The critical thermal maximum of diving beetles (Coleoptera: Dytiscidae): a comparison of subterranean and surface-dwelling species

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A B S T R A C T

Thermal tolerance limits in animals are often thought to be related to temperature and thermal variation in their environment. Recently, there has been a focus on studying upper thermal limits due to the likelihood for climate change to expose more animals to higher temperatures and potentially extinction. Organisms living in underground environments experience reduced temperatures and thermal variation in comparison to species living in surface habitats, but how these impact their thermal tolerance limits are unclear. In this study, we compare the thermal critical maximum (CTmax) of two subterranean diving beetles (Dytiscidae) to that of three related surface-dwelling species. Our results show that subterranean species have a lower CTmax (38.3-39.0°C) than surface species (42.0-44.5°C). The CTmax of subterranean species is ~10°C higher than the highest temperature recorded within the aquifer. Groundwater temperature varied between 18.4°C and 28.8°C, and changes with time, depth and distance across the aquifer. Seasonal temperature fluctuations were 0.5°C at a single point, with the maximum heating rate being ~1000x lower (0.008°C/hour) than that recorded in surface habitats (7.98°C/hour). For surface species, CTmax was 7-10°C higher than the maximum temperature in their habitats, with daily fluctuations from ~1°C to 16°C and extremes of 6.9°C and 34.9°C. These findings suggest that subterranean dytiscid beetles are unlikely to reach their CTmax with a predicted warming of 1.3-5.1°C in the region by 2090. However, the impacts of long-term elevated temperatures on fitness, different life stages and other species in the beetle's trophic food web are unknown.

1. Introduction

Ectotherms live in a range of habitats that differ in thermal variation and extremes both temporally and spatially. The variation in temperature a species experiences can be related to its thermal tolerance limits (Stevens, 1989; Sunday et al., 2011; Shah et al., 2017). Upper thermal limits have been the focus of recent studies due to the potential for climate change to expose species to higher temperatures, impacting their survival (Hoffmann et al., 2013; Sunday et al., 2014). Subterranean environments are thermally stable, and in groundwater systems, water temperatures are similar to mean land surface temperatures, and at depths >5 m, vary by <2°C over a year (Freeze and Cherry, 1979; Benz et al., 2017; Di Lorenzo and Galassi, 2017). The climate variability hypothesis suggests that taxa living in thermally stable environments should have a narrower thermal tolerance breadth, i.e. the difference between upper and lower thermal limits, than those taxa living in thermally variable environments (Stevens, 1989; Calosi et al., 2006a; Mermillod-Blondin et al., 2013; Sunday et al., 2014). Additionally, higher maximum temperatures are correlated with higher upper thermal limits (Kaspari et al., 2015; Shah et al., 2017). It would, therefore, be expected that taxa living in subterranean environments will have a lower upper thermal limit, closely matching the temperatures of their environment, compared to surface living (henceforth, epigean) counterparts that experience more variable and higher temperatures. The potential
for lower upper thermal limits may mean these species are susceptible to higher temperatures related to climate change (Mammola et al., 2019a; Mammola et al., 2019b). However, low upper thermal limits are not always observed in subterranean fauna. Many subterranean species, showing reduced thermal tolerance relative to epigeic species, have upper thermal limits that exceed what they experience in their current environment (Bull and Mitchell, 1972; Mermilod-Blondin et al., 2013; Eme et al., 2014; Rizzo et al., 2015; Raschmanová et al., 2018; Pallarés et al., 2019).

Western Australia (WA) has the largest assemblage of aquatic subterranean (henceforth, stygobiotic) diving beetles (Dytiscidae) in the world, with more than 100 described species from approximately 50 isolated calcareous aquifers, shallow (~10 m deep) carbonate deposits containing groundwater (Balke et al., 2004; Watts and Humphreys, 2009). The ancestors of these beetles are thought to have transitioned from living in surface waters, to water-filled gravels and sands (interstitial habitats), and then into aquifers as Australia became more arid 5 to 3 million years ago (Cooper et al., 2002; Leys et al., 2003; Guzik et al., 2009; Leys et al., 2010; Watts et al., 2016). Diving beetle species found in each distinct aquifer are unique and the majority of known species (~75%; Lejs et al., 2012) represent independent incursions by epigeic/interstitial species into subterranean environments. Here, they have evolved unique adaptive traits, such as cutaneous respiration for living permanently in groundwater and regrased traits such as loss of eyes, pigment and wings (Leys et al., 2003; Watts and Humphreys, 2006; Watts and Humphreys, 2009; Lejs et al., 2012; Jones et al., 2019). However, evidence from phylogenetic and molecular evolutionary analyses of photoreceptor genes indicates that as many as 26 species may have evolved from stygobiotic ancestors (Leys et al., 2003; Lejs et al., 2012; Langille et al., 2021).

