powers et al. (2010), with a much lower slope, and a
354 slightly higher intercept. This supports the argument for the development of
355 calibrations specific to the cave context.

356 A reported potential problem of application of the TEX_{86} proxy is input from
357 methanogenic and methanotrophic archaea. As this has been found to render the
358 proxy problematic in terrestrial contexts (Weijers et al., 2006), it is necessary to
359 identify if this has been the case here. Blaga et al. (2009) proposed that a ratio of
360 GDGT-0/crenarchaeol >2 signifies an important contribution of methanogenic
361 archaea to the pool of GDGTs. Application of this threshold to our data shows no
362 sample with a ratio >2 (thirty-one samples have a ratio of ≤ 0.5 , while sample MD3
363 has a ratio of 1.1 and POS-1 a ratio of 0.8). Methanogenic input does not therefore
364 seem to be an issue in this context. Other potential influences on the proxy such as
365 pH could not be determined in this study due to the unavailability of background data,
366 but should be considered in future work.

367

368 **3.4 MBT/CBT proxy**

369

370 Two samples, Turk-1 and LBM-S3 were removed from the data set for
371 MBT/CBT calculations due to branched GDGT peaks below the limit of quantitation.
372 This provided a total sample set of 28 samples from 14 sites for correlation with

373 surface MAT, and 17 samples from 8 sites for correlation with measured cave MAT.

374 Table 2 displays the main statistics for the various correlations.

375 Figure 7 shows the results of plotting MBT and CBT separately against the
376 measured surface and cave MAT. CBT has previously been proposed as being driven
377 by the pH conditions, and MBT by a combination of pH and temperature (Weijers et
378 al., 2007). In this study we were unable to obtain sufficient pH data for all sites,
379 which means that, until modern process work is completed for a range of caves, we
380 cannot assess whether CBT acts as a proxy for pH as in soils. At one site (Buchan),
381 where we have been able to make pH measurements, the soil pH range is 6.8 – 7.5,
382 while the cave drip-water pH is 6.9 - 8. The CBT derived pH for this site is 8,
383 suggesting that CBT may indeed reflect pH. It can also be seen that there is one
384 outlier in the data set compared with surface temperature (SC-4 from Christmas
385 Island) which has an abnormally high CBT compared to the rest of the samples. This
386 CBT results in a calculated pH for this sample of 4.3, which certainly initially seems
387 unrealistic. However, this sample has an unusual calcite fabric and trace element
388 profile, and it is hypothesised that microbes may have been actively modifying the
389 microenvironment on areas of the speleothem surface, reducing the pH and causing
390 microdissolution of the calcite (Frisia, pers. comm.).

391 Figure 7 shows that there is no significant relationship between CBT and
392 either measured temperature, as with soils (Weijers et al., 2007), but a reasonable
393 correlation ($r^2 = 0.62$) between MBT and surface MAT. Cave MAT has a weak
394 correlation with MBT of $r^2 = 0.38$. Following Weijers et al. (2007) and Sun et al.
395 (2011) a multiple regression was carried out between measured MAT, CBT and MBT.
396 The 3D plot for the surface MAT data set is shown in figure 8a, and gives an equation
397 of:

398

399 Surface MAT = 5.38 + (27.1 * MBT) - (4.8 * CBT) [Eq. 7]

400 ($r^2 = 0.73$, $p < 0.001$, standard error of the estimate = 2.7 °C)

401

402 The multilinear regression was then repeated for the reduced sample set with available
403 cave temperature data (Fig 8b). This gave an equation of:

404

405 Cave MAT = 6.06 + (21.3 * MBT) - (5.4 * CBT) [eq. 8]

406 ($r^2 = 0.56$, $p = 0.003$, standard error of the estimate = 2.5°C)

407

408 In both regressions MBT and CBT both contributed significantly to the regression
409 model.

410 The improved correlation of surface MAT with both CBT and MBT compared
411 to MBT alone suggest, like in soils (Weijers et al., 2007), that a multilinear regression
412 is needed. The degree of correlation of the surface MAT calibration of Eq. 7, and
413 particularly the standard error of the estimate compares favourably with those of
414 MBT/CBT calibrations in other contexts. In the Weijers et al. (2007) the original soil
415 calibration had an r^2 of 0.77 with a standard error of 5 °C (Weijers et al., 2007b). In a
416 global lakes calibration, Sun et al. (2011) reported $r^2 = 0.62$, with a standard error of
417 5.2 °C across the whole data set, and $r^2 = 0.73$ with an error of 4.3 °C when highly
418 alkaline lakes were excluded. A recent recalibration of the MBT/CBT for soils
419 reported an r^2 of 0.59 with a standard error of 5 °C (Peterse et al., 2012). Figure 9
420 shows plots of measured vs calculated MAT using our speleothem based calibration,
421 with the relevant regression lines from the equations of Sun et al., 2011 and Weijers et
422 al., 2007. The speleothem correlations have a lower slope than either of the other

423 calibrations, and produces intermediate calculated temperatures, which begin to
424 converge with the lakes calibration in colder samples (<10 °C) and the soils
425 calibration in hotter climates (>20 °C).

426 As lake studies have shown that weighted branched GDGT calibration might
427 be more suitable than the MBT/CBT, we tested the equations of Tierney et al. (2010),
428 and Pearson et al. (2011) on our data set. Both these calibrations give a much poorer
429 correlation in stalagmites than the MBT/CBT calibration, with r^2 values of 0.32 and
430 0.45 respectively against surface temperature (data not shown). We therefore propose
431 that in cave contexts, a non-weighted MBT/CBT based calibration is currently more
432 appropriate for branched GDGT data.

433

434 **3.5 Local variability within cave deposits**

435

436 Caves are potentially heterogeneous environments, with micro-environmental
437 variations dependent on position within the cave (depth, airflow, moisture), and
438 external source input varying with drip pathway and flow rate. To examine the
439 variations in GDGT composition within cave sites, and the effect on the temperature
440 correlations, we took multiple samples at four sites, Tartair (Scotland), Lower Balls
441 Mine (England), Bats Ridge (Victoria, Australia), and Wellington (NSW, Australia).
442 For Tartair, nine separate broken straw stalagmites from the same chamber of a single
443 cave were analysed for MBT/CBT, and ten for TEX_{86} . At Lower Balls Mine, two
444 stalagmite samples were collected from beneath different drip points. Both produced
445 sufficiently abundant GDGTs to calculate TEX_{86} values, but only LBM-S2 contained
446 all the required branched GDGTs above the limit of quantitation. At Bats Ridge,
447 straw stalactites encrusted with moonmilk were taken from three caves on the same

448 property (BR-36, BR-10, and BR-5) with a modern stalagmite sample also taken from
449 BR-36. At Wellington, two caves on the same reserve were sampled, with straw
450 stalactites being collected from Gaydon Cave, and separate samples of straw
451 stalactites, natural flowstone, and flowstone overgrowth from a drip-collection bottle
452 collected from Cathedral Cave.

