UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS

Patrones de distribución y ecofisiología de *Typhlatya* spp. (Atyidae) endémicas de Yucatán.

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS

PRESENTA:

Efraín Miguel Chávez Solís

TUTORA PRINCIPAL DE TESIS: Dra. Maite Mascaró Miquelajauregui
UMDI Sisal, Facultad de Ciencias, UNAM
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Ciudad Universitaria, Cd. Mx., Marzo 2022

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Ciudad Universitaria, Cd. Mx. **2022**

COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS

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ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión virtual del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día 9 de agosto del 2021 se aprobó el siguiente jurado para el examen de grado de **DOCTOR EN CIENCIAS** del estudiante **CHÁVEZ SOLÍS EFRAÍN MIGUEL** con número de cuenta **300501733** con la tesis titulada "**PATRONES DE DISTRIBUCIÓN Y ECOFISIOLOGÍA DE *Typhlatya spp.* (ATYIDAE) ENDÉMICAS DE YUCATÁN**", realizada bajo la dirección de la **DRA. MAITE MASCARO MIQUELAJAUREGUI,** quedando integrado de la siguiente manera:

Presidente:	DRA. GUILLERMINA ALCARAZ ZUBELDÍA
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Vocal:	DRA. GABRIELA RODRÍGUEZ FUENTES
Secretario:	DR. CARLOS ROSAS VÁZQUEZ

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 09 de noviembre de 2021

COORDINADOR DEL PROGRAMA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

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Índice

Resumen general

Los camarones de cueva del género *Typhlatya* son especies restringidas a vivir en cuevas inundadas –denominadas especies estigobíticas– y se encuentran en las aguas subterráneas dulces, salobres y marinas, conocidos también como sistemas anquihalinos o estuarios subterráneos. En la Península de Yucatán *T. mitchelli*, *T. pearsei* y *T. dzilamensis* son los representantes endémicos del género. Por su abundancia, talla y amplia distribución, estas especies son modelos biológicos ideales para realizar estudios ecológicos y fisiológicos. Sin embargo, sus patrones de distribución, aspectos ecológicos y características fisiológicas han sido poco estudiados y no han sido completamente reconocidos.

Esta tesis se divide en dos capítulos, el primero relacionado con la ecología de estas especies y el segundo con su fisiología. En el primer capítulo de esta tesis, se describe la abundancia de tres especies de *Typhlatya* en diferentes escalas temporales y espaciales, observando los cambios en las condiciones ambientales y las posibles fuentes de carbono como una indicación del origen de sus alimentos. Principalmente se observó que la composición y abundancia de las especies varió notablemente en el espacio y el tiempo, revelando patrones que difieren entre especies y con respecto a la salinidad de los microambientes del ecosistema anquihalino. El análisis isotópico mostró que cada especie refleja una huella particular de $\delta^{13}C$ y $\Delta^{14}C$, lo que sugiere que se alimentan en diferentes proporciones de las fuentes de carbono disponibles en cada microhábitat. Nuestros hallazgos sugieren una partición de nicho, en términos de hábitat y fuentes de alimentación entre las tres especies de *Typhlatya*, donde las características ambientales y las diferencias fisiológicas podrían desempeñar un papel importante en los patrones de distribución.

Una vez reconocida la partición del nicho entre estas especies, el segundo capítulo, caracteriza las capacidades fisiológicas de cada especie en su ambiente nativo. Se evaluó el potencial metabólico aerobio (PMA) de las tres especies y la respuesta del sistema antioxidante al hacer ejercicio, manteniendo a cada especie en la salinidad de su hábitat particular; dulceacuícola para *T. mitchelli* y *T. pearsei*, y salado para *T. dzilamensis*. Además, se exploraron las temperaturas críticas máxima y mínima, así como el impacto de estos procedimientos sobre el sistema antioxidante y el daño celular. Las especies mostraron

diferencias en características fisiológicas que coinciden con la ocupación del hábitat subterráneo dulceacuícola o salado. De las tres especies estudiadas, *T. dzilamensis*, presentó el mayor PMA, el mayor rango de temperaturas críticas y también el mayor cambio en los indicadores del sistema antioxidante. Los procedimientos experimentales en *T. mitchelli* y *T. pearsei*, en general tuvieron menor impacto tanto en el PMA como en los cambios observados en el sistema antioxidante. La comparación interespecífica indicó que *T. dzilamensis*, quien habita en agua salada subterránea, es la especie con mayor PMA y ventana térmica, con un sistema antioxidante reactivo y robusto, en tanto que el incremento metabólico no implicó daño celular por radicales libres. Estas características están relacionadas con una mayor plasticidad fenotípica, que a su vez está relacionada con mayor capacidad de reacción a variaciónes ambientales y resiliencia. Por ello, se sugiere que los cambios ambientales tendrían mayores impactos en aquellas especies con menor plasticidad en comparación con aquellas de mayor plasticidad.

General Abstract

Cave shrimp of the *Typhlatya* genus are cave restricted species – Stygobitic – which are found in fresh and marine groundwater, also known as subterranean estuaries. Because of their abundance, size and wide distribution throughout the Yucatan Peninsula, *T. mitchelli*, *T. pearsei* and *T. dzilamensis* are ideal biological models for ecologic and physiologic studies. Nevertheless, their distribution patterns, ecological aspects and physiology have not been studied and remain to be recognized.

This thesis is divided into two chapters, the first one is dedicated to understanding aspects of *Typhlatya* cave shrimp ecology and the second one to understanding their physiology. The first chapter describes the abundance of three *Typhlatya* species in different time and spatial scales, linking environmental variables and possible carbon sources as indicators of their food origin. The species composition and abundance varied markedly in space and time, showing differential patterns among species in accordance to a salinity gradient, revealing a series of microhabitats within the anchialine environment. The isotopic analysis showed that each species reflects a particular trace of $\delta^{13}C$ and $\Delta^{14}C$, suggesting each species feeds in different proportions from each of the available carbon sources within each microhabitat. Our findings suggest a niche partitioning among the three *Typhlatya* species in terms of habitat and feeding sources, where environmental variables and physiological differences could play an important role in the determination of such distribution patterns.

