

LIFE IN THE HYPOXIC AND ANOXIC ZONES: METABOLISM AND PROXIMATE COMPOSITION OF CARIBBEAN TROGLOBITIC CRUSTACEANS WITH OBSERVATIONS ON THE WATER CHEMISTRY OF TWO ANCHIALINE CAVES

*R. E. Bishop, B. Kakuk, and J. J. Torres*

(REB, correspondence) Pennsylvania State University, Worthington Scranton, 120 Ridge View Drive, Dunmore, Pennsylvania 18512, U.S.A. (reb20@psu.edu);

(BK) Caribbean Marine Research Center, 100 North U.S. Highway 1 #202, Jupiter, Florida 33477, U.S.A. (bkakuk@cmrc.org);

(JJT) University of South Florida, College of Marine Science, 140 Seventh Avenue South, St. Petersburg, Florida 33701, U.S.A. (jtorres@usf.edu)

A B S T R A C T

The interaction of freshwater, seawater, and limestone creates the unique environment of the anchialine cave. Anchialine cave systems are characterized by the presence of several strong density interfaces that isolate the cave system into components: regions of freshwater at the surface, a hydrogen sulfide layer, and a warm oceanic intrusion that is devoid of oxygen. These unusual physical parameters provide a challenging environment for the organisms that reside there. Within the hypoxic and anoxic regions of the cave system, a unique community of macrofauna has adapted to cope with constant low oxygen conditions and a depauperate food supply. We examined respiration rates and proximate compositions of individuals representing seven orders of troglobitic crustaceans collected from two Bahamian anchialine caves. In addition, we examined the physical conditions of both caves to determine ambient oxygen concentrations. Mass-specific respiration rates of the troglobites were more than an order of magnitude lower than those of comparably sized pelagic crustaceans. When compared to organisms residing in oxygen minimum zones, respiration rates of the troglobites were similar, or in some cases, lower. Enzymes of the troglobites were anaerobically poised with lactate dehydrogenase activities exceeding those of citrate synthase in all species. Malate dehydrogenase activities were greater than lactate dehydrogenase activities, indicating further evolutionary adaptations to an anaerobic environment.

Anchialine caves are regions where freshwater, seawater, and limestone interact to create a very unique ecosystem. Anchialine caves are submerged caves found in drowned coastal karst that include a surface opening inland and subsurface connections with seawater. They are most numerous and most extensive along the margins of the Pleistocene limestone platform bordering the eastern rim of the Great Bahama Bank. The caves were most likely formed from joints in the Pleistocene limestone that is covered with a thin veneer composed of Holocene carbonate reefs and sediment (Jaume *et al.*, 1998).

Hydrology of the anchialine systems is complex. The marine influence typically consists of a hypoxic layer of sea water ( $0.23\text{--}0.25\text{ mL}^{-1}\text{O}_2$ ) beneath one or more layers of limnetic to polyhaline water (Yager *et al.*, 1994). The lens of freshwater and the hypoxic sea water beneath are separated by a layer of hydrogen sulfide. Thickness of the hydrogen

sulfide layer varies with the inorganic input of each cave system (Kakuk, personal observation).

Food may be a limiting resource in marine caves. Primary productivity is only possible at the periphery of the cave system and as a result of the density interfaces, unlikely to penetrate deeply into the system (Pohlman *et al.*, 1997). With few exceptions, only small amounts of organic matter enter caves, and most is of low food quality such as detritus and fecal material. However, methanotrophic and sulfide-oxidizing bacteria may be significant food sources in the anchialine cave systems (Pohlman *et al.*, 1997).

Anchialine cave communities are dominated by crustaceans (Holsinger, 1989). Residents can be divided into two categories: those with members found throughout various marine habitats such as isopods, amphipods, and ostracods, and those orders containing only troglobites: Thernosbaenacea, Remipedia, and Leptostraca. The troglobites, remipedes in particular, appear to have been isolated for many millions of years.

Remipedes are thought to have been present prior to the formation of the Atlantic Ocean (Yager, 1991). The presence of these unique organisms in a habitat that is so extreme poses a major question: After the many years of isolation, have the troglobitic crustaceans evolved physiological adaptations to compensate for the extremely low oxygen levels and the potentially limiting food resources found in the cave systems?

As a consequence of their harsh habitat, anchialine troglobites are very unusual organisms. Permanent cave residents are notable for their small size, lack of pigment, and highly developed nonvisual sensory organs that compensate for the lack of eyes (Barr, 1968). They have a vast array of diverse setae and sensilla enabling them to inhabit an environment devoid of light (Yager, 1991).

Troglobites, either terrestrial or aquatic, have evolved a suite of adaptations that enable them to survive in ecosystems that are severely limited in organic resources (Culver and Poulson, 1971; Culver, 1982; Poulson, 2001). Adaptations include reduced metabolic rates, fewer eggs per clutch, and infrequent reproduction. Those adaptations have not been examined in the anchialine ecosystem.

The objectives of this project were threefold. The first was to determine oxygen content and water quality parameters of two anchialine caves in the Bahamas to determine oxygen concentrations within the troglobites' habitat. The second objective was to examine the troglobites' metabolic rates via direct measurement of  $O_2$  consumption. Measurements of metabolism at the whole-organism level were corroborated with determinations of aerobic enzyme activities and glycolytic enzyme activities (as an index of burst swimming capacity). Oxygen consumption and enzyme activities of troglobites were then compared to the metabolism of crustaceans inhabiting oxygen minimum layers. The final objective was to examine the proximate composition of troglobitic crustaceans, in the form of proteins and lipids, and to compare the lipid and protein contents and compositions to crustaceans of similar size in aerobic environments.

#### MATERIALS AND METHODS

Collections and water chemistry profiles were made in two cave systems in the Exuma Cays, Bahamas. The Exuma Cays are situated along the eastern rim of the Great Bahama Bank bordering Exuma Sound.

Because of the delicate balance of organisms in the cave environment, only a small number of specimens of each order were collected per dive. Anchialine cave systems are

currently imperiled by many anthropogenic impacts (Yager and Spokane, 1997). It was vital to the investigators to make as small an impact on the ecosystem as possible. As a result, total numbers of specimens representing each order are smaller than optimal. We chose to place our emphasis on the diversity within both cave systems, rather than an extensive sampling regimen that would more completely characterize the two cave systems.