453 As seen in Figs. 3 and 4, there is variation in the broad GDGT composition
454 within Tartair and Wellington, especially with respect to the relative proportions of
455 crenarchaeol and the branched GDGTs, although not GDGT-0. Figure 10 and table 3
456 show the variation in the different sample groups when our TEX_{86} , and MBT/CBT
457 equations (calibrated to surface MAT) are applied. It can be seen that there is
458 variation in the precision of the two proxies, with TEX_{86} showing a smaller standard
459 deviation at Wellington Caves, while the MBT/CBT proxy has a lower standard
460 deviation at Bats Ridge. The standard deviation for the Tartair samples is similar in
461 both proxies. For the MBT/CBT proxy, the standard deviation at each site is within
462 the standard error of the estimate for the calibration ($2.7\text{ }^{\circ}\text{C}$). For TEX_{86} the standard
463 deviations at Bats Ridge ($3\text{ }^{\circ}\text{C}$) falls just outside the error for the calibration ($2.3\text{ }^{\circ}\text{C}$).
464 In terms of accuracy of the temperature calculation, the TEX_{86} calibration places the
465 mean for each site slightly nearer the 1.1 line. These results suggest that there is some
466 within cave variability of the proxy values which causes part of the scatter in our
467 proxy calibrations. We hypothesise that this variability is due to a combination of
468 small scale variability in microbial populations and temperature within a given site,
469 and variations in drip input. However, this variability is largely within the error of the
470 calibration, and does not therefore have major implications for palaeo applications.
471 At present it is not simple to identify characteristics in the broader GDGT data set that
472 might indicate which proxy would be most applicable at a specific site. For example,

473 it might be thought that BIT values could indicate whether TEX_{86} or the MBT/CBT
474 proxy would be more appropriate, with high BIT values potentially biasing TEX_{86} .
475 However, these results suggest that a low BIT does not in itself indicate applicability
476 of TEX_{86} or contra-indicate use of MBT/CBT, with Bats Ridge (where MBT/CBT
477 gives the best repeatability) having a lower range of BIT values than Wellington (0.03
478 - 0.1, as opposed to 0.05 – 0.28).

479

480 **3.6. Implications for application of GDGT proxies in stalagmites**

481

482 The results indicate that both TEX_{86} and MBT/CBT have potential for use as
483 temperature proxies in speleothems, showing significant correlations with measured
484 temperature. Overall, the TEX_{86} proxy appears more generally suitable with a higher
485 r^2 value (0.78 against surface MAT) when measured temperature is correlated against
486 proxy temperature, and a lower standard error of estimate (2.3 °C), a finding that can
487 probably be attributed to the predominance of crenarchaeol in the samples. However,
488 the MBT/CBT proxy also has an r^2 (0.73) and a standard error of estimate (2.7 °C)
489 comparable to the proxy calibrations in other contexts, and our site specific repeats
490 indicate that at some sites, such as the Bats Ridge cave system, the MBT/CBT proxy
491 may be more suitable. At this early stage of proxy development, while we do not
492 have prior indicators to identify the most applicable proxy, we would suggest
493 measuring and reporting both to be the most conservative and robust approach.

494 An outstanding issue to be resolved is whether in the long-term speleothem
495 GDGT proxies are best calibrated against surface air temperature or against cave
496 temperature. Given that we have demonstrated that the GDGT signal in speleothems
497 is dominated by crenarchaeol, and appears to have an in situ cave / aquatic character,

498 it would be logical to hypothesise that cave temperature will provide the better
499 correlation. The results here show consistently stronger correlations with surface air
500 temperature, however, this may be a function of sample size, with only a limited
501 number of cave temperature measurements being available compared to surface MAT.
502 To test this hypothesis, we correlated TEX_{86} against surface MAT (fig. 11), but this
503 time using a restricted data containing only samples for which we have both cave and
504 surface MAT measurements ($n=17$, from 7 sites). Our hypothesis was that if cave
505 temperature is a better comparator, then the r^2 for the cave MAT correlation should be
506 higher than the r^2 for the surface MAT correlation on the same sample set. Indeed,
507 the cave correlation of $r^2 = 0.73$ ($p < 0.001$; standard error of the estimate = $1.9\text{ }^\circ\text{C}$) is
508 higher than that of the surface MAT correlation with $r^2 = 0.65$ ($p < 0.001$; standard
509 error of the estimate = $2.3\text{ }^\circ\text{C}$). A similar exercise rerunning the multi-linear
510 regression for the MBT/CBT proxy ($n = 15$, from 7 sites) gives an r^2 for the cave
511 correlation of 0.56 ($p = 0.007$; standard error of the estimate = $2.7\text{ }^\circ\text{C}$), and a surface
512 correlation of $r^2 = 0.44$ ($p = 0.031$; standard error of the estimate = $3.2\text{ }^\circ\text{C}$). The
513 correlations are much less robust than the original calibrations of the full cave and
514 surface temperature data sets. However, in the case of both proxies, the results
515 suggest that the poorer performance of the cave calibrations in the full sample sets
516 may indeed be caused by the reduced size of the available data-set. Given the
517 constraints of the currently available data sets, we consider the full data set correlation
518 with surface MAT to be the most robust and useful calibration available at present, as
519 it is based on the largest number of samples and sites. However, the development of a
520 larger data-set based on internal cave MAT should be a priority for future work in this
521 area, so that the calibrations can be revisited in future and made statistically more
522 robust.

523 Another issue is the degree of error, not in the GDGT proxies, but in the
524 surface and cave temperature measurements used for the calibration. The cave
525 temperatures are generally the result of direct monitoring projects. However, due to
526 the constraints arising from using a largely archive derived sample set, the surface
527 temperatures have been derived from a range of sources ranging from local
528 monitoring in the direct vicinity of the cave through to published regional weather
529 station measurements. The latter in particular may not be completely representative
530 of the microclimate in the cave or in the soil directly above. Future modern
531 monitoring work could significantly reduce uncertainty in measured MAT and
532 improve our understanding of the limitations of the current calibrations, although such
533 measurements are unlikely to ever be available for all speleothem sites. There is also
534 a degree of error inherent in the use of modern or historical climate measurements in
535 comparison with results from speleothem samples which will be time-averaged over
536 potentially a few hundred years. Due to the amount of calcite required to obtain a
537 measurable organic signal, this error is unavoidable at all but sites with exceptional
538 growth rates. Targeting new sample collection at such sites will help ensure the
539 robustness of the calibration, and the impact of factors such as seasonality.

540

541 **4. Conclusions**

542

543 This study has shown that GDGTs are recoverable at measurable levels from
544 speleothem samples from a wide range of environments and climates, and that
545 significant correlations with temperature are observed with both TEX₈₆ and
546 MBT/CBT proxies, allowing us to create the first speleothem based proxy
547 calibrations. The correlation coefficients and standard errors for both proxies

548 compare favourably with the use of GDGT proxies in marine, lacustrine and terrestrial
549 contexts. The unique mixed aquatic / terrestrial environment of caves is probably
550 responsible for the unusual situation of both TEX_{86} and MBT/CBT based proxies
551 being useable, although the dominance of crenarchaeol in this context suggests that
552 TEX_{86} is likely to be the most effective proxy in the long-term. The considerable
553 potential shown here now needs to be built on by further work which should be aimed
554 at identifying, and if possible reducing the scatter in the correlation; identifying any
555 conflicting controls on the signal, which may include other climatic factors such as
556 precipitation, as well as variations in the microbial communities, and increasing the
557 calibration sample set, especially to increase the number of samples for which
558 measured cave temperature is available. An increased data set will also enable a
559 reappraisal of the most appropriate GDGT index to use in speleothems, including
560 investigation of new equations developed specifically for the cave context. These
561 advances will help in the development of a strong and robust novel terrestrial
562 paleotemperature proxy.