The temperature within the aquifers, where the water table is 1.9-4.0 m below the surface, average 23.8°C, but can vary by ~3°C over a year (Watts and Humphreys, 2006; Allford et al., 2008; Saccò et al., 2020b). In the habitats of epigeic species, temperature may vary by 10°C or more within a day (Nilsson and Svensson, 1995; Jones, pers. obs.). Therefore, these diving beetles provide an excellent system to test whether taxa that have lived for millions of years in a thermally stable environment with lower maximum temperatures have lost thermal tolerance capacities, and if they are potentially susceptible to future climate change.

The upper thermal limits of epigeic dytsicids in air are 41.8-46.9°C (Calosi et al., 2008a; Calosi et al., 2008b; Sánchez-Fernández et al., 2010; Sánchez-Fernández et al., 2012; Bilton and Foster, 2016; Verberk et al., 2018). However, while adults of epigeic dytsicids usually come out of water, either for dispersal or secretion grooming, stygobiotic species never or rarely do (Dettner, 1985; Miller and Bergsten, 2016; Jones et al., 2019).

In this study, we compare the critical thermal maximum (CTmax, the temperature at which an animal can no longer escape lethal conditions) of two stygobiotic dytsicid species and three epigeic dytsicid species. We also measure water temperatures in stygobiotic (calcrete aquifer) and epigeic habitats to understand the temperature change and variation that the dytsicids may experience. We hypothesise that epigeic habitats will be more thermally variable with higher maximum temperatures than stygobiotic habitats, and that the CTmax of stygobiotic dytsicids will be lower than the CTmax of epigeic species, reflecting these habitat differences. Our results will help to better understand how susceptible adult dytsicids might be to future climate warming, particularly its likely impact on stygobiotic species, which remains a fundamental question in subterranean biology (Mammola et al., 2020). Additionally, our study is the first investigation into the thermal tolerance of the diverse array of dytsicids which inhabit the calcrete aquifers of WA, and provides a basis on which future studies can explore the thermal diversity that may exist among these beetle species.

2. Material and methods

2.1. Collecting stygobiotic diving beetles and measuring water temperature in aquifers

Live specimens of stygobiotic dytsicid beetles Paroster macrosturtensis (Watts and Humphreys, 3.6-4.1 mm long) and P. mesosturtensis (Watts and Humphreys, 2.05-2.3 mm) were collected from a calcareous aquifer at the Sturt Meadows Pastoral Station in WA (Table 1). These two species are most likely to have evolved from a stygobiotic common ancestor (Lejs et al., 2012; Langille et al. 2021). A 1.4 km x 3.5 km (3.5 km²) region of the calcarete was accessible via a grid of approximately 100 mineral exploration boreholes (5 to 11 m in depth and 100 mm in diameter). The bores were unlined within the groundwater section of the bore, but lined with a PVC pipe approximately 0.5 m from the surface and capped (Allford et al., 2008). A 75 mm diameter weighted plankton net (250 μm mesh size) was lowered down boreholes into the aquifer and drawn through the water column to collect beetles into a collection tube at the bottom of the net. Stygobiotic beetles were then placed into small containers of water with sand and small stones collected from the aquifer and transported back to Adelaide, South Australia (SA) by road in a modified temperature-controlled car refrigerator maintaining temperature between 24 and 25°C. Specimens were then placed in a constant temperature cabinet at 25°C and kept dark until they were utilised for experiments.

In order to determine the temperature variation that stygobiotic beetles may experience within a calcarete aquifer, we obtained temperature data from individual boreholes both spatially and temporally across the bore grid of the Sturt Meadows aquifer. These data include previously unpublished data, collected using a Level Troll® 500 temperature logger (In-Situ Inc.; https://in-situ.com) from one borehole (at a water depth of 5.26 m) between June 2006 and March 2008. Water temperature was also measured from multiple boreholes using a Hydrolab Quanta Multi- Probe Meter® (n=116), some at multiple times and different depths (n=41), during March/April 2005 and May 2011 (n=8). Additionally, water temperature was obtained from 30 boreholes during July 2017, November 2017 and March 2018 using the Hydrolab Quanta. All temperature datasets were characterised according to rainfall in the preceding 30 days, determined from Australian Bureau of Meteorology data from Sturt Meadows. Rainfall categories were divided into low rainfall (<10 mm, LR), moderate rainfall (10-30 mm, MR), and high rainfall (>30 mm, HR), which corresponded to the rainfall’s affect on aquifer recharge, with <10 mm resulting in negligible recharge, 10-30 mm moderate recharge and >30 mm a major recharge event (Hyde et al., 2018).

Data were categorised as follows, March/April 2005 LR (4.4-6.6 mm cumulative rainfall), May 2011 MR (11.2 mm), July 2017 LR (4.4 mm), November 2017 LR (0.8 mm) and March 2018 HR (37.8 mm).