After recognizing the niche partitioning among these *Typhlatya* species, the second chapter characterizes the physiological capacities of each species within their native microhabitat. We evaluated the aerobic scope of the three species and the response of their anti-oxidant system to induced exercise, keeping each species at the salinity of their particular habitat; fresh groundwater for *T. mitchelli* and *T. pearsei*, and marine groundwater for *T. dzilamensis*. We also explored the critical thermal maximum and minimum of each species, as well as the impact of such procedures on their anti-oxidant system and cellular damage.

The *Typhlatya* species showed differences in their metabolic potential, which coincides with fresh or marine groundwater occupation. *T. dzilamensis*, which inhabits marine groundwater, was the species with the greatest aerobic scope, the widest critical temperature range, and

also showed the greatest response in anti-oxidant system. The experimental procedures in *T. mitchelli* and *T. pearsei* had generally a lower impact, both in the aerobic scope and the anti-oxidant system.

The interspecific comparison shows that *T. dzilamensis* is the species with the greatest aerobic scope, thermal window and has a reactive and robust anti-oxidant system because the metabolic increase did not cause any stress due to free oxygen radicals. These characteristics are related with a greater phenotypic plasticity, which in turn is related to a greater capacity to respond to environmental conditions and resilience. Thus, we suggest that environmental changes would have greater impacts on those species with less plasticity, as compared to those with greater plasticity.

Introducción general

Los acuíferos y sistemas anquihalinos de la Península de Yucatán (PY) albergan una biodiversidad única, fascinante y desconocida en muchos sentidos. La estigofauna (especies que desarrollan su ciclo de vida dentro de cuevas inundadas) de Yucatán está compuesta en su mayoría por crustáceos endémicos (Álvarez & Iliffe, 2008). Las características ambientales de estos ecosistemas demandan que sus habitantes posean un conjunto particular de adaptaciones que les permiten florecer en un ambiente característicamente oligotrófico, afótico y verticalmente estratificado por haloclinas. En particular, las especies del género *Typhlatya* que están presentes en los sistemas anquihalinos de la Península de Yucatán, son de las más frecuentes y ampliamente distribuidas (Álvarez et al., 2015; Angyal et al., 2020). A pesar de su ocurrencia y abundancia, algunos autores sostienen que estas especies no presentan patrones de distribución distinguibles (Benítez et al., 2019), lo que conduce a pensar que las *Typhlatya* se distribuyen al azar dentro de las cuevas. El primer capítulo de esta tesis estudia la abundancia de estas especies en diferentes regiones del sistema anquihalino, muestra las fuentes de alimentación que cada especie consume y finalmente, describe los patrones de comportamiento asociados con la presencia de luz. El conjunto de estos resultados indica una partición de nichos que permite la coexistencia de estas tres especies hermanas.

La ocupación del hábitat marino y dulceacuícola de estas especies levanta interrogantes sobre su origen evolutivo, su historia adaptativa y las características fisiológicas de cada una de ellas. Consecuentemente, el segundo capítulo versa sobre las características bioenergéticas de cada especie desde la perspectiva de la fisiología, buscando su relación con las diferencias ecológicas observadas en el primer capítulo. En este capítulo se caracterizó el campo aerobio y la respuesta del sistema antioxidante de *Typhlatya mitchelli*, *T. pearsei* y *T. dzilamensis* bajo las condiciones nativas de temperatura, salinidad y oxígeno disuelto presentes en el microhábitat particular de cada especie.

El conjunto de los resultados obtenidos, representa una caracterización ecofisiológica sin precedentes de este grupo de camarones estigobíticos. Se conjuntan características ecológicas y fisiológicas que probablemente son compartidas por otros crustáceos estigobíticos del

mundo, contribuyendo con ello a profundizar nuestro conocimiento sobre la evolución y la capacidad de adaptación de estas especies características de acuíferos costeros.

Artículo 1: Distribution patterns, carbon sources and niche partitioning in cave shrimps (Atyidae: Typhlatya).

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Distribution patterns, carbon sources and niche partitioning in cave shrimps (Atyidae: *Typhlatya*)

E. M. Chávez-Solís[1,2], C. Solís[3], N. Simões[2,4,5] & M. Mascaró[2,4]✉

Cave shrimps of the *Typhlatya* genus are common and widespread in fresh, brackish and marine groundwater throughout the Yucatan Peninsula (Mexico). These species are ideal models to test niche partitioning within sympatric species in oligotrophic systems. Nevertheless, their food sources remain unidentified, and despite their frequency and functional importance, distribution and abundance patterns of these species within caves have not been fully recognized. Here, we describe the abundance of three *Typhlatya* species in different temporal and spatial scales, investigate changes in water conditions, and potential sources of carbon as an indication of food origin. Species composition and abundance varied markedly in space and time revealing patterns that differed from one system to another and in relation to environmental parameters. Isotope analysis showed that each species reflects a particular $\delta^{13}C$ and $\Delta^{14}C$ fingerprint, suggesting they feed in different proportions from the available carbon sources. Overall, our findings suggest a niche partitioning of habitat and feeding sources amongst the three *Typhlatya* species investigated, where environmental characteristics and physiological differences could play an important role governing their distribution patterns.

The lack of photosynthesis in caves and the resulting limitation in food sources is one of the strongest selection pressures and drivers of evolution for life in caves[1]. Competition for nutrients in oligotrophic environments, such as anchialine ecosystems—defined as subterranean estuaries that extend inland to the limit of seawater penetration[2], certainly requires a unique set of specialization traits that allow for niche partitioning amongst stygobionts (aquatic species strictly bound to the subterranean habitat). A closer look at stygobiont biodiversity reveals that co-occurrence of sympatric species within the same system is rare but can be occasionally observed[3,4]. This work gathers evidence that suggests how three sympatric cave shrimp species that coexist in groundwater ecosystems have partitioned their niche to avoid competitive exclusion.