563

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583

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- 778 List of figures and tables
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783 text and figures.
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792 Figure 3. Ternary plot showing the relative distributions of GDGT-0, crenarchaeol,
793 and the sum of branched GDGTs I, II, & III.

794

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796 were measured in duplicate and error bars represent the difference between the two
797 measurements.

798

799 Figure 5. Scatter graphs showing the correlations between TEX_{86} and a) surface
800 MAT, and b) cave MAT. The black dashed line is the marine calibration line for Kim
801 et al., 2008, the grey dotted line is the lakes calibration line for Powers et al., 2010.

802

803 Figure 6. Scatter graphs showing TEX_{86} correlations with samples with $\text{BIT} \geq 0.5$
804 excluded, a) surface MAT, b) cave MAT.

805

806 Figure 7. Linear correlations between surface MAT and a) MBT, b) CBT; and
807 between cave MAT and a) MBT, b) CBT.

808

809 Figure 8. 3D correlations between a) MBT, CBT, and surface MAT; b) MBT, CBT,
810 and cave MAT.

811

812 Figure 9. Scatter plots showing the relationship between a) surface MAT and
813 MBT/CBT calculated temperature using eq. 7; b) cave MAT and MBT/CBT
814 calculated temperature using eq. 8. Black dashed line shows the regression trend
815 using the soils calibration of Weijers et al., 2007, grey dotted line shows the
816 regression trend using the lakes calibration of Sun et al., 2011.

817

818 Figure 10. Plots showing the within site variability in the MBT/CBT surface

819 calibration (a) and $\text{TEX}_{86}^{\text{H}}$ (b) against surface MAT. Error bars are +/- 1 SD (except

820 for Lower Balls Mine which shows the range), grey dotted line is the 1:1 line for

821 measured and calculated temperature.

822

823 Figure 11. Scatter plots showing the relationship between surface MAT (a) and cave

824 MAT (b) and $\text{TEX}_{86}^{\text{H}}$ for the reduced dataset for which both cave and surface

825 temperature data are available.

826

827

Table 1.

Sample no.	Sample type	Location	Age	Cave MAT °C	Surface MAT °C	Reference for MAT °C
Tar-1						
Tar Aa						
Tar Ab						
Tar Ac						
Tar Ae	Broken straw	Uamh am Tartair, Scotland	Recent / Holocene	7.2	7.1	Fuller et al., 2008
Tar Af						
Tar Ag						
Tar Ah						
Tar Ai						
Tar Aj						
MD-3						
Net-1	Straw			8.2	-	
Pos-1	Stalagmite	Postojna cave, Slovenia	Forming at collection (base max 15 years)	8.4	7.8	Dominguez-Villar pers comm
PC-1	Stalagmite	Pooles Cavern, England	Past 10 years	7.9	9	Hartland pers comm
LBM-S2	Stalagmite	Lower Balls Mine, England	Forming at collection (max 100 years)	9.8	10	Dominguez-Villar pers comm
LBM-S3	Stalagmite					
Buchan	Stalagmite	Buchan Caves, Victoria, Australia	Forming at collection	-	12	http://www.bom.gov.au
BR-10-A	Straw / moonmilk	Bats Ridge Cave System, Victoria, Australia	Forming at collection	-	13	http://www.bom.gov.au
BR-36	Stalagmite					
BR-36-A	Straw / moonmilk					
BR-5-A	Straw / moonmilk					
WM-4	Stalagmite	Wombeyan Caves, New South Wales, Australia	Past 40 yrs	14.5	13.7	McDonald pers comm
EC-2	Stalagmite	Eagle Cave, Spain	Forming at collection (base max 150 years)	15.4	14	Dominguez-Villar pers comm
Turk-1	Stalagmite	Turkey	Forming at collection	12.7	8	Jex pers comm
PDS-5	Stalagmite	Tuscany, Italy	Forming at collection	-	16	Blyth, 2007
Wel-C-1	Straw	Cathedral Cave, Wellington, NSW, Australia	Recent deposits, including on manmade artefacts	-	16	http://www.bom.gov.au
Wel-C-2	Flowstone					
Wel-C-3	Flowstone on bottle					
Wel-G-1	Straw	Gaydon Cave, Wellington NSW, Australia				
Lab-S2	Stalagmite	Labyrinth Cave, Western Australia	Forming at collection	-	16.4	Treble pers comm
Ach-1	Stalagmite	Achere Cave, Ethiopia	Holocene	-	21	Asrat et al., 2008
Merc-1	Stalagmite	Rukessia Cave, Ethiopia	Past 100 years	18.9	21	
SC-4	Stalagmite	Christmas Island	Past 600 years	-	25.4	http://www.bom.gov.au

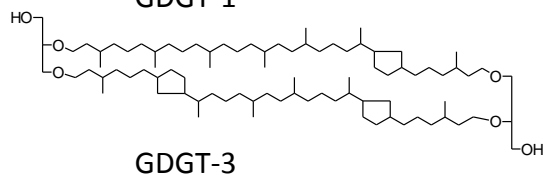
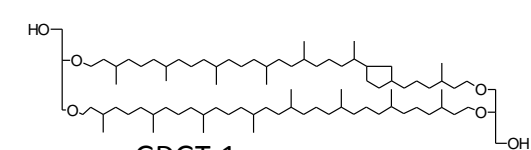
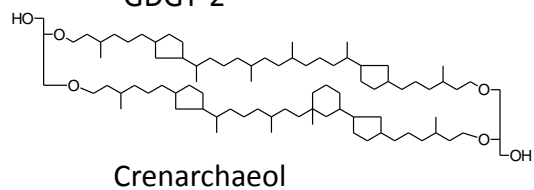
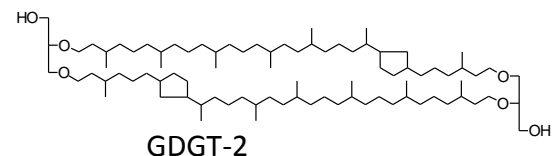
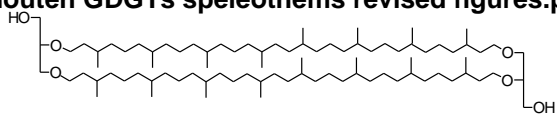
Table 2.