2.2. Collecting epigeic dytsicids and water temperature in epigeic habitats

Epigeic dytsicids were collected with a kitchen sieve (1.5 mm mesh size) from pools, ponds and dams in the Adelaide Hills, SA (n=7 sites, Table 1) and placed in small containers of water with leaf litter or plant matter to be transported back to the laboratory. At a subset of sites (n=4, Table 1), temperature data loggers (Onset, model UX120-014M, United States of America) with T-type thermocouples measured water temperature at different depths every 30 min for two to three weeks. These sites were a pond in Balhannah, SA, approximately 4 m x 2 m and up to 60 cm deep with dense macrophyte growth and well shaded by surrounding trees; a dam in Verdun, SA (~770 m²) with no macrophyte growth and little algal growth, but with a layer of leaf litter on the substrate and some shade from surrounding trees, and two dams at Mt Torrens, SA (~1000 m², 3200 m²) which had moderate amounts of macrophyte and algal growth, but little shade from surrounding trees (Table 1). At these sites, adult and larval dytsicid species were collected and placed in
ethanol when data loggers were deployed and retrieved. Adult and larval beetles were identified according to Watts and Hamon (2014) with the assistance of Chris Watts (South Australian Museum). Of the dytiscids caught, *Parastor nigroadumbra* (Clark, 3.2 mm long), *Limobedus amabilis* (Clark, 3.0 mm) and *Alloedes bistrigatus* (Clark, 3.3 mm) were selected for experiments due to their phylogenetic relatedness to the stygobiotic species (Leijs et al., 2012) and their similarity in body size (Table 1; Watts, 1978).

### 2.3. Feeding regimes of dytiscid specimens and laboratory conditions

Dytiscids were kept in small containers of water in a constant temperature (CT) cabinet between 24-26°C. Stygobiotic species were kept in darkness and fed small (~2-4 mm long) pieces of fresh blackworm (*Lumbriculidae*) twice a week, while epigean species, on a 12:12h light:dark cycle, were fed *ad libitum* with live blackworms. All beetles were maintained between 24-26°C for at least one week prior to experiments to reduce the effects of variable thermal history (Terblanche et al., 2005; Calosi et al., 2007; Calosi et al., 2012).

### 2.4. Determining critical thermal maximum (CTmax)

The CTmax is defined as the maximum temperature at which an animal can no longer escape lethal conditions due to unorganised locomotion or paralysis but can recover when returned to favourable conditions (Cowles and Bogert, 1944). In this study, we have defined the CTmax as the temperature at which the beetles no longer move (e.g. leg movements) and no longer respond to stimuli (e.g. gentle movements of the beetle with a small spatula or wire). Therefore, an experimental set up was constructed to allow manipulation of the temperature, while observing the beetles’ behaviour (Fig. 1). The set up consisted of a temperature controllable water bath (Model F20-HC, Julabo Labortechnik GmbH, Seelbach, Germany), which circulated water through a plastic container (27 cm x 19 cm x 12.5 cm high with a lid) with a clear Perspex window. Air-equilibrated water was pumped through the container at 41.7-44.5 ml s⁻¹. Water was either reverse osmosis (RO) water with rock salt (salinity 21-22 ppt) for the stygobiotic dytiscids, or pure RO water for epigean dytiscids, to match the water of their natural environment. Six plastic vials (5 cm tall, 1.5 cm dia., ~8.8 ml vol.) were mounted in the container and viewable through the window. For the stygobiotic dytiscids, each vial had a layer of plastic mesh (1.5 mm x 1.2 mm holes) and fine fabric (~1 mm x 0.5 mm holes) around half the circumference to allow water circulation and to stop the beetles escaping. Vials were placed so there was ~2 cm of water in the bottom, the remaining being air-filled, with the top closed with foam stoppers. As stygobiotic species use cutaneous respiration, where O₂ diffuses through the exoskeleton, a lack of water circulation could affect these beetles’ ability to obtain O₂ and influence their thermal tolerance (Verberk and Bilton, 2015; Jones et al., 2018; Jones et al., 2019). Food dye was used to ensure water was refreshed in the vials every few minutes. To test the beetles’ reaction to stimuli, the small spatula was placed into the vial if beetles were not observed to move in the preceding 2 min period. For the three epigean species, vials were enclosed, apart from a small air tube (3.5 mm I.D.) to allow gas exchange with the atmosphere. A plastic covered wire (2.5 mm O.D.) was inserted in to the tube to allow the beetles to be moved gently to test their reaction to stimuli without opening the vial. In the vial containing ~3.5 ml of water floated a small ~0.12 cm³ piece of wood for the beetles to climb, with the remainder of the vial being air-filled.

To measure CTmax, individual beetles were placed into vials and left for one hour at 25°C to adjust to conditions before the temperature was increased at 0.1°C every 2 min using the water bath controller (mean rate of 0.05°C min⁻¹). Ramping rate was informed by field measurements, which showed maximum rates of temperature increase in epigean habitats was 0.03-0.13°C min⁻¹ (see results). Temperature was recorded in the water surrounding the vials containing the stygobiotic beetles with two T-type thermocouples, one connected to a digital thermometer (Fluke 52, John Fluke MFG Co. Inc., Washington, United States of America) and the other to a data logger (Squirrel data logger, type OQ610, Grant Instruments Ltd, Cambridge, England). For epigean species, water and air temperature were measured in a replicate vial, which simulated the vials that beetles were in. Temperature was manually recorded from the digital thermometer every 2 min, and with the data logger, every 30 s. Beetles in the vials were recorded with a video camera (XA-20, Canon Inc., Tokyo, Japan) placed in front of the window. During experiments, beetles were observed for movement such as swimming, crawling, and leg movement. The CTmax was the water temperature (used as a proxy for body temperature to 0.1°C) where the beetles stopped moving and were unresponsive to stimuli, which involved being gently swirled with the spatula or metal wire. Once beetles were unresponsive, they were removed from the experiment and placed into a water container in a CT cabinet set to 25°C, for 24 h. The number of beetles that were found moving and responding to stimuli (i.e. survived) that 24 h period was then recorded.