All diving was conducted under the standards of the U.S. National Speleological Society Cave Diving Section. Organisms were collected by a diver using hand-held squeeze bottles. Water chemistry in both caves was monitored using a YSI minisonde (Yellow Springs Instruments) held in front of the diver to reduce turbulence as the diver penetrated the cave system. The following parameters were determined: Time, temperature ( $\pm 0.10^\circ\text{C}$ ), dissolved  $O_2$  as percentage air saturation and  $\text{mL}^{-1}$  ( $\pm 0.1 \text{ mL}^{-1}$ ), depth ( $\pm 0.3 \text{ m}$ ), pH ( $\pm 0.2$  units), and specific conductivity ( $\pm 0.001 \text{ mS cm}^{-1}$ ). The instrument recorded data every 30 seconds and stored it internally until the completion of the dive. Concurrent water samples were collected in Winkler bottles to confirm YSI minisonde data.

Oven Rock Cave is located on Great Guana Cay in the Exuma Cays. It is an inland, lens-based cave. Around the circumference of the breakdown is a pool of water with a freshwater lens. The underwater passages extend from the cavern pool, in a south-southwest direction for approximately 233 m. The main passage is a series of five large, round caverns attached at each end by connecting fractures. Heavy bacterial growth covers the walls of the caverns furthest from the entrance.

Basil Minns Bluehole is located on Great Exuma Island, Bahamas. The entrance to the cave lies beneath a large, inland saline pond, where the salinity varies with season. The surface pond was formed by a karst depression/collapse that occurred during the last low sea level stand, and the cave entrance allows access into the remaining passages below. The entrance leads down through jumbled boulders to a depth of 43 m where a large passage, highly decorated with speleothems (cave formations), leads in an easterly direction for a distance of 240 m. At this point, a small opening at a depth of 48 m leads into a large, circular and ascending dome room, a chamber with a large pocket of air above. The chamber had a well-developed fresh-water lens on the surface with stratification of fresh water, sea water, hydrogen sulfide layer, and anoxic sea water zone, respectively. All specimens were collected in this chamber.

#### Oxygen Consumption

In the laboratory, active and apparently undamaged troglobitic crustaceans were placed in modified 10 mL plastic syringe barrels with the tops removed (Ikeda *et al.*, 2001). Clark-type, microcathode, polarographic oxygen electrodes (Clark, 1956) were specifically designed to be inserted with an airtight seal into the modified syringe barrels. Syringes were filled with filtered ( $0.45 \mu\text{m}$ ) cave water. Syringe volumes ranged from 1 to 3 mL, depending upon the size of the organism. The syringes were maintained at an experimental temperature of  $20 \pm 0.2^\circ\text{C}$  by a circulating, refrigerated waterbath. Oxygen partial pressure,  $\text{PO}_2$ , was monitored continuously as the individuals reduced the oxygen levels to low partial pressures. Electrodes were calibrated before and after each incubation using air- and nitrogen-saturated cave water at the experimental temperature.

Data were recorded continuously for the duration of the respiration analysis using a data-logging system that scanned each of 15 channels for a period of 1 sec every minute.

Respiratory determinations were of the routine type where activity was not controlled. After an acclimation period of 30 min, respiratory rates were obtained by regressing the change in oxygen content in the respirometers on the time elapsed.

Following the incubations, specimens were removed from the syringes and photographed for identification purposes. They were then rinsed with deionized water, blotted dry, and frozen in liquid nitrogen. Frozen specimens were maintained at  $-80^{\circ}\text{C}$  until enzyme and proximate composition analyses were conducted.

To determine the potentially large impact of microbial respiration on the overall decrease in oxygen concentration within the syringes, selected syringes were filled with filtered cave water and sealed with an electrode. The oxygen concentration was monitored during the entire respiration analysis and ended when the final respiration incubation was completed. In several series of incubations, the oxygen consumption of the microorganisms was significant relative to the oxygen consumption of the troglobite ( $0.0108\text{--}0.0879\text{ mL}\cdot\text{L}^{-1}\text{ O}_2\text{ hr}^{-1}$ ). For the corresponding incubation series, the rate of oxygen consumption of the microorganisms was subtracted from the individual oxygen consumption rates to obtain the oxygen consumption of the individual crustaceans.

#### Enzyme Activity and Proximate Composition

Because of homogenate volume limitations, particularly in the very small troglobites, the total suite of analyses was not conducted on every specimen. Individual specimens were introduced frozen into the homogenizing medium, ice-cold distilled water, at varying dilutions (1:10 to 1:99) depending upon the mass of the organism. Samples were homogenized at  $0\text{--}4^{\circ}\text{C}$  using a sonifier and by hand using conical glass homogenizers with ground-glass contact surfaces.

Citrate synthase [CS, EC 4.1.3.7; citrate: oxaloacetate-lyase (CoA-acylating)] activity was assayed using the methods of Torres and Somero (1988). L-Lactate dehydrogenase (LDH, EC 1.1.1.27; lactate: NAD<sup>+</sup> oxidoreductase) activity was assayed in the pyruvate reductase direction also using methods described by Torres and Somero (1988). Malate dehydrogenase activity (MDH, EC 1.1.1.37; malate) was assayed as it catalyzed the formation of malate from oxaloacetate using the methods of Walsh *et al.* (1987). All enzyme activities were assayed in triplicate on a spectrophotometer at  $20 \pm 0.1^{\circ}\text{C}$ . Means are reported in  $\mu\text{mol}$  of substrate converted to product per minute.

Specimens were also assayed for protein and lipid levels following the methods described in Donnelly *et al.* (1993). Proximate composition was expressed as concentration, a percentage of wet mass, and is the component's proportion of the organism's total mass.

## RESULTS

### Oven Rock Cave

The cavern pool was approximately 3 m deep and had a mean temperature of  $23.0^{\circ}\text{C}$ . The surface of the pool was brackish with a specific conductivity of  $46.81\text{ mS cm}^{-1}$ , roughly 29 ppm. The mean dissolved oxygen content was  $4.69\text{ mL}\cdot\text{L}^{-1}$ , a value corresponding to 97% air saturation. Profiles of the oxygen concentration ( $\text{mL}\cdot\text{L}^{-1}$ ), depth (m), and temperature ( $^{\circ}\text{C}$ ) of Oven Rock Cave recorded with the distance traversed (m) are illustrated in Fig. 1. The

oxycline occurred at a depth of 2.0–3.5 m, corresponding to the transition from the brackish pool to the first chamber.