Correlation	Equation	r^2	p	Standard error of the estimate
TEX ₈₆ vs surface MAT	MAT = -7.4 + (33.3 * TEX ₈₆)	0.78	<0.001	2.3 °C
TEX ₈₆ vs cave MAT	MAT = -7.3 + (32.2 * TEX ₈₆)	0.68	<0.001	2.0 °C
TEX ₈₆ vs surface MAT (BIT <0.5 excluded)	MAT = -7.1 + (32.1 * TEX ₈₆)	0.77	<0.001	2.2 °C
TEX ₈₆ vs cave MAT (BIT <0.5 excluded)	MAT = -9.2 + (35.2 * TEX ₈₆)	0.70	<0.001	1.9 °C
TEX ₈₆ vs surface MAT (cave & surface dataset, fig. 11)	MAT = -8.1 + (34.2 * TEX ₈₆)	0.65	<0.001	2.5 °C
TEX ₈₆ vs cave MAT (restricted dataset, fig. 11)	MAT = - 8.3 + (34.4 * TEX ₈₆)	0.73	<0.001	1.9 °C
MBT vs surface MAT	MAT = 22.8 * MBT + 5.6	0.62	<0.001	3.2 °C
MBT vs cave MAT	MAT = 21.9 * MBT + 4.3	0.38	0.009	2.9 °C
CBT vs surface MAT	MAT = 0.3 * CBT + 12.3	0.00	0.898	5.1 °C
CBT vs cave MAT	MAT = -5.8 * CBT + 10.8	0.20	0.069	3.3 °C
MBT vs CBT vs surface MAT	MAT = 5.4 + (27.1 * MBT) - (4.8 * CBT)	0.73	<0.001	2.7 °C
MBT vs CBT vs cave MAT	MAT = 6.1 + (21.3 * MBT) - (5.4 * CBT)	0.56	0.003	2.5 °C
MBT vs CBT vs surface MAT (cave & surface data-set)	MAT = 6.0 + (20.9 * MBT) - (4.5 * CBT)	0.44	0.031	3.2 °C
MBT vs CBT vs cave MAT (cave & surface data-set)	MAT = 6.6 + (20.2 * MBT) - (5.8 * CBT)	0.56	0.007	2.7 °C
MBT/CBT calculated MAT vs surface MAT	Calc MAT = 3.4 + (0.7 * surface MAT)	0.73	<0.001	2.3 °C
MBT/CBT calculated MAT vs cave MAT	Calc MAT = 4.0 + (0.6 * cave MAT)	0.56	<0.001	1.8 °C

Table 3.

Site	Sample number	Surface MAT °C	Mean MAT °C TEX ₈₆	Standard deviation TEX ₈₆	Mean MAT °C MBT/CBT	Standard deviation MBT/CBT
Tartair (TEX ₈₆)	10	7.1	8.4	1.44	8.6	1.01
Lower Balls Mine (TEX ₈₆)	2	10	9.0	1.20 (range / 2)	-	-
Bats Ridge (TEX ₈₆)	4	13	13.2	3.02	11.6	0.40
Wellington (TEX ₈₆)	4	16	16.4	0.83	14.8	2.34

Isoprenoid GDGTs



Branched GDGTs

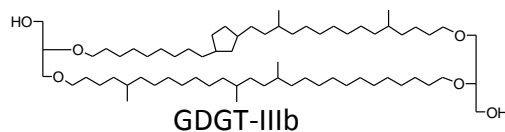
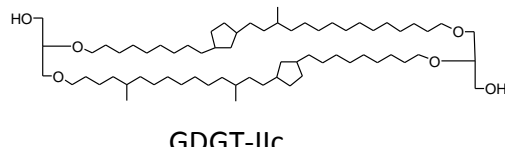
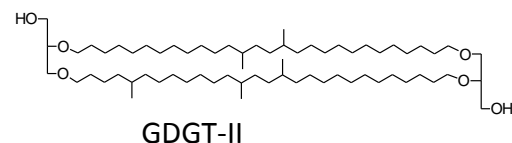
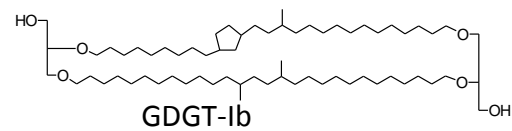
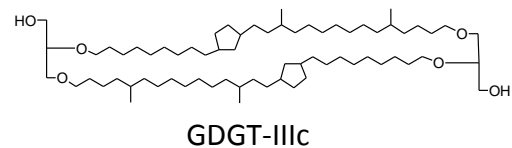
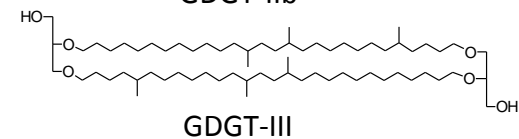
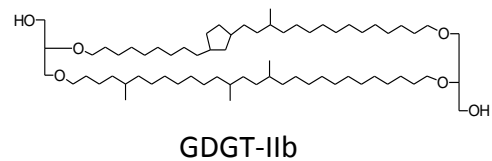
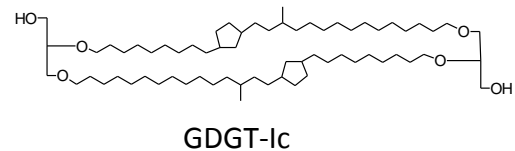
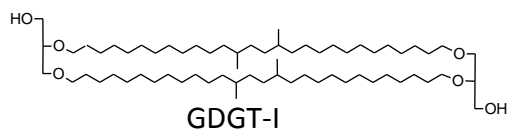


Fig. 1

Figure 2.



Figure 3.

Crenarchaeol

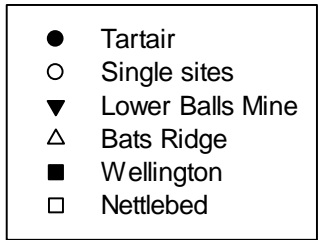
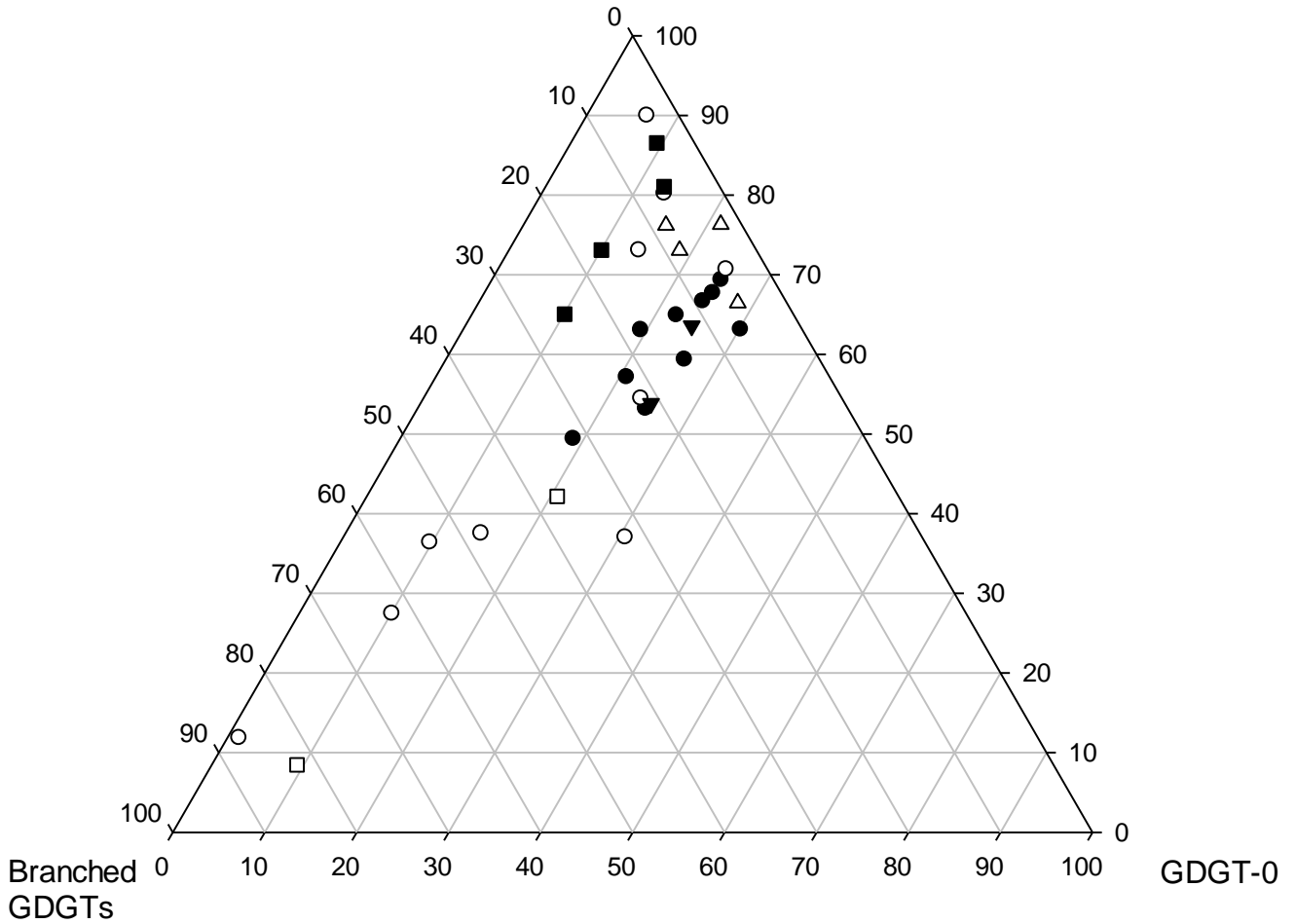