The vials were designed to avoid O₂-limitation in both the stygobiotic and epigean beetles, which use different modes of respiration, because O₂-limitation may affect their CTmax (Verberk and Bilton, 2013; Verberk and Bilton, 2015; Jones et al., 2019). Once O₂ in the air store becomes depleted, epigean dytiscids generally return to the water’s surface and exchange gas stored under the elytra with the atmosphere (Calosi et al., 2007; Kehl, 2014). Epigean species may use a compressible gas Gill to gain O₂ from the water, which would be influenced by a lack of convection in the vials reducing O₂ diffusion. However, O₂ is gained primarily from the atmosphere, and they should not become O₂-limited with access to air (Verberk and Bilton, 2015). Experiments show that the aquatic bug *Ilyocoris cimicoides*, which uses an air store and gas Gill does not have reduced CTmax when aquatic O₂-partial pressure is low (which also reduces O₂ diffusion) if it has access to air (Verberk and Bilton, 2015). The stygobiotic dytiscids do not appear to undertake aerial gas exchange as they have tightly locked elytra and rarely go to the sur-

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**Table 1**

Locations and collection dates for stygobiotic dytiscids from Western Australia, and epigean dytiscids from the Adelaide Hills, South Australia.

| Site                     | Collection dates       | Coordinates       |
|-------------------------|------------------------|-------------------|
| Sturt Meadows, WA¹      | May 2019               | 28.68’S, 120.97’E |
| Baldinna, SA²           | Nov/Dec 2018, Feb/March 2019 | 34.99’S, 138.82’E |
| Verdon, SA²             | Nov/Dec 2018, Feb/March 2019 | 35.01’S, 138.80’E |
| Mt Crawford, SA¹        | Aug/Sept 2019          | 34.75’S, 138.96’E |
| Mt Torrens (big dam), SA¹ | Dec 2018, March 2019   | 34.93’S, 138.97’E |
| Mt Torrens (small dam), SA² | Dec 2018, March 2019   | 34.92’S, 138.97’E |
| Harrogate, SA²          | Nov 2019               | 34.91’S, 138.99’E |
| Graffers West, SA³      | Nov 2019               | 35.00’S, 138.69’E |

Superscripts indicate where ¹ P. macrosturtensis and P. mesosturtensis, ² P. nigroadumbra, ³ A. bistrigatus, and ⁴ L. amabilis were collected for ramping experiments. ⁵ Indicates sites where temperature data loggers were deployed.
face; rather, they rely on cutaneous exchange where $O_2$ diffuses from the water through an $O_2$-boundary layer and the cuticle, and into the respiratory gas or tissues in the beetle (Watts and Humphreys, 2006; Jones et al., 2019). The $O_2$-boundary layer is a fluid layer above a respiratory surface that is deficient in $O_2$ and provides resistance to $O_2$ diffusion. The thickness of the boundary layer is inversely related to water convection, and thinner boundary layers are required to satisfy higher $O_2$ demand (Seymour et al., 2015). Therefore, the likelihood of the stygobiotic beetles becoming $O_2$-limited in their vials is reduced with well-convected air-equilibrated water flowing through, relative to stagnant water. A second cohort of beetles was used as a control to ensure beetles could survive in the experimental setup for the duration of the ramping experiments, and for 24 h afterwards. In control experiments beetles were kept at 25°C for approximately 30 min longer than beetles in temperature ramping experiments.

As stygobiotic and epigean dytiscids could crawl out of the water onto the mesh or wooden block, respectively, the amount of time they spent above the water was measured. One video frame every 5 s was removed from the recording of the beetles with a media player (VLC media player, Version 3.0.11, USA), and the number of frames an individual was above the water during the experiment was counted (to the nearest integer temperature below the CTmax). This number was divided by the total number of frames viewed and multiplied by 100 to get a percentage of time spent above the water. These percentages were then used to calculate a grand mean of the percentage of time each species spend above the water during the entire experiment.

Temperature gradients developed inside the vials in the transition between the water and air. At 25°C there was little difference between temperatures, but for stygobiotic beetles at 39°C, air temperature was ~3°C lower, while for epigean beetles at 43°C, air temperature was ~2.5°C lower.

2.5. Statistical analysis

ANCOVA analyses coupled with Tukey’s post-hoc tests were performed in R software version 4.0.5 (R Core Team, 2013; package “emmeans”; Lenth et al., 2021) to compare differences between CTmax of dytiscid species and incorporate the influence of the percentage of time spent out of the water as a covariate. Means are reported with 95% confidence intervals.