Chamber 1 had a continuous decline in oxygen concentration with depth to its maximum depth of 7.8 m and minimum  $\text{O}_2$  concentration of  $2.10\text{ mL}\cdot\text{L}^{-1}$ . A reverse thermocline was concurrent with the oxycline as the temperature increased from  $23.0^{\circ}\text{C}$  to  $26.2^{\circ}\text{C}$ .

Intrusion of marine water into the second chamber resulted in an increase in the specific conductivity to  $55.417\text{ mS cm}^{-1}$  (approximately 37 ppm). Oxygen continued to drop in chamber 2, and as the diver passed into chamber 3, the oxygen concentration dropped to  $0.69\text{ mL}\cdot\text{L}^{-1}\text{ O}_2$  or a mean percentage air saturation of 15.5%.

All crustaceans were collected below the freshwater/seawater interface. Ostracods, amphipods, and remipedes were collected in the second chamber. All other organisms were collected in the third chamber. Oxygen concentrations where organisms were collected ranged from  $2.45\text{ mL}\cdot\text{L}^{-1}$  to a minimum of  $0.66\text{ mL}\cdot\text{L}^{-1}\text{ O}_2$ .

### Basil Minns Bluehole

Figure 2 is a profile of Basil Minns Bluehole cave including oxygen concentration ( $\text{mL}\cdot\text{L}^{-1}$ ), depth (m), and temperature ( $^{\circ}\text{C}$ ) with distance covered (m) in the dive. The mean temperature of the extensive surface pool of Basil Minns Bluehole was  $24.9^{\circ}\text{C}$ . Mean specific conductivity in the pool was  $54.83\text{ mS cm}^{-1}$ .

The passage to the dome room had a maximum depth of 48.35 m, with oxygen concentrations approximately  $1.8\text{ mL}\cdot\text{L}^{-1}$ . The passage ascended as the diver proceeded from the passage to chamber. The oxycline occurred at 27 m, with the transition from the passage to the dome room at 5.3 m. The oxygen concentration decreased from more than  $6.8\text{ mL}\cdot\text{L}^{-1}$  in the surface pool to  $1.3\text{ mL}\cdot\text{L}^{-1}$  at a depth of 9 m. In the dome room, at a depth of 26 m, the oxygen concentration fell below detectable limits ( $< 0.1\text{ mL}\cdot\text{L}^{-1}$ ), and the temperature rose to  $27.76^{\circ}\text{C}$  with little variation. The specific conductivity in the dome room, where all organisms were collected, was  $53.64\text{ mS cm}^{-1}$ . All organisms were collected in the anoxic layer.

### Respiration Rates

The 72 troglobites respired spanned seven orders and included 17 species (Table 1). Each cave system contains its own endemic crustacean populations making identification of the organisms to species level difficult and occasionally

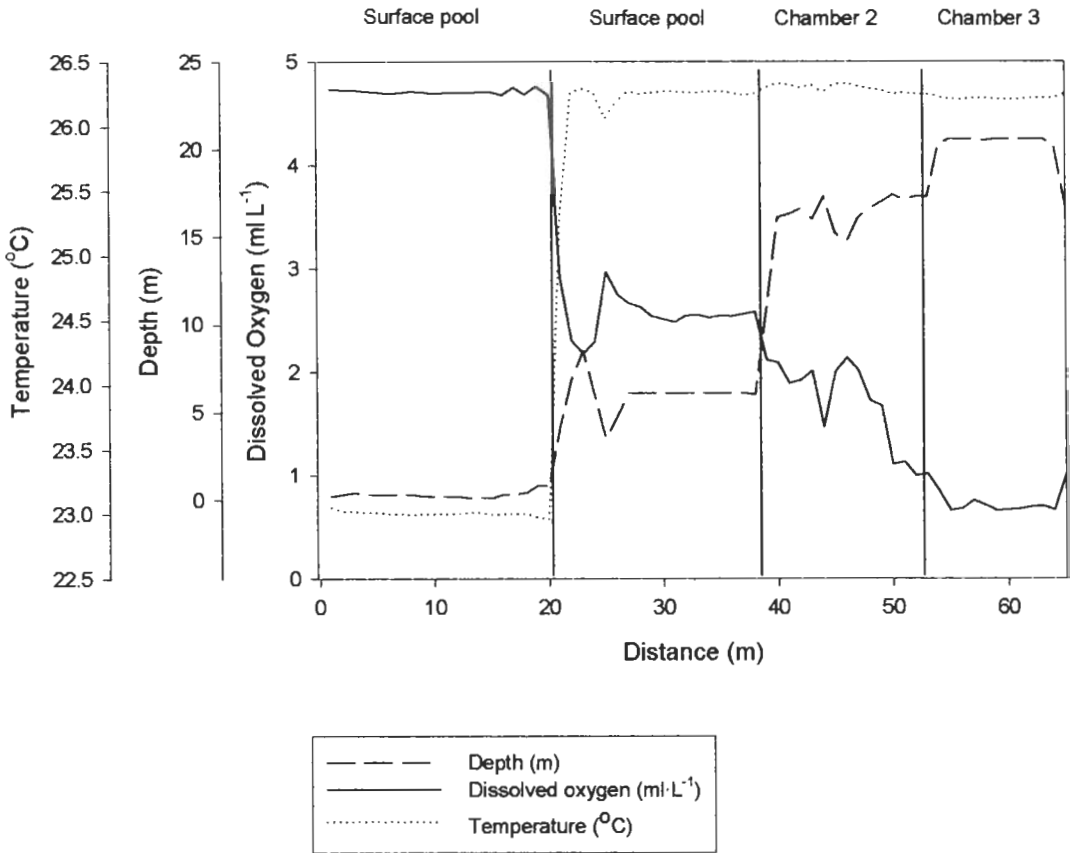


Fig. 1. Profile of Oven Rock Cave with Temperature ( $^{\circ}\text{C}$ ), Depth (m), and dissolved Oxygen ( $\text{mL}\cdot\text{L}^{-1}$ ) against Distance (m). Vertical lines indicate transitions to the next chamber.

not possible. Many of the crustaceans inhabiting Oven Rock cave have been identified, but Basil Minns Bluehole cave is still under exploration. Identifications and naming of the species are in progress (T. Illiffe, personal communication). Identifications of the organisms collected in this study were made by consulting the literature and identifying live organisms, examination of photographs of preserved specimens, and in several cases using the genus identification of previously examined organisms collected from the same cave. Thermosbaenaceans were collected in both caves, but species identifications have not been published for specimens from either system. Because two caves rarely have the same species, *Tulumella* sp. from Oven Rock is designated as species 1, and Basil Minns Bluehole specimens are species 2. Identifications of the Oven Rock ostracod species became available after initial collections were made for this study. Consequently, ostracods collected from Oven Rock cave are referred to as *Danielopolina* sp.