Groundwater in the anchialine systems of Yucatan is vertically stratified by the intrusion of marine water from the coast which inserts below the freshwater that constantly flows towards the coast[2]. This marked halocline, along with changes in temperature, dissolved oxygen, pH and redox potential has been considered a physicochemical barrier that separates freshwater and saline communities[5,6]. This stratification is expected in coastal systems, and as distance from the coast increases, the marine intrusion is found deeper and only a few deep cenotes inland may reach the saline layers[7].

In terms of energy production and food webs, the sun-influenced *cenote pools*—cenote is a Mayan derived name for local sinkholes—are the only sites where photosynthesis can take place and allochthonous organic matter can enter the system. Therefore, these ecosystems have been historically regarded as dependent of

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allochthonous input. However, recent investigations have changed the oligotrophic perspective of the aphotic cave passages and recognize chemosynthesis by microbes in caves[8], suggesting a vital role to the stygobitic trophic structure.

Stable isotope analysis has been used to track the origin, flow and transformations of energy through trophic webs[9–11]. We used the isotope fractionation principle to identify carbon sources in groundwater and relate them to the carbon composition in *Typhlatya* species collected from fresh and marine groundwater habitats. Stable carbon ($\delta^{13}C$) signatures from terrestrial and aquatic photosynthesis, however, may overlap and preclude the discrimination of carbon sources[12].

Plants maintain a constant radiocarbon content as they incorporate carbon from the atmosphere. When they die, ^{14}C begins to decay at a rate governed by a half-life of 5,730 years[13,14]. While "modern" radiocarbon content (atmospheric or recently incorporated through photosynthesis) in a sample is similar to atmospheric concentration, complex soil dynamics can hold chemically labile organic carbon that, despite its age (lower ^{14}C content than modern carbon), may still be available for microbial catabolism[12]. Dissolved inorganic carbon (DIC)—carbon dioxide (CO_2) and carbonates (CO_3)—in karstic groundwater habitats is most likely to be derived by the weathering of the calcareous limestone, which is ^{14}C depleted (considered "radiocarbon-dead carbon")[12]. This leads to a contrasting radiocarbon content in autotrophs that incorporate DIC (i.e. algae and chemosynthesis by microbes) in contrast with plants incorporating atmospheric CO_2, which will be reflected in their consumers. Therefore, the use of radiocarbon together with stable isotopes in food sources and consumers can shed light on the structure of food webs. Species feeding on different sources are likely to have contrasting stable isotopes or radiocarbon content, a condition that may help explain the ecological niche and distribution patterns of *Typhlatya* populations in groundwater ecosystems.

In this work we used the *Typhlatya* genus as a biological model to study distribution patterns within caves and feeding sources which are available in the groundwater. *Typhlatya* species are some of the most common and widespread stygobionts in the Yucatan Peninsula. They have been observed in fresh, brackish and marine groundwater, both in coastal and inland systems[3,15]. They are key in the energy transfer to higher trophic levels as they graze or filter feed on small particles or microbial mats[10], and have been observed in cenote pools and aphotic cave passages where the absence of light precludes photosynthesis. They are sufficiently large to be conspicuous and may be identified underwater by the unaided eye. Notwithstanding their frequent occurrence and their trophic importance, patterns in their distribution within caves and factors associated have not been fully recognized.

The aims of this work were to describe the distribution and abundance patterns of *Typhlatya mitchelli, T. pearsei* and *T. dzilamensis* in two spatial scales (depth and geography of the Yucatan Peninsula) and two temporal scales (daily and seasonal). The purpose was to correlate these features with changes in physical and chemical water conditions, as well as potential sources of carbon as an indication of food origin. In addition, we aimed to test if these species had daily movements within sunlight influenced regions and whether meteorological seasons influenced their populations. Linking distribution patterns and feeding sources with environmental data will help understand the community structure, functional role and niche partitioning of ecologically similar sympatric species of this widespread *Typhlatya* genus in Yucatan.

Results

Tza Itza, Nohmozon and Kankirixche are all freshwater systems, whereas Ponderosa is a vertically stratified anchialine system with the presence of a halocline (Fig. 1). Parameters in Tza Itza and Nohmozon were constant throughout the water column and differed among systems. Overall, temperature was highest in Tza Itza, followed by Nohmozon and finally, Ponderosa had the lowest thermal values. Dissolved oxygen was highest in Nohmozon and was similar in Ponderosa and Tza Itza. Observed pH was greatest in Nohmozon, with values close to 10, whilst Tza Itza and Ponderosa had values closer to 9. Ponderosa was vertically stratified in all parameters with a marked cline around 13 m of depth (Fig. 1). Salinity increased slowly from 3 psu at the surface to 6 psu at 13 m, and raised abruptly to 36 psu at 14 m, resulting in a marked halocline. Temperature was slightly under 25 °C at the surface and showed an abrupt increase of 1 °C across the halocline. Dissolved oxygen increased at the upper limit of the halocline from 3.4 to 4 mg/l and decreased in the saline layer to 2.8 mg/l. Finally, pH changed from the freshwater to the saline layer, by first dropping slightly then increasing from 9.2 to 9.4.

Spatial distribution of *Typhlatya* species within anchialine systems. The abundance and presence of *Typhlatya* species changed between hydro-regions in a way that differed from one system to another (Fig. 2A, B), as indicated by the significant interaction term in Table 1 (Sy × Hr). *Typhlatya mitchelli* was present in all three systems, but was most abundant in the cavern of Tza Itza. Here, its abundance decreased in the cave and was rarely found in the cenote pool. In Nohmozon and Ponderosa, *T. mitchelli* was consistently observed in relatively low abundance both in the cavern and in the cenote pool (Fig. 2A). This suggests *T. mitchelli* is mostly distributed in the cavern and cave as close to the cenote pool, but avoiding it (Fig. 2B). *Typhlatya pearsei* was only observed at night. This species was primarily found in Nohmozon, it occurred only once in Ponderosa and was never registered in Tza Itza. The highest abundance of all species registered in this study corresponded to *T. pearsei* in the cenote pool of Nohmozon, and decreased in numbers through the cavern and cave (Fig. 2A). *Typhlatya dzilamensis* was found mostly under the halocline in the cave of Ponderosa (Fig. 2B). It was only rarely observed in the cavern, and never observed in the cenote pool (Fig. 2A). *Typhlatya dzilamensis* was also the species with the lowest abundance of all three species in this study.