3. Results

3.1. Field data: water temperature, temperature change, and dytiscid species collected from epigean habitats

Groundwater temperature in the Sturt Meadows aquifer averaged between 23.0°C and 26.2°C depending on when temperatures were recorded, and the borehole measured. An overall maximum of 28.8°C and minimum of 18.4°C, corresponding to a maximum difference of 10.4°C was recorded across time and space within the aquifer (Table 2, Fig. 2). Within bore hole variation (max. 3.60°C) was lower than the variation across the aquifer (Table 2, Fig. 2 and 3). Long term measurements in a single borehole showed seasonal variation in water temperature, ranging from 25.01°C to 25.55°C, with a maximum rate of heating of 0.008°C h⁻¹ and cooling -0.009°C h⁻¹ (Fig. 4).

Water temperature in epigean habitats ranged from 8.7°C at Balhanah (Nov./Dec.) to 34.9°C at Verdun (Feb./Mar., Fig. 5, Table S1). Maximum rates of heating varied between 0.033°C min⁻¹ to 0.133°C min⁻¹, with mean rates being between 0.005°C min⁻¹ to 0.024°C min⁻¹ (Table S1). Maximum rates of cooling were -0.024°C min⁻¹ to -0.100°C min⁻¹, with mean rates -0.005°C min⁻¹ to -0.013°C min⁻¹ (Table S1). Temperatures were recorded near the water’s surface to 34 cm deep. Additionally, individual water temperature measurements were made in ephemeral pools where P. nigroaureus was caught on 23/9/2019 (Mt Crawford, Table 1), and were 6.9°C at 8:22 am, 9.7°C and 8.8°C at 9:17 am in one pool (heating 0.05°C min⁻¹), and 10.0°C at 9:35 am in a separate pool (water depth <15cm).

Eleven epigean dytiscid species with adult specimens and six species with larvae were collected from four field sites (Table S1). The only species collected in the larval stage and not collected as an adult was
Table 2
Temperature variation between and within boreholes in the Sturt Meadows aquifer during different sampling and rainfall periods.

| Sampling period/ Rainfall period | Temp. (°C) | Within borehole variation (°C) | Water depth (m) | n bore holes |
|----------------------------------|------------|---------------------------------|-----------------|-------------|
|                                  | Mean | Max. | Min. | Mean diff. | Max. diff. | Min. diff. | Mean | Min. | Max. |
| March/April 2005 MR              | 26.2 | 28.8 | 24.0 | 1.5 | 3.6 | 0.15 | 1.1 | 0.0 | 8.1 | 116 (41) |
| May 2011 LR                      | 26.0 | 27.3 | 24.4 | 1.0 | 1.54 | 0.1 | 2.0 | 0.1 | 7.1 | (8) |
| Jul 2017 LR                      | 23.0 | 25.2 | 18.4 | - | - | - | 3.5 | 0.3 | 7.3 | 30 |
| Nov 2017 LR                      | 23.4 | 25.1 | 22.1 | - | - | - | 3.5 | 0.3 | 7.5 | 30 |
| Mar 2018 HR                      | 26.2 | 27.0 | 25.1 | - | - | - | 3.3 | 0.1 | 7.2 | 30 |

Mean temperature represents the average temperature of all recordings made in that sampling period; Max. temperature represents the single highest record; Min. represents the single lowest record. Water depth is that at which temperature recordings were made. n is the number of boreholes sampled, with numbers in parentheses indicating the number of boreholes with multiple measurements at different depths. For July 2017, Nov 2017 and March 2018, only single measurements were taken, generally halfway through the water column. LR indicates a low rainfall period (<10 mm in the preceding 30 days), MR, a medium rainfall period (10-30 mm), and HR, a high rainfall period (>30 mm).

Figure 2. Examples of spatial temperature variation across the Sturt Meadows aquifer bore field. (A) shows borehole positions (black circles) overlain onto a satellite image. (B and C) each square represents a different location within the aquifer bore field. Bold values indicate single temperature measurements within a borehole, while non-bold values are means of multiple measurements at different depths. Colours indicating temperature may be intermediaries between those shown in the temperature scale. (B) shows temperatures during a medium rainfall period in March/April 2005. Mean temp. for boreholes with single measurements = 26.8°C, mean depth=0.1 m, max. depth = 0.2 m, min. depth = 0 m. Mean temp. for boreholes with multiple measurements = 25.6°C, mean depth = 2.1 m, max. depth = 8.1 m, min. depth = 0 m. (C) shows single temperature measurements made in each borehole during a low rainfall period in July 2017. Mean depth of measurements = 3.5 m, max. = 7.3 m, min. = 0.3 m.

Onychohydrus scutellaris (Germain). Adult O. scutellaris are strong swimmers and unlikely to be caught with a sieve.