The cirrolanaid isopod from Basil Minns Bluehole had the lowest mass specific respiration rate ( $0.0005 \mu\text{L O}_2\text{mg}^{-1}\text{hr}^{-1}$ ) (Table 1). Of the species with more than one representative respired, *Godzilligonomous frondosus* Yager, 1989, had the lowest mean mass-specific respiration rate ( $0.0966 \pm 0.04756 \mu\text{L O}_2\text{mg}^{-1}\text{hr}^{-1}$ ) (Table 1). The unidentified copepods from Basil Minns Bluehole had the greatest mean mass-specific rate of all troglolobites ( $0.4064 \pm 0.0818 \mu\text{L O}_2\text{mg}^{-1}\text{hr}^{-1}$ ). Table 1 shows the mean mass specific respiration rates for all troglolobitic species.

#### Enzyme Activities

Citrate synthase (CS), located at the beginning of the Krebs's citric acid cycle, is an indicator of an organism's maximum aerobic potential. The greatest mass specific CS activities were observed in the copepods ( $5.28 \pm 2.733 \mu\text{mol}$  of substrate converted to product per minute) (Table 1). The lowest CS rates were consistently

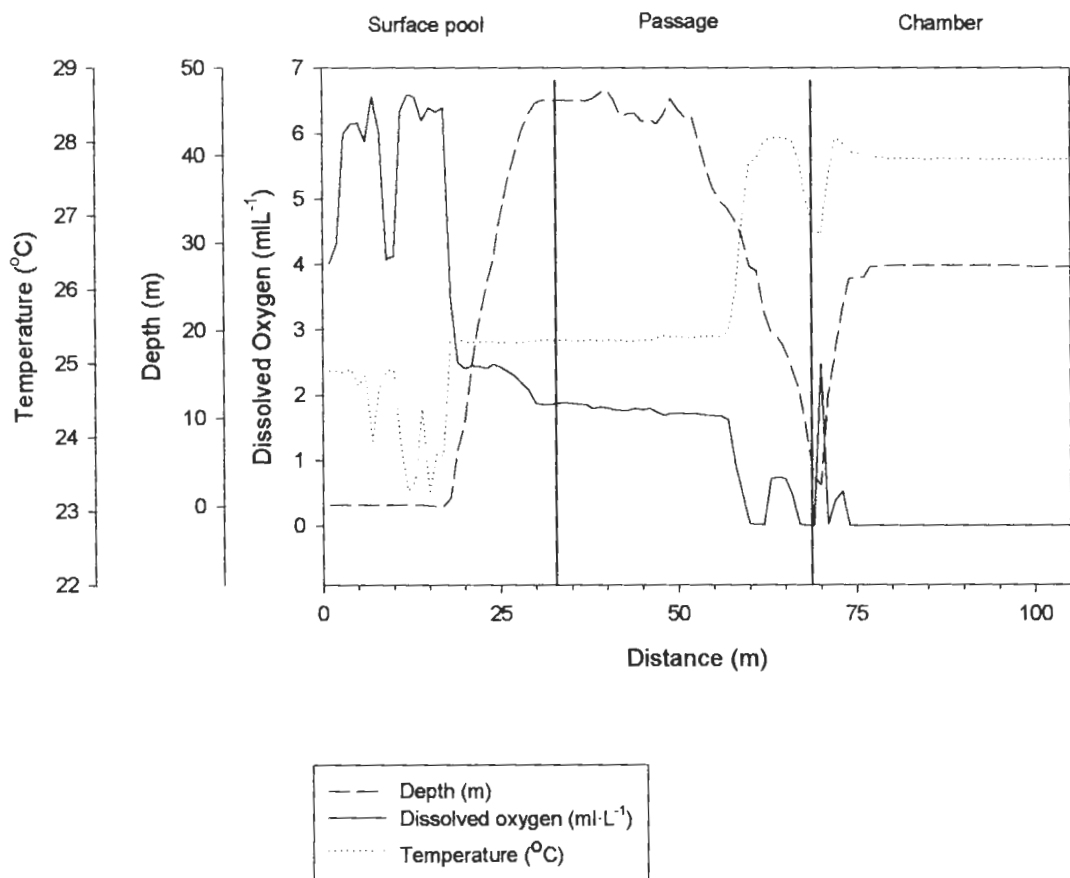


Fig. 2. Profile of Basil Minns Bluehole with Temperature ( $^{\circ}\text{C}$ ), Depth (m), and dissolved Oxygen ( $\text{mL}\cdot\text{L}^{-1}$ ) against Distance (m). Vertical lines indicate transitions to the next chamber.

found in the remipedes ( $0.43\text{--}0.47$  units  $\text{min}^{-1}$ ) and *Tulumella* sp. ( $0.23 \pm 0.093$   $\mu\text{mol min}^{-1}$ ).

Lactate dehydrogenase (LDH) is the terminal enzyme in glycolysis that contributes to both aerobic and anaerobic metabolic pathways and serves as an indicator of glycolytic potential. Mass-specific activities for LDH were highly variable for all of the troglobites. The greatest LDH rates were found in *Danielopolina* sp. ( $37.80 \pm 19.657$   $\mu\text{mol min}^{-1}$ ) and the lowest were in the copepod *Enatronoidea bahamensis* ( $0.76 \pm 0.206$   $\mu\text{mol min}^{-1}$ ) (Table 1).