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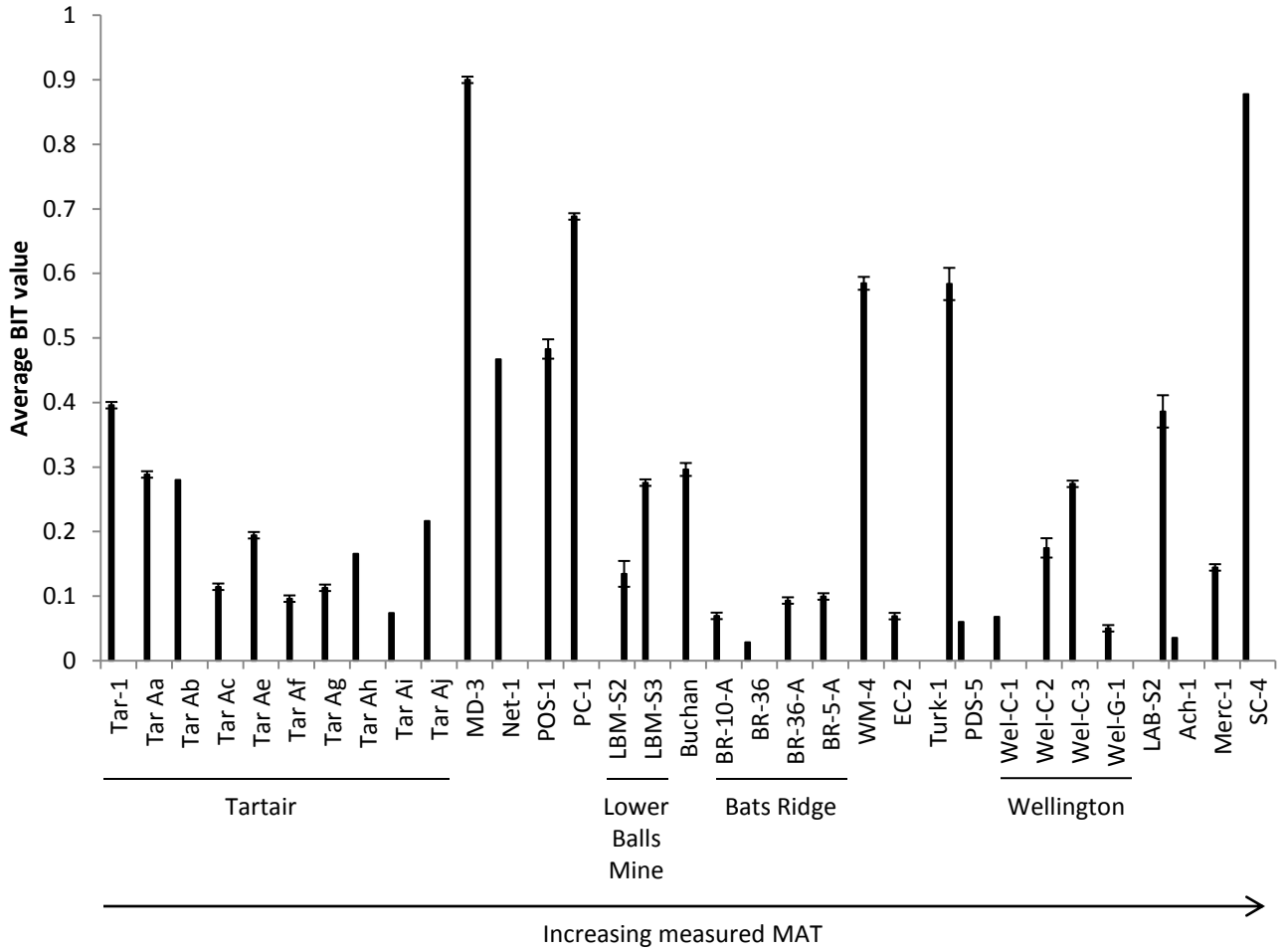