3.2. CTmax and time spent above the water

Mean CTmax of stygobiotic P. macrosturtensis was 38.3±0.2°C (n=10), significantly lower than P. mesosturtensis at 39.0±0.2°C (n=10, ANCOVA with Tukey’s multiple comparisons test, P<0.05), and both were significantly lower than the mean CTmax of all epigean species (ANCOVA with Tukey’s multiple comparisons test, P<0.001, Fig. 6). CTmax of the epigean species P. nigroadumbratus (42.7±0.6°C, n=6) and L. amabilis (42.0±0.8°C, n=6) were not significantly different (ANCOVA with Tukey’s multiple comparisons test, P=0.8863), but both had a significantly lower CTmax than A. bistrigatus (44.5±0.5°C, n=5; ANCOVA with Tukey’s multiple comparisons test, P<0.05). Survival was lower in the stygobiotic beetles and L. amabilis 24 h after the ramping experiments (60% for P. macrosturtensis and P. mesosturtensis, n=10 each, and 83% for L. amabilis, n=6) compared to the controls (100%, P. macrosturtensis n=6, P. mesosturtensis n=5 and L. amabilis n=6), but for P. nigroadumbratus and A. bistrigatus, all beetles survived ramping (n=6 and n=5, respectively) and control experiments (n=5 and n=6, respectively). All beetles spent some time above the water during ramping experiments. On average P. macrosturtensis spent 1.9±1.0% of the time above the water, P. mesosturtensis 5.0±2.8%, P. nigroadumbratus 2.4±2.6%, L. amabilis 1.5±0.8% and A. bistrigatus 1.0±0.8%. All beetles were in water at the 1°C temperature interval below their CTmax, except A. bistrigatus which averaged 6% at the 43-43.9°C interval (See Supplementary file S2). The incorporation of the covariate “% of time spent above the water” within the ANCOVA modelling did not reveal statistically significant results (ANCOVA, P=0.1467), indicating that differences in measured CTmax were not modulated by time spent above the water.

4. Discussion

4.1. CTmax

In this study, we show that stygobiotic dytiscids had reduced thermal tolerance (3-6°C lower) and experience less thermal variation and lower maximum temperatures in their aquifer environment compared to epigean species (Fig. 2-6). These findings align with the predictions made under the climate variability hypothesis (Stevens, 1989; Calosi et al., 2008a; Merrimill-Blondin et al., 2013; Sunday et al., 2014). Our results also suggest that either the stygobiotic dytiscids have lost some thermal tolerance capacity or their epigean ancestors had
lower thermal tolerances than modern epigean species. It is also possible, however, that thermal limits may be set by $O_2$ limitation where the more restrictive cutaneous respiration in the stygobiotic dytiscids (Jones et al., 2019) results in a lower CTmax than the epigean species, which have access to air. Furthermore, whether thermal tolerance is set by variation in temperature, or maximum environmental temperature is unclear and confounded by epigean habitats having both greater variation and higher temperatures than stygobiotic habitats.

The CTmax of the two stygobiotic dytiscid species (38.3-39.0°C, Fig. 6) is substantially higher than the temperatures recorded in the Sturt Meadows aquifer (18.4-28.8°C, Table 2). Therefore, despite increases in temperature related to climate change (1.3-5.1°C predicted warming by 2090 in the region where the Sturt Meadows aquifer is located; RCP4.5 and RCP8.5, Watterson et al., 2015), the beetles are unlikely to reach their CTmax. However, this suggestion does not consider thermal plasticity, the ability of the beetles to adapt to increasing temperatures over time, nor that prolonged exposure to elevated, but lower temperatures than CTmax, may result in death (Rezende et al. 2014; Semsar-Kazerouni and Verberk, 2018). We also do not know how other life history traits, such as breeding or foraging, may be affected, nor the responses to increased temperatures of other life stages, like larvae and eggs. Furthermore, these dytiscids are at a high trophic level within the aquifer (Bradford et al., 2014; Saccò et al., 2019; Saccò et al., 2020a; Saccò et al., 2021), and the responses of species in their trophic food web are unknown and could impact them indirectly.

There are several possible explanations, or combinations of them, on why the CTmax of the stygobiotic dytiscids do not closely match their environment. First, changes in upper thermal limits may require significant molecular changes and might be constrained by phylogenetic inertia (Addo-Bediako et al., 2000; Sunday et al., 2011; Kellermann et al., 2012; Hoffmann et al., 2013). Second, CTmax could be in disequilibrium with the current environment, requiring more time for the beetles to adapt, assuming a stable environment (Sunday et al., 2014; Pallarés et al., 2019). Third, previous climate cycles that resulted in variation in temperature may prevent narrowing of thermal tolerance (Eme et al., 2014). Finally, CTmax may have returned to a “default state” where selection is not strong enough to reduce it further (Rizzo et al., 2015). A further consideration is that selection may be stronger for sublethal thermal limits resulting in improved performance in traits such as reproduction, metabolism, growth rate and development at temperatures the beetles currently experience (Angilletta Jr et al., 2002; Dallas and Ross-Gillespie, 2015). One possibility that can be excluded is gene flow between populations from thermally different aquifers contributing to variation (Mermillod-Blondin et al., 2013). The Sturt Meadows aquifer is isolated from other aquifers both geologically and by unhabitable epigean environments due to aridity (Cooper et al., 2002; Lyes et al. 2003). In addition, although phylogenetic data show genetic differentiation over short distances in both P. macrosturtensis and P. mesosturtensis, suggesting limited dispersal within the aquifer (Guzik et al., 2009), there are no consistent changes in temperature across the aquifer (Table 2, Fig. 2; Watts and Humphreys, 2006). It is also noteworthy that the CTmax of epigean dytiscids in this study is 7-10°C higher (Fig. 6) than the maximum temperatures recorded in their habitats (Table S1, Fig. 5). This may indicate that a major difference between temperatures experienced in natural habitats and experimental CTmax may be a more common phenomenon irrespective of whether the beetles live in epigean or stygobiotic habitats, as seen with other taxa (e.g. Collembus using LD50, Raschmanová et al., 2018). However, the highest water temperatures epigean dytiscids have been recorded in are 35-42°C from habitats associated with hot springs (Brues, 1927; Mason, 1939; Stark et al., 1976).