Malate dehydrogenase plays a dual role in facultative anaerobes. In the presence of oxygen, phosphoenolpyruvate (PEP) is converted to pyruvate and via the Krebs' citric acid cycle is oxidized completely to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  (Lehninger *et al.*, 1993). In anaerobic conditions, PEP is carboxylated to oxaloacetate, which is then reduced to malate by malate dehydrogenase (Hochachka *et al.*, 1973). Mass specific

malate dehydrogenase (MDH) activities were greatest in *Spelaeonicippe provo* Stock and Vermeulen, 1982 ( $48.48 \pm 22.909$   $\mu\text{mol min}^{-1}$ ) and the ostracods, *Deeveya medix* Kornicker, 1990 and *Danielopolina* sp., exhibited the lowest activities ( $0.45$  and  $0.47 \pm 0.457$   $\mu\text{mol min}^{-1}$  respectively) (Table 1).

Mean protein concentrations were lowest in *Danielopolina* sp. ( $3.60 \pm 0.972\%$ ) and greatest in the single leptostracan, *Speonebalia* sp. ( $11.06\%$ ) (Table 1). Lipid concentrations ranged from  $2.01\%$  to a mean of  $12.72 \pm 0.979\%$  in *Cirolana troglaxuma* Botasaneanu and Iliffe, 1997, and *Speleonectes* sp., respectively (Table 1).

## DISCUSSION

The water chemistry profiles of Oven Rock cave and Basil Minns Bluehole were similar to the results obtained by Yager *et al.*, (1994) from two anchialine ecosystems in Cuba. A hydrogen

Table 1. Wet mass (mean, g), mean oxygen consumption ( $\mu\text{L O}_2 \text{mg}^{-1} \text{h}^{-1}$ ) at 20°C, enzyme activities for citrate synthase (CS), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH), all expressed as units  $\text{mg}^{-1}$  and proximate concentrations (% wet mass) for each species of troglobites, SE represents the standard error of the mean and  $n$  is the number of organisms assayed. Where only one organism was assayed, SE values are not reported. Species are identified by cave system: OR = Oven Rock Cave, and BM = Basil Minnis Blue Hole. NM = not measured.

Organism	Wet mass (range, g)	Oxygen consumption $\pm$ SE, $n$	Mean enzyme activity			% Protein $\pm$ SE, $n$	% Lipid $\pm$ SE, $n$
			CS $\pm$ SE, $n$	MDH $\pm$ SE, $n$	LDH $\pm$ SE, $n$		
<b>Amphipoda</b>							
<i>Speleocricippe provo</i> Stock and Vermeulen, 1982 (OR)	0.0025–0.0164	0.259 0.0646 17	0.806 0.1519 14	48.485 22.9099 17	2.886 0.7335 16	6.226 0.6244 14	3.657 0.3295 7
<i>Sokarhops catacumbae</i> (BM)	0.0001–0.0024	0.336 0.0202 3	3.778 1.3778 3	7.583 3.7698 3	7.583 3.7698 3	NM	NM
<b>Copepoda</b>							
<i>Enatironaidea bahamensis</i> (OR)	0.0015–0.0003	0.170 0.0317 9	5.288 2.7328 3	32.678 11.3302 5	0.760 0.2058 2	NM	NM
<i>Bomburietta gigas</i> Fosshagen, Boxshall and Iliffe, 2001 (OR)	0.0020–0.0025	0.221 0.0099 2	1.632 1 NM	5.298 1.1426 2	7.213 1 20.405	NM	NM
Unknown species (BM)	0.0002–0.0027	0.406 0.0818 3	NM	24.903 7.0392 2	20.405 1 1	NM	NM
<b>Isopoda</b>							
<i>Cirolana troglodyma</i> Botasaneanu and Iliffe, 1997 (OR)	0.00296–0.0272	0.228 0.1933 6	1.458 0.8690 3	17.949 5.2454 5	5.082 1.8480 5	4.92 0.623 4	2.011 1 NM
<i>Cirolana</i> sp. (BM)	0.0003–0.0579	0.001 1	0.0488 1	0.284 1	14.129 1	NM	NM
<b>Remipedia</b>							
<i>Plecometura</i> sp. (OR)	0.0051–0.0056	0.113 1	0.446 0.1269 2	3.927 3.6724 2	3.296 3.0799 2	NM	4.01 1
<i>Speleomectes</i> sp. (OR)	0.0038–0.0055	0.208 0.0783 7	0.432 0.1080 3	13.054 2.7692 3	20.043 3.1204 3	4.880 1.0101 3	12.723 0.9797 2
<i>Godzilligononius frondosus</i> Yager, 1989 (OR)	0.0018–0.0440	0.097 0.0476 4	0.475 0.1324 4	10.666 2.2659 4	15.155 11.0754 3	5.781 0.7291 4	NM

Table 1. Continued.

Organism	Wet mass (range, g)	Oxygen consumption $\pm$ SE, <i>n</i>	Mean enzyme activity			% Protein $\pm$ SE, <i>n</i>	% Lipid $\pm$ SE, <i>n</i>
			CS $\pm$ SE, <i>n</i>	MDH $\pm$ SE, <i>n</i>	LDH $\pm$ SE, <i>n</i>		
Ostracoda							
<i>Deveya mediv</i> Kornicker, 1990 (BM)	0.0001–0.0014	0.050	1.383 0.5641 3	0.451 1 0.477 0.4572 2	26.413 16.9489 3 5.293 3.2724 4	NM NM	NM 6.282 1 NM
<i>Danielopolina exuma</i> (OR)	0.0011–0.0029	NM	1.167 1.0412 4				
<i>Danielopolina</i> sp. (OR)	0.0005–0.0019	0.2945 0.0546	NM	4.379 1.2177 6	37.801 19.6571 8	3.604 0.9724 4	1 NM
<i>Deveya exleyi</i> Kornicker and Iliffe, 1998 (OR)	0.0004–0.0038	0.368 0.0991 4	0.051	7.671 2.7567 5	35.9125 11.9387 5	5.561 1.3802 3	3.212 1
Thermosbaenacea							
<i>Tulumella</i> sp. 1 (OR)	0.0011–0.0071	0.191 0.0861	0.232 0.0934 6	25.119 7.8228 6	37.70237	5.433 0.9868 4	3.071 0.2228 3 NM
<i>Tulumella</i> sp. 2 (BM)	0.0012–0.00324	0.275 0.0861	0.262 0.1313	14.037 8.2886	28.0381	NM	NM
Leptostracea							
<i>Specnebalia</i> sp. (OR)	0.0010–0.0030	0.0156	1.069 0.5275 3	6.3531 5.9382 3	9.931 5.2501 3	11.064	NM 1

sulfide layer separated the surface pools from deeper marine water. In all caves, the marine intrusion was substantially warmer than the surface pools. Basil Minns Bluehole, however, was the only system with a saltwater surface pool. Oxygen minimum levels between the four pools were very similar. The Cuban systems examined by Yager *et al.* (1994) had oxygen levels of approximately  $0.28 \text{ mL}\cdot\text{L}^{-1} \text{ O}_2$ , intermediate between the levels found at Oven Rock and Basil Minns Bluehole.