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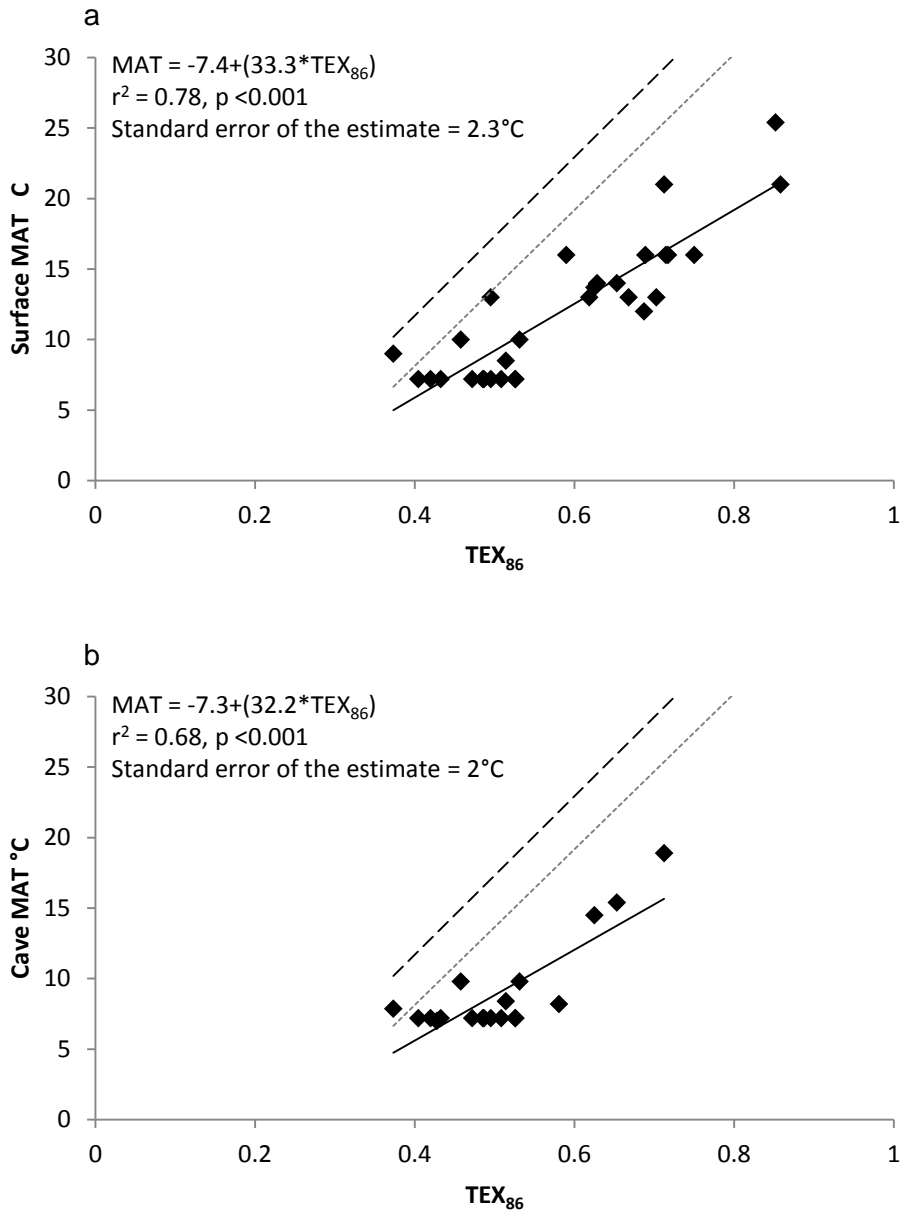