Species composition did not vary markedly from one season to another (Table 1) and spatial differences in the abundance of all three *Typhlatya*, both between hydro-regions and systems were remarkably constant throughout

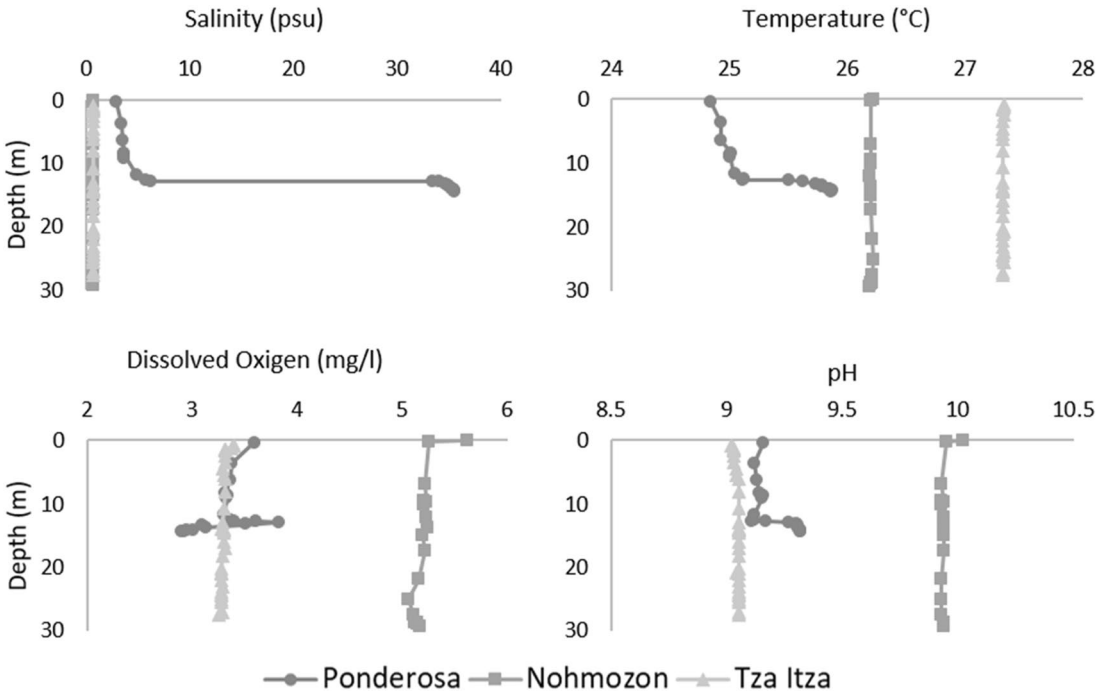

Figure 1. Vertical profile of physical and chemical hydrological parameters of Ponderosa Nohmozon and Tza Itza Systems. Data obtained using a Hydrolab DataSonde5.

the year (Se, Sy × Se, Hr × Se; Table 1). Because the random factor "observer" was nested within the combinations of all three fixed factors, results of the permutational multivariate ANOVA are fully interpretable (Obs; Table 1).

Typhlatya species composition was unique in each hydro-region at each system, as further confirmed by pair-wise t-test comparing hydro-regions within each system which showed consistent statistical differences (Table 2 and Fig. 2).

Carbon tracing in *Typhlatya* tissue. The Carbon (C) and Nitrogen (N) percent in tissue composition of all three *Typhlatya* species was similar and ranged from 48 to 63.6% and 3.6 to 12.4% for C and N, respectively (Supplementary Figure SF1). *Typhlatya mitchelli* showed a consistently lower C/N mean ratio of 4.7 (± 0.6) in comparison to *T. pearsei* and *T. dzilamensis*, which resulted in 9.8 (± 6.2) and 8.2 (± 2.4) respectively.

A broad range of accelerator mass spectrometry (AMS)-$\delta^{13}C$ and $\Delta^{14}C$ values were obtained from the biomass of *Typhlatya* species. *Typhlatya mitchelli* from Tza Itza (n = 6) had $\delta^{13}C$ values ranging from – 23.0 ± 2 to – 26.3 ± 1.4‰. *Typhlatya pearsei* from Nohmozon showed lower values ranging from $\delta^{13}C$ – 35.0 ± 1 to – 41.0 ± 1‰ (n = 3) (Fig. 3). Similarly, *T. dzilamensis* collected from the saline groundwater layer in Ponderosa showed $\delta^{13}C$ values that varied from – 31.0 ± 0.3 to – 44.0 ± 0.6‰ (n = 4) (Fig. 3). Values of $\delta^{13}C$ obtained via AMS were always within the ranges obtained using IRMS $\delta^{13}C$, hence were used as indicators in the analysis of food sources in *Typhlatya* biomass contribution (Supplementary Table ST1).

The $\delta^{13}C$ in dissolved inorganic carbon (DIC) in fresh groundwater (FGW) ranged from – 16.1‰ (± 0.8) in a sample taken at Nohmozon to an average – 10.8‰ (± 0.1) at Tza Itza and – 10.3‰ (± 2.5) at Ponderosa. $\delta^{13}C$ in DIC in saline groundwater (SGW) taken from Ponderosa showed average value of – 11‰ (± 3.1) (Fig. 3).