Oven Rock cave and Basil Minns Bluehole cave were dominated by crustaceans (cf. Yager and Spokane, 1997). Excluding the leptostracans, members of all groups were found in each cave system in our study. All organisms were observed below the freshwater/saltwater interface in water with oxygen contents below  $0.64 \text{ mL}\cdot\text{L}^{-1} \text{ O}_2$ .

Many organisms are capable of obtaining energy in the absence of oxygen; however, few are able to survive indefinitely without a return to oxygen (Hochachka and Somero, 2002). Anaerobiosis is most often used to compensate for ATP demand if the aerobic energy supply becomes inadequate; this is known as facultative anaerobiosis. Facultative anaerobiosis results from one of two situations: 1, physiological activity raises ATP demand in an organism until it exceeds aerobic capacity (functional anaerobiosis), or 2, oxygen levels become too low, preventing the maintenance of cell functions aerobically (Kamp, 1993). The latter is referred to as environmental anaerobiosis (Hochachka and Lutz, 2001).

Organisms are able to conserve their energy stores during periods of environmental anaerobiosis by a dramatic depression of their ATP demand combined with a loss in physiological functions such as motility, ingestion, and digestion (Hochachka and Lutz, 2001). It is readily apparent that the troglobites must have evolved physiological adaptations for surviving in their extreme habitat. An interesting paradigm, "the low tissue  $\text{O}_2$  strategy" (Massabuau, 2001), may help shed light on the evolution of survival in low oxygen environments. Three groups of organisms (fish, molluscs, and crustaceans) have been identified as having low arterial  $\text{O}_2$  partial pressures independent of the ambient water (Forgue *et al.*, 1989; Massabuau *et al.*, 1991; Fogue *et al.*, 1992; Tran *et al.*, 2000). Massabuau (2001) suggests that this strategy is a remnant of organisms evolving in the primitive earth's atmosphere when aerobic life began in a hypoxic environment.

A comparison of metabolism and proximate composition in troglobites to other similarly sized crustaceans is difficult. There are no previous metabolic rate measurements for marine troglobites and three of the orders are found only in marine caves. Metabolic rates have been determined for freshwater, troglobitic crayfish (Gannon *et al.*, 1999), but the size difference is large and their benthic lifestyle is not directly comparable. Troglobitic organisms tend to be more diminutive than nontroglobites within the same order. Finally, organisms residing in low-oxygen environments that have been examined reside at much lower temperatures than are found in anchialine systems. All of these factors complicate the comparison of troglobitic enzymes and metabolic rates to other organisms. Regardless, Table 2 illustrates the oxygen consumption, enzyme activities, and proximate composition of the four orders of crustaceans found in the deep sea,  $\text{O}_2$  minima zones, Gulf of Mexico, Hawaiian Islands, and freshwater caves.

Examination of Table 2 indicates that the mass-specific respiration rates of the troglobites are similar to or higher than rates observed in the other reported regions, while in fact they are distinctly lower when we take into account the small size of the troglobites. By inserting the mass values of the organisms from Table 2 into the allometric relationships reported in Table 2, we can see that the predicted mass-specific respirations of the cave organisms would be consistently lower, in some cases more than an order of magnitude lower than reported. For example: a 0.2 g *Phronima sedentaria* Forskål, 1775, from an oxygen minimum zone would have a respiration rate of  $0.001 \mu\text{L O}_2\text{mg}^{-1}\text{hr}^{-1}$ , substantially lower than the  $0.0129\text{--}0.061 \mu\text{L O}_2\text{mg}^{-1}\text{hr}^{-1}$  Childress (1975) reported at  $10^\circ\text{C}$ .

Mass-specific activities of CS and LDH in troglobites were higher than those of Thuesen *et al.* (1998) for the deep sea copepods analyzed at  $20.0^\circ\text{C}$  (Table 2). The deep sea copepods' CS activities ranged  $0.747\text{--}3.478 \text{ units g}^{-1}$ , and mean LDH activities ranged  $0.086\text{--}70.027 \text{ units g}^{-1}$ , substantially lower than the enzyme activities of the organisms analyzed in our study.

All orders of troglobites were anaerobically poised, with higher LDH activities than CS activities, suggesting that glycolytic energy is critical to their metabolism. Those organisms with the greatest LDH : CS ratios were the themosbaenacean and *Deeveya exleyi*. Numerous studies

Table 2. Comparison of mean wet mass (g), mean oxygen consumption ( $\text{mL} \cdot \text{gWm}^{-1} \cdot \text{hr}^{-1}$ ), citrate synthase and lactate dehydrogenase activities ( $\text{units} \cdot \text{g}^{-1}$ ), lipid and protein concentrations (%WM) for troglotic (T), deep-sea (D), Gulf of Mexico (GOM), Hawaiian Islands (HI), and oxygen minimum zone (OM) residing crustaceans. Specimens for comparison were selected based upon size and available data. Where available, published  $Q_{10}$  values were used to adjust the mean oxygen consumption values to 20°C (designated by \*). Assay temperatures are reported (°C) following oxygen consumption and enzyme activities, R indicates those values that are reported as ranges not means. — indicates variables not measured.