The CTmax of epigean dytiscids in this study are within the range of the CTmax of dytiscids from Europe and the Mediterranean (41.8-46.9°C), despite differences in methodology (Calosi et al., 2008a; Calosi et al., 2008b; Sánchez-Fernández et al., 2010; Sánchez-Fernández et al., 2012; Bilton and Foster, 2016; Verberk et al., 2018). In the current study, beetles were primarily in water with a 0.05°C min$^{-1}$ ramping rate and acclimated to 25°C, while in most previous studies beetles were in air with a 1°C min$^{-1}$ ramping rate and generally acclimated to 14.5°C or 20.5°C (range 10.0-24.3°C; Calosi et al., 2008a; Calosi et al., 2008b; Sánchez-Fernández et al., 2010; Sánchez-Fernández et al., 2012; Bilton and Foster, 2016; Verberk et al., 2018). In Deronectes latus, CTmax has been recorded in air at 46.9°C (1°C min$^{-1}$, acclimation 20.5°C; Calosi et al., 2008b) and in water at 37.5°C (ramping 0.25°C min$^{-1}$, acclimation 10°C; Verberk et al., 2018), indicating the CTmax of dytiscids measured in water may be lower than air. However, the interaction between higher acclimation temperatures and higher ramping rate, might have led to a higher CTmax (Sánchez-Fernández et al., 2010; Terblanche et al. 2011). In our study, a relatively lower ramping rate may have been offset by a higher acclimation temperature. The difference in methodology is primarily because the stygobiotic dytiscids have rarely been observed to leave water (Jones et al., 2019) and are unlikely to experience high rates of temperature change in their environment. A comparison between methodologies (air/water, ramping rates, acclimation temperature) using the same dytiscid species would be valuable to better understand the effect of varying methodology on dytiscid CTmax and the interaction between duration and intensity of heat stress on CTmax.
Figure 5. Temperature change and variation in water bodies containing epigean diving beetles in the Adelaide Hills, South Australia. Water temperature is shown at different depth ranges (given in the top left-hand corner of each graph), recorded every 30 min for up to 14 days. Dark grey areas show night-time, while light grey shows change in sunrise and sunset over the duration of temperature recording. Start date for when the temperature data loggers were deployed are shown. Locations indicate the dam at Verdun, the pond at Balhannah, and the two dams at Mt Torrens, one being the smaller dam (~1000 m²), and two the larger (3200 m²).

Figure 6. Critical thermal maximum temperature (CTmax) of stygobiotic (grey shaded) and epigean diving beetles. CTmax is defined as the temperature at which the beetles no longer move and do not respond to stimuli, i.e. unable to escape lethal conditions. Species include, stygobiotic *P. macrosturtnensis* and *P. mesosturtnensis*, and epigean *P. nigroadumbratus*, *L. amabilis* and *A. bistrigatus*. Letters indicate significant differences (P<0.05). Means are shown with 95% confidence intervals.

Significant differences between CTmax in the epigean species used in this study may represent differences in habitat, distribution, temperature preferences, and/or behaviour during experiments (Fig. 6). *Paroster nigroadumbratus* and *L. amabilis* were found in more shaded, and thus likely cooler habitats than *A. bistrigatus*, and are restricted to mesic environments with distributions confined to south-eastern Australia, while *A. bistrigatus* is found throughout arid Australia (Watts, 1978). In experiments, *A. bistrigatus* spent more time above the water at temperatures close to their CTmax, which may indicate the species disperses at high temperature or uses evaporative cooling (Prange, 1996). However, due to the small temperature gradient that developed in vials, *A. bistrigatus* may have experienced slightly lower temperatures for periods while in air. Whether these species experience temperature close to their CTmax is unclear due to temperature heterogeneity in their habitats and the potential for behavioural avoidance.