Typhlatya mitchelli from Tza Itza showed $\Delta^{14}C$ values ranging from – 3.5 to 19.2‰ (percentage of modern carbon (pMC) of 99.0 ± 0.3 to 103.0 ± 0.3%) corresponding to a modern age. *Typhlatya pearsei* collected in FGW at Nohmozon had $\Delta^{14}C$ values from – 202 to – 166‰ (pMC of 80.4 ± 0.3% to 91.6 ± 0.3%), equivalent to an apparent age of – 1,750 to – 1,400 years before present (yBP). *Typhlatya dzilamensis* collected in SGW at Ponderosa showed $\Delta^{14}C$ values from – 91.5 to – 135.5‰ (pMC of 91.6 ± 0.3% to 87.1 ± 0.3%), equivalent to a range of 700 to 1,100 yBP (Fig. 3). These results indicate there is a difference in $\Delta^{14}C$ trace corresponding to each of the observed species. While *T. mitchelli* incorporates carbon from a modern source, *T. pearsei* and *T. dzilamensis* are partially incorporating carbon from an old source.

DIC in FGW of Tza Itza showed an average of $\Delta^{14}C$ = – 203‰ (pMC of 82.2 ± 0.3%), corresponding to an apparent age of 1759 yBP. DIC in FGW in the cenote pool of Nohmozon had a $\Delta^{14}C$ of – 247‰ (pMC of 75.9 ± 0.3), corresponding to an apparent age of 2,213 yBP. DIC in FGW of Ponderosa had a $\Delta^{14}C$ of – 185‰, corresponding to an apparent age of 1576 yBP, while SGW had $\Delta^{14}C$ of – 260‰ (pMC of 74.7 ± 0.3), with an apparent age of 2,765 yBP (Fig. 3). Despite the fact that *T. mitchelli* inhabits groundwater that is ^{14}C depleted in Tza Itza, it has a modern $\Delta^{14}C$ trace, suggesting it is feeding on a modern source. *Typhlatya pearsei* and *T. dzilamensis* show older apparent ages, which are closer to the $\Delta^{14}C$ of DIC of their specific habitat, suggesting that they are feeding on sources that can uptake DIC available in groundwater.

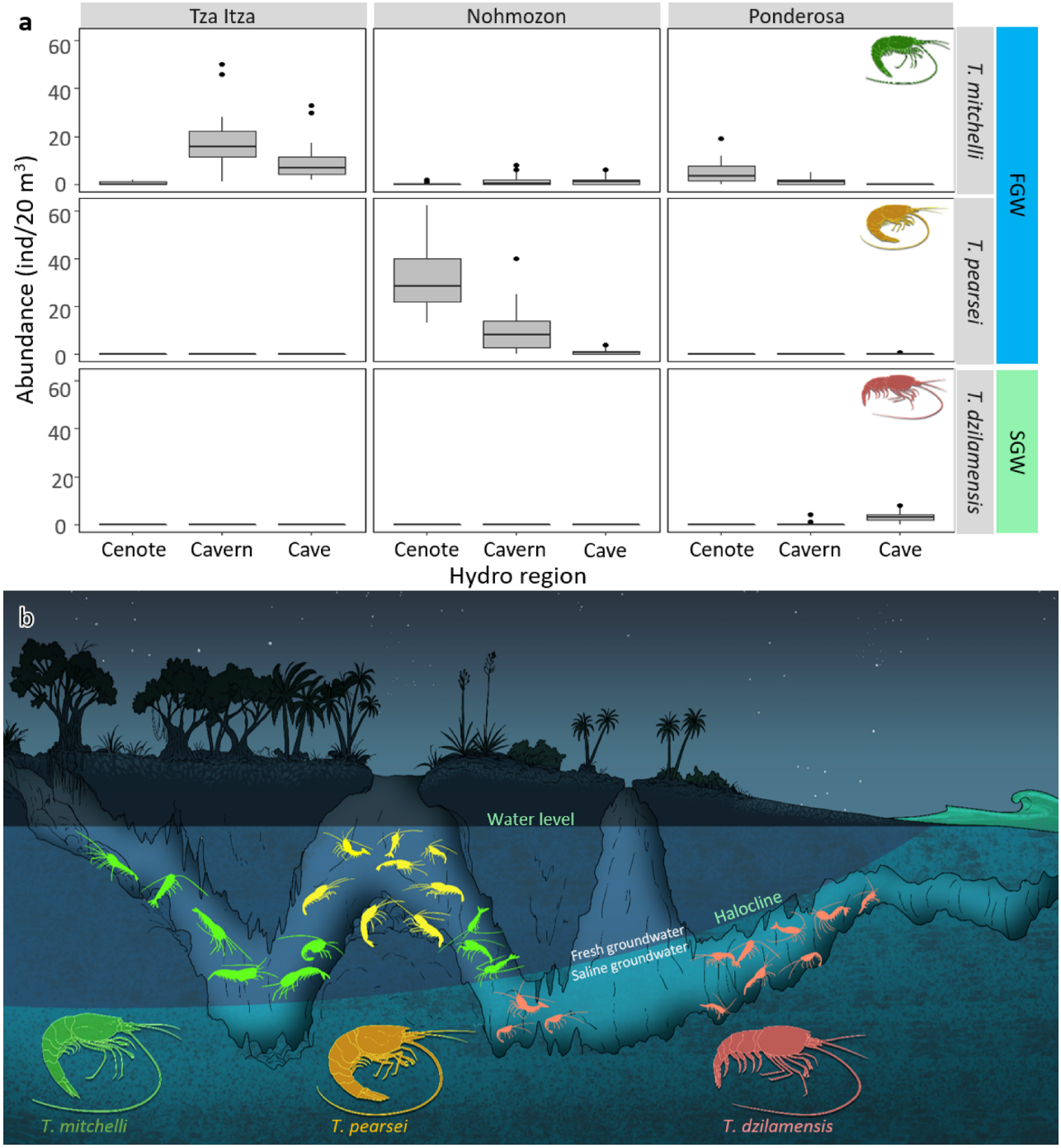

Figure 2. *Typhlatya* hydro region distribution in Yucatan underground submerged systems. (**A**) Amount of observed individuals per transect of 20 m^3 at the cenote pool, cavern and cave hydro regions of Nohmozon, Tza Itza and Ponderosa systems. The boxplots indicate the first and third quartile of the data, the black line indicates the median and the whiskers extend to the most extreme data which is no further than 2 standard deviations. (**B**) Visualization of species distribution in a theoretical continuous underground system, showing the freshwater cenotes farther from the coast and the saline intrusion being shallower as closer to the coast. Artwork by Alberto Guerra.