Figure 6

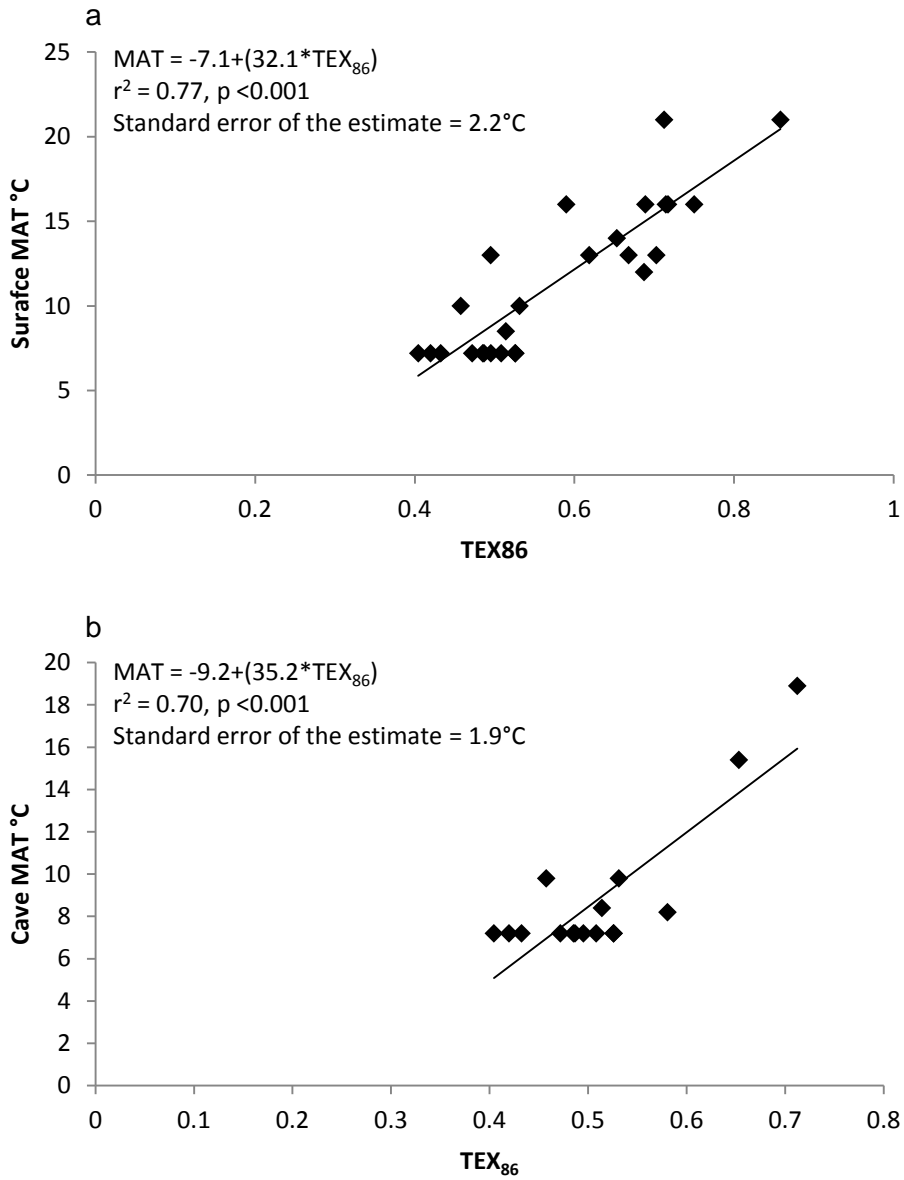


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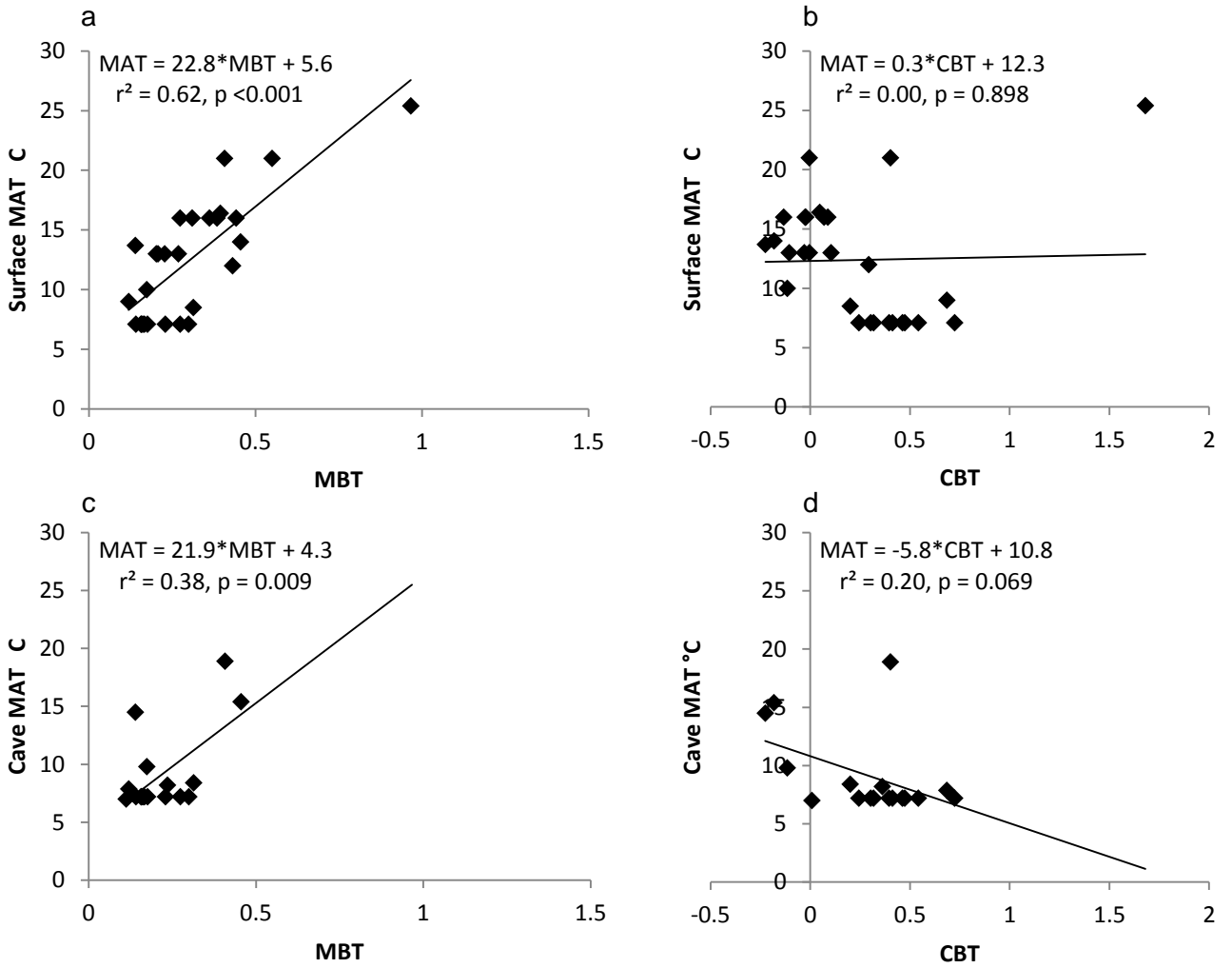


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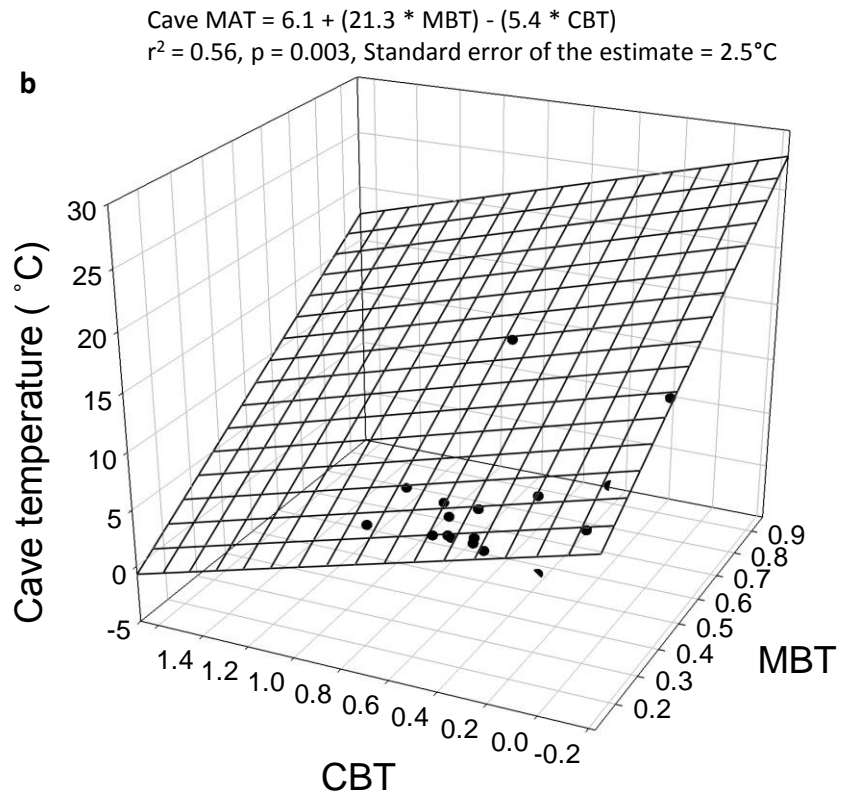
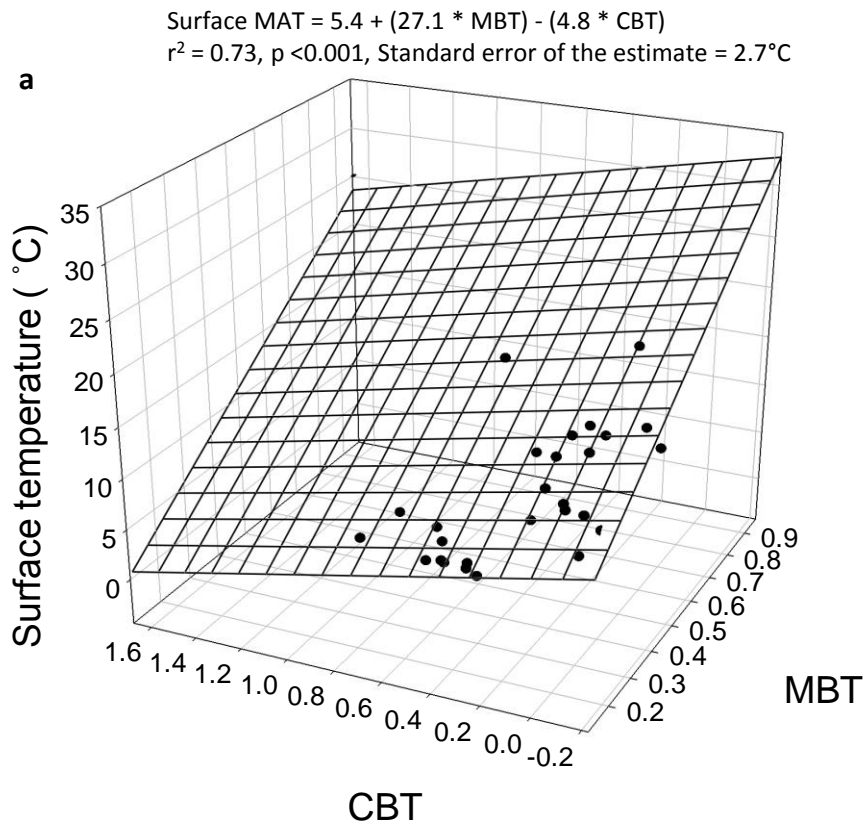