4.2. Oxygen limitation and thermal tolerance

The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis suggests that thermal tolerance is set by a mismatch between
O₂ delivery and O₂ supply at temperature extremes in ectotherms (Pörtner, 2001; Verberk and Milton, 2015). With an increase in temperature, O₂ consumption rate increases and could increase to the point that O₂ supply is insufficient for survival. The universality of the OCLTT hypothesis has been questioned (Jutfelt et al., 2018), but the strongest support comes from water breathing insects that use plastrons and tracheal gills where capacity to obtain O₂ is limited (Verberk and Milton, 2015; Verberk et al., 2016). Stygobiotic diving beetles obtain their O₂ underwater using cutaneous respiration, where O₂ diffuses from the water through the body surface (Jones et al., 2019). This form of respiration enables stygobiotic beetles to stay permanently under water without returning to the surface to collect an air bubble as do epigean dytiscids. However, cutaneous respiration is restrictive and limits beetles to a small size, <5 mm long (Jones et al., 2019).

O₂ diffusion through plastrons and compressible gas gills is mostly limited by diffusion through the boundary layer, the fluid layer above a respiratory surface that provides resistance to O₂ diffusion. However, boundary layer resistance can be reduced through water convection over the respiratory surface, either through movement of the animal or flowing water. In the stygobiotic dytiscids, the cuticle provides an additional and more significant resistance to O₂ diffusion along the O₂ diffusion pathway (Jones et al., 2019). This means that these species have more stringent requirements for high O₂-partial pressure (PO₂) of the surrounding water to satisfy their O₂ demands when compared to plastron and gas gill breathers.

Our results conform to what would be expected if O₂-limitation does set CTMax. The epigean dytiscids with a less restricted mode of respiration have a higher CTMax than the stygobiotic species, and P. macrosturtensis has a lower CTMax than P. mesosturtensis, aligning with the higher surface area to volume ratio in the latter. These results also complement other studies where a species’ capacity to increase O₂ uptake rates was correlated with improved heat tolerance in other aquatic insects, including dytiscids (Verberk and Milton, 2013; Verberk and Bilton, 2015; Verberk, 2018).

Jones et al. (2019) modelled O₂ diffusion into P. macrosturtensis and P. mesosturtensis in relation to water convection and increasing metabolic rate to determine the conditions under which beetles become O₂-limited. At 25°C, well-convected (i.e. a 100 μm thick O₂-boundary layer) air-equilibrated water, the metabolic rate of P. mesosturtensis could increase more than ten times above resting without becoming O₂-limited, while in P. macrosturtensis metabolic rate could increase 4-5 times above resting. The values provide a hypothetical metabolic scope, above which the beetles could become O₂-limited. Assuming a Q₁₀ of 2, the factor by which O₂ consumption rate increases with a 10°C rise in temperature (Chown et al., 2007), the resting metabolic rate of P. mesosturtensis would be 2.6 times higher at their CTMax, and in P. macrosturtensis, 2.5 times higher. This suggests the stygobiotic beetles may not be O₂-limited at the CTMax measured in this study. However, CTMax may still be set by O₂-limitation if the conditions outlined within the model differ from those experienced in the CTMax experiments; i.e., if Q₁₀ is different from 2 or varies with temperature, or if boundary layer and convective conditions differed from those in the model. Even if the stygobiotic beetles’ CTMax is not set by O₂-limitation in air saturated water, it is possible that the beetles may become O₂-limited when O₂ levels within the ground water are low (O₂ saturation has been measured between 50-100% in the Sturt Meadows aquifer; Jones et al., 2019). Further experimental studies would be required to test the relationship between O₂ consumption, O₂-limitation and temperature for the stygobiotic beetles to determine under what conditions CTMax may be set by O₂-limitation.

4.3. Conclusion

In this study, we have shown that the CTMax of stygobiotic and epigean dytiscid beetles are significantly different. These results correlate positively with both increased thermal variation, and temperature extremes in their habitats, as well as increased capacity to obtain O₂. We also show that there are moderate levels of thermal variation spatially and temporally within the Sturt Meadows aquifer. Our study adds to the growing number of studies showing that thermal tolerances in subterranean animals do not necessarily closely match the temperatures they experience, a pattern that was also found for the epigean species we studied. Additionally, we provide the first thermal tolerance data for dytiscids from the calcrete aquifers of Western Australia and broaden understanding of their thermal environment. Given the high dytiscid species richness and evidence for multiple independent incursions by epigean species into the subterranean environment in these aquifers, there is an opportunity to investigate the diversity and patterns of thermal tolerance evolution in these systems.

Author contributions

Karl K. Jones: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review and Editing, Visualization, Project administration. William F. Humphreys: Investigation, Writing – Review and Editing, Funding acquisition. Mattia Saccò: Investigation, Formal analysis, Writing – Review and Editing. Terry Bertozzi: Writing – Review and Editing. Funding acquisition. Andy D. Austin: Writing – Review and Editing, Funding acquisition. Steven J. B. Cooper: Conceptualization, Writing – Review and Editing, Supervision, Project administration, Funding acquisition.

Data availability

Data for CTMax, time spent above the water, habitat water temperature and depth, and dytiscid species caught from epigean habitats are available with this paper as supplementary material (see supplementary Table S1 and supplementary file S2).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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