Mixing models. Biomass values of $\delta^{13}C$ and $\Delta^{14}C$, as well as values published for potential sources were incorporated into our mixing model under two scenarios. Organic matter (OM) was established as $\delta^{13}C_{OM} = -25.3‰$ and $\Delta^{14}C_{OM} = -4.3‰$, obtained from the average values of *T. mitchelli* in Tza Itza, which represent the closest value to the photosynthetic and modern organic matter from terrestrial and aquatic sources measured in this study (Table 3). The oldest DIC measured in this study was from the saline groundwater of Ponderosa, thus, our second scenario was modelled with an ancient methane of $\Delta^{14}C_{AM2} = -260‰$ (Table 3).

Source	df	SS	MS	Pseudo-F	*p*	Unique permutations
System (Sy)	2	99,424	49,712	96.7	0.0001***	9,944
Hydro region (Hr)	2	21,191	10,596	20.6	0.0001***	9,944
Season (Se)	2	2,110	1,055	2.2	0.0822	9,959
Sy × Hr	4	89,856	22,464	43.8	0.0001***	9,933
Sy × Se	4	2,519	630	1.4	0.2359	9,947
Hr × Se	4	1858	464	1.0	0.4174	9,959
Sy × Hr × Se	8	1723	215	0.5	0.8665	9,946
Obs (Sy × Hr × Se)	38	15,310	403	1.7	0.0037***	9,857
Residuals	169	40,086	237			
Total	233	274,000				

Table 1. Permutational Multivariate ANOVA (PERMANOVA) results on distribution data of *Typhlatya* species within underground systems of Yucatan. Sources are: System (Sy), Hydro region (Hr), Season (Se) and Observer (Obs). Asterisks refer to the probability of that *p* value. ***$p < 0.001$.

	Cenote pool	Cavern
Tza Itza		
Cavern	15.1***	
Cave	13.8***	2.9*
Nohmozon		
Cavern	2.4**	
Cave	6.7***	2.6**
Ponderosa		
Cavern	3.0*	
Cave	10.5***	7.6***

Table 2. Pair-wise t-tests for system and hydro region terms with each factor level. t-values contrasting each hydro region within the same cenote are provided. Symbols refer to the probability of t-values. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

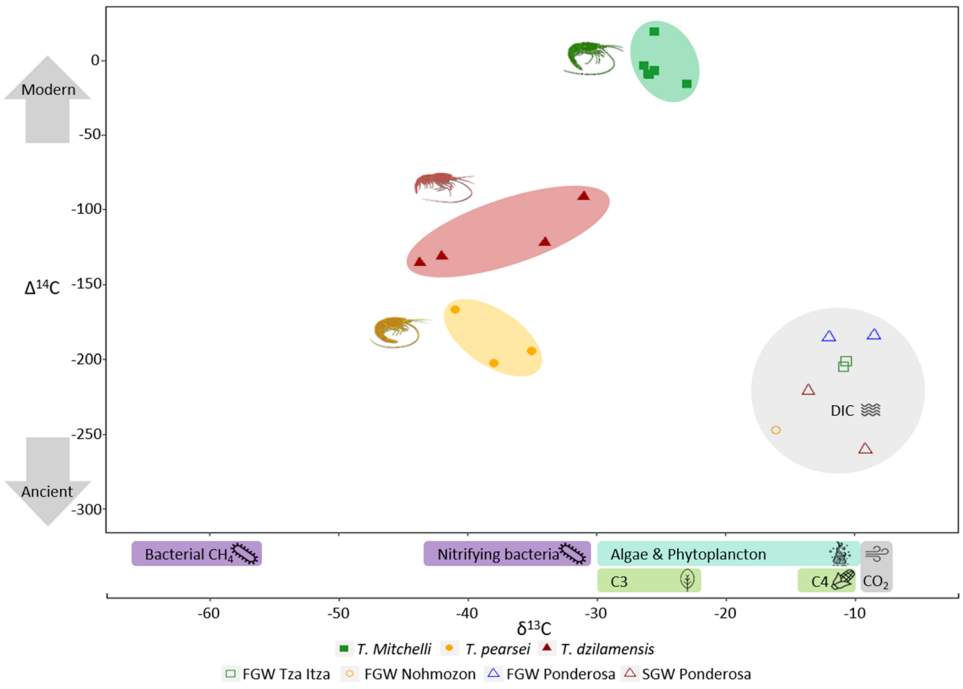

Figure 3. Carbon isotopic analysis indicating the $\Delta^{14}C$ and $\delta^{13}C$ bulk composition of *T. mitchelli* (sampled in Tza Itza), *T. pearsei* (sampled in Nohmozon) and *T. dzilamensis* (sampled in Ponderosa), and DIC contained in fresh groundwater (FGW) and saline groundwater (SGW), accordingly. $\delta^{13}C$ values of potential carbon sources are indicated as ranges (source data from[8,11,16,17]). Artwork by Alberto Guerra.

	Scenario	1	2
	$\delta^{13}C$ (‰)	$\Delta^{14}C$ (‰)	$\Delta^{14}C$ (‰)
OM	−25.4	−4.3	−4.3
MM	−66.3	0	0
AM	−56.3	−1,000	−260

Table 3. Two mixing model scenarios considering accelerator mass spectrometry (AMS) $\delta^{13}C$ and $\Delta^{14}C$ of modern organic matter (OM), modern methane (MM) and ancient methane (AM) as endmember potential sources that contribute to *Typhlatya* diet affecting their tissue composition. $\delta^{13}C$ remains constant in all scenarios, whilst AM was assigned as ^{14}C-carbon dead in the first scenario and the oldest $\Delta^{14}C$ obtained in this study for the second scenario.