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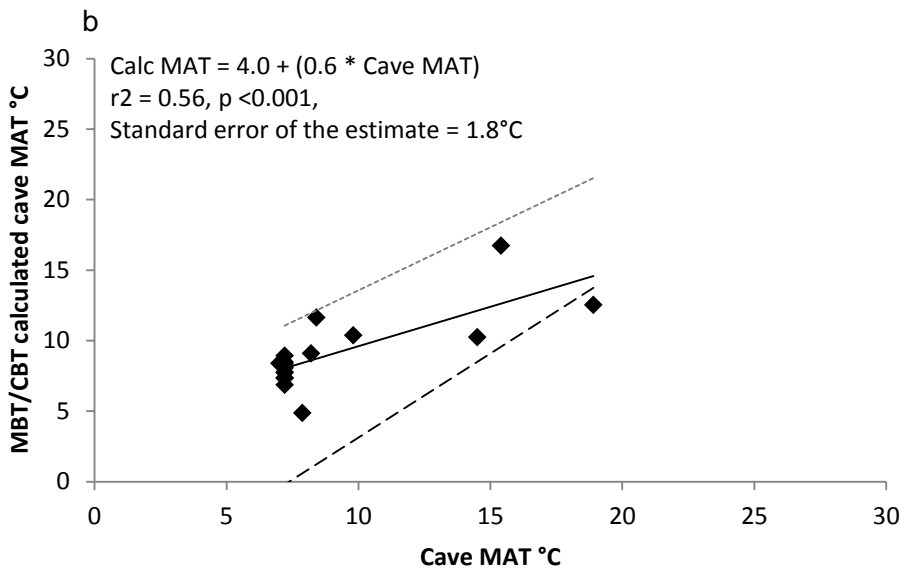
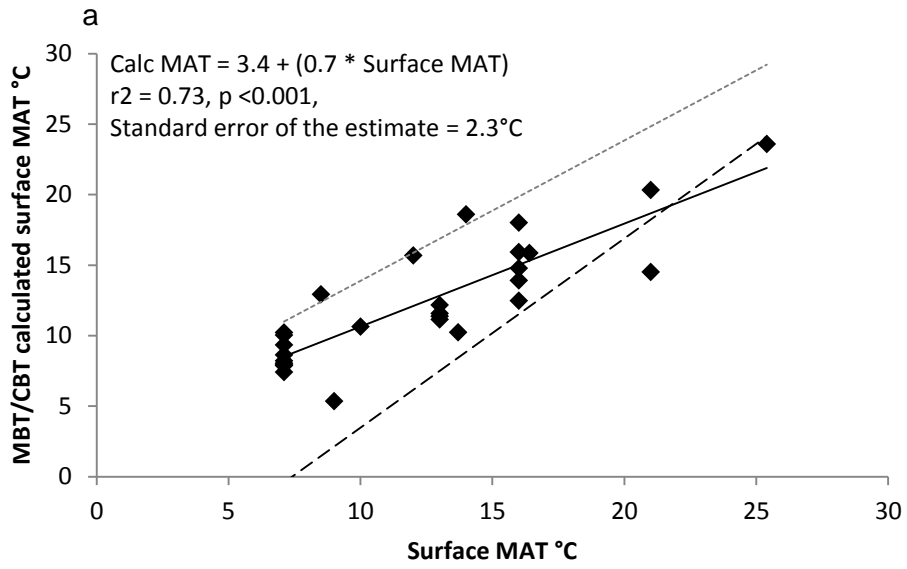


Figure 10.

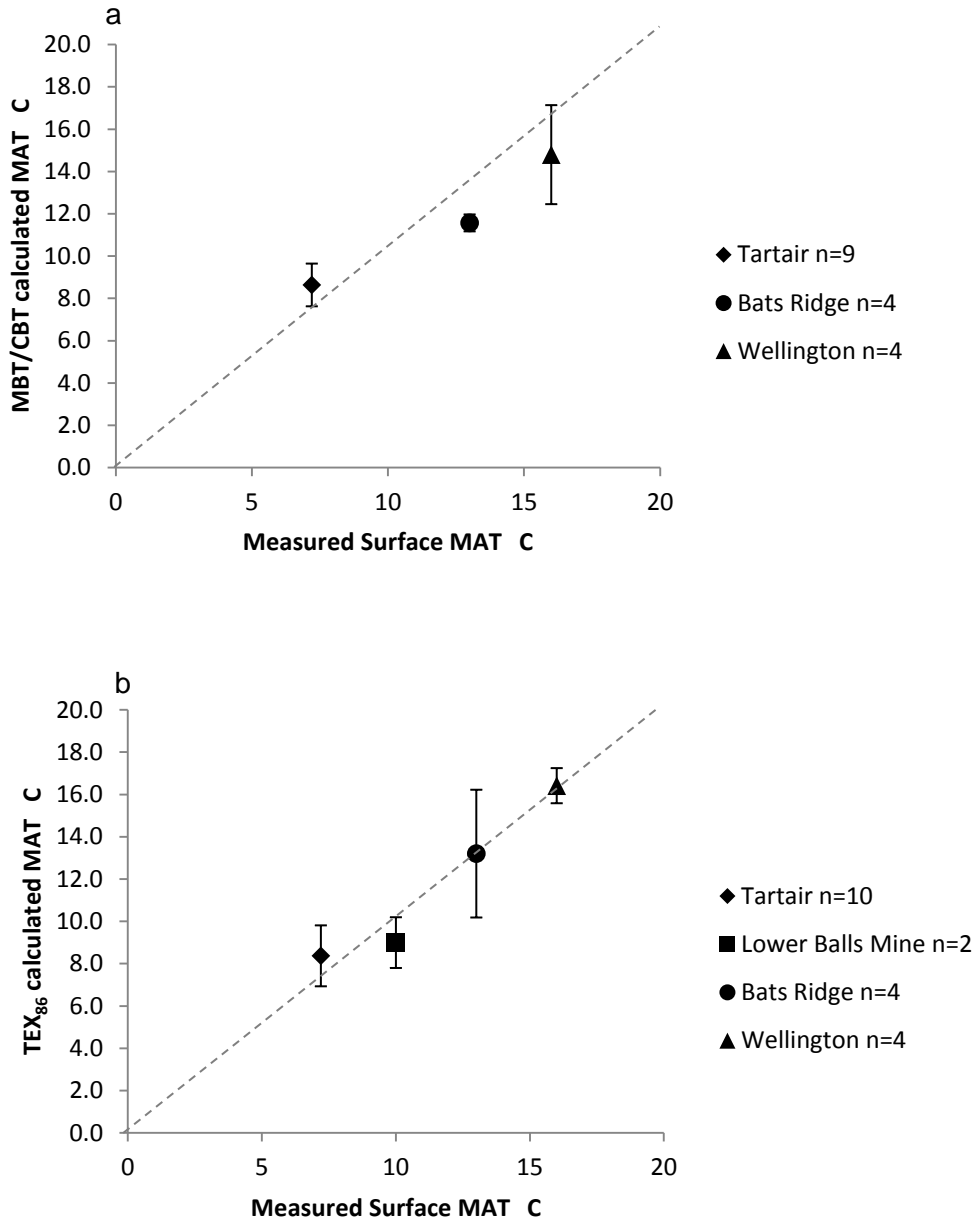


Figure 11.