Species	Wet mass (g)	Mean $O_2$ ( $\text{mL} \cdot \text{gWm}^{-1} \cdot \text{hr}^{-1}$ )	CS Activity ( $\text{units} \cdot \text{g}^{-1}$ )	LDH Activity ( $\text{units} \cdot \text{g}^{-1}$ )	Protein (%WM)	Lipid (%WM)	Authors
<b>Amphipoda</b>							
<i>Ploronima sedentaria</i> Forskål, 1775 (OM)	0.2	— 0.0129–0.061 (10°C) R	—	—	11.84	0.88	Childress and Nygaard, 1974 Childress, 1975
<i>Hypenetia galba</i> Montagu, 1813 (OM)	0.09	— 0.0872 (10°C)	—	—	4.83	2.95	Childress and Nygaard, 1974 Childress, 1975
<i>Paracallincha coecus</i> (OM)	0.1	— 0.0360 (5.5°C)	—	—	0.62	—	Childress and Nygaard, 1974 Childress, 1975
<b>Copepoda</b>							
<i>Arietellus plumifer</i> Sars, 1905 (D)	0.0081–0.0112	0.04082 ± 0.0095 (5°C)	3.478 ± 0.211 (20°C)	12.093 ± 4.257 (20°C)	—	4.90	Thuesen <i>et al.</i> , 1998
<i>Lucicutia maxima</i> Steuer, 1904 (D)	0.0084–0.0130	0.2500 ± 0.0283*	0.747 ± 0.075 (20°C)	14.477 ± 1.668 (20°C)	2.23 ± 0.11	—	Thuesen <i>et al.</i> , 1998
<i>Paracuchaeta birostrata</i> Brodsky, 1950 (D)	0.0116–0.0143	0.1074 ± 0.0081*	0.737 ± 0.102 (20°C)	1.178 ± 0.095 (20°C)	5.45 ± 0.43	—	Thuesen <i>et al.</i> , 1998
<i>Paracuchaeta tonsa</i> Giesbrecht, 1895 (D)	0.0062m–0.0070	0.0989 ± 0.00621 (5°C)	1.187 ± 0.062 (20°)	1.071 ± 0.149 (20°)	—	—	Thuesen <i>et al.</i> , 1998
<i>Bathycalanus bradyi</i> Wolfender, 1905 (OM)	0.03	— 0.2430–0.6660 R (4°C)	—	—	1.45	2.78	Childress and Nygaard, 1974 Childress, 1975
<i>Bathycalanus princeps</i> Brady, 1883 (OM)	0.05	— 0.0203 (5.5°C)	—	—	2.39	2.13	Childress and Nygaard, 1974 Childress, 1975
<i>Goussia princeps</i> Scott, 1894 (OM)	0.04	— 0.0152–0.0775 R (7°C)	—	—	3.60	1.53	Childress and Nygaard, 1974 Childress, 1975
<i>Labidocera jollae</i> Easterly, 1906 (OM)	2.2	—	—	—	5.53	—	Childress and Nygaard, 1974
<b>Mysidacea</b>							
<i>Gnathophausia ingens</i> Dohrn, 1870 (OM) (GOM)	—	0.0348 (5.5°C)	—	—	—	—	Childress, 1975
<b>Isopoda</b>							
<i>Anuropus bathypelagicus</i> Menzies and Dow, 1958 (OM)	8.2	—	—	—	0.85	—	Childress and Nygaard, 1974

Table 2. Continued.

Species	Wet mass (g)	Mean O <sub>2</sub> (mL g Wm <sup>-1</sup> hr <sup>-1</sup> )	CS Activity (units g <sup>-1</sup> )	LDH Activity (units g <sup>-1</sup> )	Protein (%WM)	Lipid (%WM)	Authors
Ostracoda							
<i>Gigantocypris agassizii</i> (OM)	0.6	0.0056 (5.5°C)	-	-	4.77	-	Childress, 1975
Decapoda							
<i>Janicella spinicauda</i>							Childress and Nygaard, 1974
Milne Edwards, 1883 (HI)	0.35	0.3712 (20°C)	-	-	-	-	Childress, 1975
<i>Parapandalus richardi</i>	0.249	-	-	-	9.7	-	Cowles <i>et al.</i> , 1991
Coutière, 1905 (GOM)	0.284	0.245 (17°C)	-	-	-	-	Donnelly <i>et al.</i> , 1993
<i>Sergestes pectinatus</i> Sund.							Donnelly and Torres, 1988
1920 (GOM)	0.039	-	-	-	12.6	-	Donnelly <i>et al.</i> , 1993
<i>Sergestes armatus</i> Krøyer,							Donnelly and Torres, 1988
1855 (GOM)	0.029	0.217 (14°C)	-	-	-	-	Donnelly and Torres, 1988
<i>Sergia grandis</i> Sund, 1920							Donnelly and Torres, 1988
(GOM)	0.713	0.251 (20°C)	-	-	-	-	Donnelly and Torres, 1988
<i>Sergia talismani</i> Barnard,							Donnelly and Torres, 1988
1946 (GOM)	0.202	0.263 (20°C)	-	-	-	-	Donnelly and Torres, 1988
<i>Troglocambarus macleani</i>							Gannon <i>et al.</i> , 1999
Hobbs, 1942 (T)	0.47-0.22	0.0398 (22°C)	-	-	-	-	Gannon <i>et al.</i> , 1999
<i>Procambarus pallidus</i>							Gannon <i>et al.</i> , 1999
Hobbs, 1937 (T)	0.83-2.28	0.031794 (22°C)	-	-	-	-	Gannon <i>et al.</i> , 1999
<i>Procambarus erythropus</i>							Gannon <i>et al.</i> , 1999
Relyea and Sutton, 1975 (T)	1.41-3.29	0.039168 (22°C)	-	-	-	-	Gannon <i>et al.</i> , 1999

have established that lactate accumulates during both functional anaerobiosis and environmental anaerobiosis (Booth *et al.*, 1982; Lallier *et al.*, 1987; Milligan *et al.*, 1989). Species that use burst swimming in their search for food or mates tend to have high LDH activities (Thuesen *et al.*, 1998). This strategy is utilized by deep sea organisms to conserve energy when food supply is low.

Anaerobic glycolysis is a fundamentally inefficient metabolic strategy, generating maximally 15% of the aerobic potential of an organism (Jackson, 1968). For organisms that sustain anoxia indefinitely it would be particularly inefficient. There is a direct correlation between an organism's ability to withstand temporary anaerobiosis and values of MDH : LDH; the higher the ratio, the greater an organism's tolerance to hypoxia. This relationship between MDH : LDH and hypoxia has been observed in crustaceans (Shapiro and Bobkova, 1975). The troglobites all exhibited a MDH : LDH greater than 1, ranging from 18.9 in the isopods to 1.6 in the remipedes, indicating an evolutionary adaptation for exposure to hypoxic and anoxic conditions.