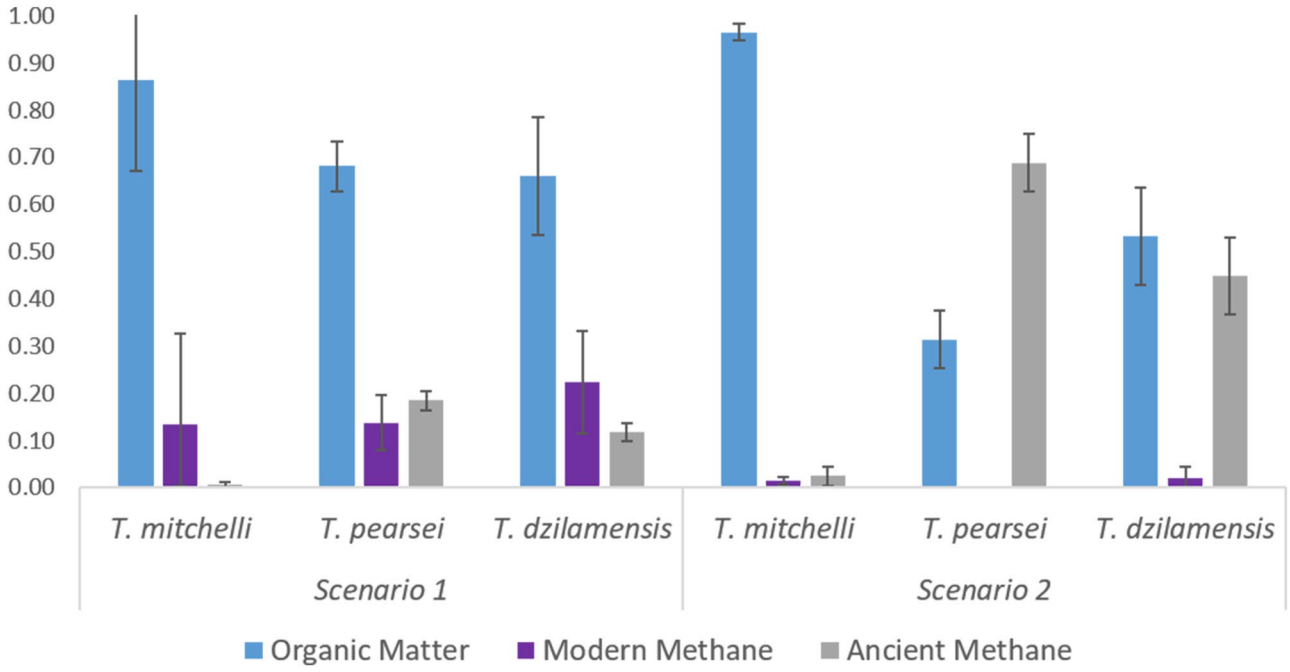

Figure 4. Relative contribution to the carbon in *Typhlatya* species as an average percentage of their carbon uptake in two mixing model scenarios, considering the $\Delta^{14}C$ and $\delta^{13}C$ of three potential feeding sources: organic matter (OM), modern methane (MM) and ancient methane (AM). Scenario 1 considered AM as ^{14}C-dead carbon ($\Delta^{14}C_{AM2} = -1{,}000$‰); scenario 2 considered AM as $\Delta^{14}C = -260$‰ corresponding to the DIC measured in the saline layer of Ponderosa cave.

Under the first scenario (Fig. 4), the OM was the greatest carbon source for all three species: 88–98% for *T. mitchelli*, 66–74% for *T. pearsei* and 53–79% for *T. dzilamensis*. Modern methane (MM) contributed 1–11% of carbon to *T. mitchelli*, 7–18% to *T. pearsei* and 12–34% in *T. dzilamensis*. Ancient methane (AM) contributed with 0–1% of carbon to *T. mitchelli,* 16–20% in *T. pearsei* and 9–13% in *T. dzilamensis* (Fig. 4). The second scenario shows that OM in this scenario was again the greatest source of carbon for *T. mitchelli* (93–98%), but for *T. dzilamensis* (44–67%) and *T. pearsei* (26–38%) this source was greatly reduced compared to the first scenario. MM contributes marginally to the carbon sources in *T. mitchelli* and *T. dzilamensis* with 1–3% and 0–5% respectively, and shows no contribution to the biomass of *T. pearsei*. AM would contribute with 0–6% of carbon to *T. mitchelli*, as much as 62–74% to *T. pearsei* and 33–51% of carbon to *T. dzilamensis* (Fig. 4).

Diel vertical distribution of *T. mitchelli*. The vertical distribution of *T. mitchelli* in the water column was determined by the presence of light and differed significantly between the two studied systems: Tza Itza and Kankirixche ($F = 6.06$; $p < 0.001$; Fig. 5). Overall, the frequency of occurrence of *T. mitchelli* in transects generally decreased as depth and distance from the cenote pool increased, with higher numbers closer to the cenote pool and lower numbers at the greatest depth of the caverns. A noteworthy exception, however, was the low occurrence of *T. mitchelli* in the shallowest transects (i.e. those immediately below the surface of the cenote pool) in both Tza Itza and Kankirixche.