The proximate composition of the troglobitic organisms reflects their adaptations to the anchialine environment. Compared to mesopelagic crustaceans (Donnelly *et al.*, 1993), the troglobites are sacrificing protein mass for increased lipid stores. Protein concentrations in the troglobites were lower than those observed for two species selected for comparison because of their small sizes, *Parapandalus richardi* Coutière, 1905 (0.14–0.45 g WM) and *Sergestes pectinatus* Sund, 1920 (0.03–0.05 g WM), both having 9.7% and 12.6% WM protein, respectively. Lipid concentrations in the troglobites, though, were higher (*P. richardi* 1.2% WM lipid and *S. pectinatus* 1.9% WM lipid). A comparison of the troglobites' protein level to that of deep-sea copepods (Thuesen *et al.*, 1998) revealed that the troglobitic crustaceans' protein content is comparable to that of deep-sea copepods living in the oxygen minimum of the California borderland.

Increased lipid stores may serve a two-fold purpose in some of the troglobites, the remipedes in particular, which are lie-in-wait predators. The lipid provides neutral buoyancy without energy expenditure. Lipid globules observed throughout the bodies of remipedes also can serve as an energy reserve when food is

limiting (Yager, 1989). The role of increased lipid may be to facilitate a substantial reduction in activity in the troglobites. The combination of lower metabolic rates and lower energy-density allows the troglobites to allocate a greater fraction of their ingested food energy to growth, much like deeper-living midwater fishes do (Childress and Nygaard, 1973).

The anchialine troglobites are obtaining energy via three potential routes. In the first strategy, they are either functioning anaerobically, potentially retreating to anoxic regions as a refugium or awaiting changing conditions in the cave system. This strategy would be energetically expensive because of the low energy return via glycolysis per meal. This is not a likely scenario in an environment with a very low food supply unless the troglobites are gaining an energy boost from a symbiotic relationship with bacteria, which is the second potential strategy. Rod-shaped and spherical bacteria, loosely scattered throughout the tissue of several species, have been observed in TEM micrographs (Yager, 1989) and as epibionts on the surface of some remipedes (Yager, 1987). This is an intriguing theory and is further substantiated by the discovery of an extensive cave system in Dobrogea, Romania. The Movile cave system is reported to be completely chemoautotrophically based. Microorganisms use the energy derived from the oxidation of  $H_2S$ , present in high concentrations in caves, to fix carbon. The bacteria essential for the functioning of the ecosystem belong to the genera *Beggiatoa*, *Thiobacillus*, and *Thiomicrospira* (Sarbu and Kane, 1995). Huge, filamentous colonies of *Beggiatoa-Thiothrix* are commonly found in anchialine caves (Yager, 1991). A similar symbiotic situation exists between bacteria and the carid shrimp residing in the deep hydrothermal vents in the mid-Atlantic Ridge (Van Dover, 1995). The third potential way the troglobites are surviving anoxia is by functioning aerobically while efficiently extracting oxygen from the very low levels in the cave environment.

We can understand how troglobitic crustaceans may function aerobically in their unique habitat by examining the physiological adaptations of organisms in oxygen minimum zones, regions where the oxygen levels fall beneath  $0.833 \text{ mg}\cdot\text{L}^{-1}$  of  $O_2$  (Schmidt, 1925; Sewell and Fage, 1948; Banse, 1964). Like the anchialine systems, these regions remain stable and possess a characteristic fauna. Despite very

low  $O_2$  levels, studies have shown that pelagic crustaceans living in the California oxygen minimum regions are able to do so aerobically by being unusually effective at extracting  $O_2$  from water (Childress, 1968, 1971, 1975; Sanders and Childress, 1990).

Childress (1968, 1971) demonstrated that *Gnathophausia ingens* is capable of living aerobically in oxygen concentrations as low as  $0.23 \text{ mL}\cdot\text{L}^{-1} O_2$ , a level falling between the oxygen concentrations at Oven Rock cave ( $0.7 \text{ mL}\cdot\text{L}^{-1} O_2$ ) and Basilman's Bluehole ( $0.14 \text{ mL}\cdot\text{L}^{-1} O_2$ ). *Gnathophausia ingens* may use its anaerobic metabolism briefly to support high activity levels at the lowest oxygen levels, but it is able to live aerobically at the lowest oxygen concentrations it encounters (Childress, 1971, 1975). Its ability to regulate its oxygen consumption to low  $PO_2$  values is due to its ability to maintain a high ventilatory flow and simultaneously remove a large fraction of the oxygen in the inhaled water (Childress, 1971). These abilities are made possible by a high gill surface area, efficient circulatory systems, and high-affinity hemocyanin (Belman and Childress, 1976; Sanders and Childress, 1988). However, unlike the troglobites, *G. ingens* has a very low tolerance for anaerobic conditions, surviving less than 30 minutes in anoxia (Sanders and Childress, 1988).

Anchialine troglobites, remipedes in particular, may be using strategies similar to *G. ingens* to efficiently extract oxygen from their environment. Remipedes do not have specialized respiratory structures such as gills, but their broad paddle-like trunk appendages could serve as respiratory surfaces. Several species of remipede, including *Godzillignomous frondosus* Yager, 1989, have been observed pumping water anally. Anal intake of water has been reported for a number of small crustaceans and is considered to be a respiratory adaptation particularly for organisms inhabiting water deficient in dissolved oxygen. Remipedes use hemocyanin to further facilitate the utilization of oxygen (Yager, 1991).

Although  $O_2$  minimum zones and anchialine caves have similar dissolved oxygen levels, there are striking dissimilarities that reveal the extreme harshness of the anchialine system. Oxygen minima are found typically below 200 m, where temperatures are well below that found in the cave systems studied here ( $5\text{--}20^\circ\text{C}$ ) (Childress, 1971, 1975), which would result in correspondingly lower metabolic rates. The

troglobites examined in this study and those observed by Yager and Spokane (1997) were residing in water in excess of  $25^\circ\text{C}$ , thereby elevating the troglobites' metabolic requirements above those of the typical  $O_2$  minimum dweller.

Anchialine cave systems are uniquely harsh environments compounding the low food availability found in all cave systems with elevated temperatures and anoxic regions. Anchialine troglobites are adapted to their environment by having very low metabolic rates and small size, and in addition, their enzymatic machinery is poised for the most efficient energy usage during times of extreme anoxia. They appear to be utilizing strategies similar to organisms that reside in oxygen minima: maximizing the efficiency of oxygen extraction from the water as well as having a reduction of protein and an increase in lipid, which can serve as an energy depot when food supply is limiting.

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