Day/night differences in shrimp abundance were evident in shallow transects, where light was most intense, but were imperceptible in deep cavern transects where light was almost absent throughout a 24 h period (Fig. 5). In Kankirixche, the first two transects were markedly affected by day-night cycles, whereas in Tza Itza these daily differences were only observed in the first transect of the cenote pool (Fig. 5). Individuals were rarely observed

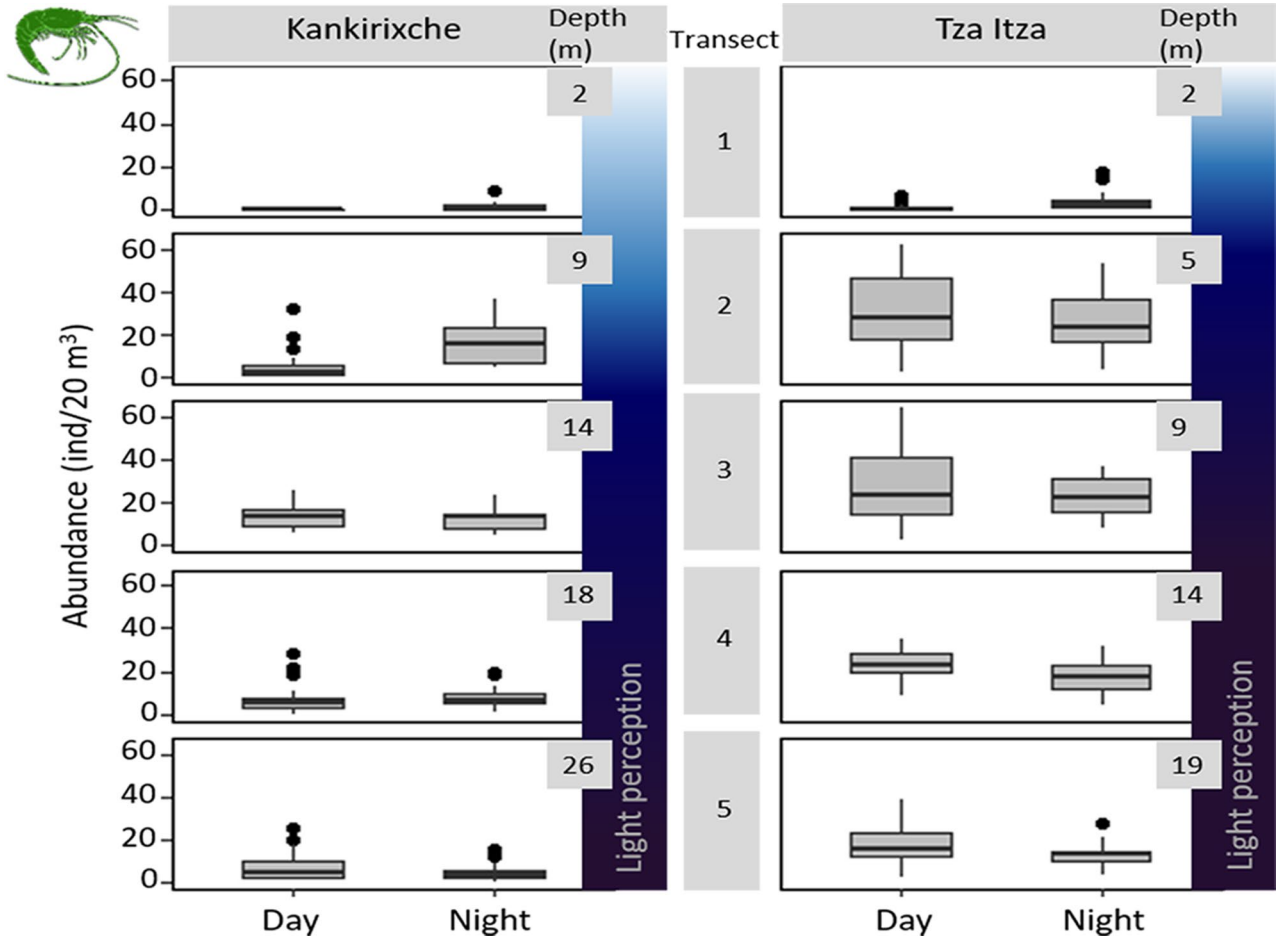

Figure 5. Distribution patterns of *T. mitchelli* through a depth gradient in day and night observations at Kankirixche and Tza Itza. Transect depth is shown in meters and relative to increasing transect number; transect 1 (under cenote pool) to 5 (end of cavern closest to cave). A light perception bar is drawn to represent the approximate depth at which light reaches in either cavern. At each transect abundance was registered during day and night in each system. The boxplots indicate the first and third quartile of the data, the black line shows the median and the whiskers extend to the most extreme data which is no further than 2 standard deviations, data beyond whiskers is shown as black dots. Artwork by Alberto Guerra.

during day observations in shallow transects of both cenotes, but numbers increased during the night, clearly indicating a preference for dark environments (Fig. 5).

Both the total abundance and vertical distribution of *T. mitchelli* varied through the year ($F = 5.71$; $p < 0.001$) and did so in a similar fashion in Tza Itza and Kankirixche ($F = 2.93$; $p = 0.087$). Total abundance of *T. mitchelli* was highest during the months of August, October and February. These months correspond to the rainy season and the end of the winter season, where precipitation is highest in the Yucatan Peninsula. April (dry season), by contrast, had the lowest overall abundance (Fig. 6).

The vertical distribution from August to December showed a consistent pattern of high abundance in the cavern nearest to the cenote pool, decreasing as depth increased (Fig. 6). This pattern, however, started to flatten during February (end of winter) and was much less evident from April to June, when shrimp were more uniformly distributed throughout all but the shallowest transects (Fig. 6).

Discussion

Typhlatya species are found throughout marine and fresh groundwater habitats and are some of the most abundant and widespread stygofauna component in the anchialine ecosystems of the Yucatan Peninsula[3,15,18–20]. In contrast with previous authors suggesting that anchialine fauna has no distribution patterns within underwater caves[21], results herein show differently. Shrimp abundance varied markedly in space and the resulting patterns differed from one system to another depending on topographic features, solar influence and geographical position (Fig. 2B). In addition, we found both diel and seasonal variations in the vertical distribution and abundance of *T. mitchelli*, one of the most common species in the study area[3,15]. Furthermore, carbon source analysis show a distinct feeding pattern across *Typhlatya* species. Overall, our observations on three *Typhlatya* species in four groundwater systems of Yucatan portray a niche partitioning where salinity appears to play a fundamental role in separating the realized niche of *T. dzilamensis* from that of *T. mitchelli* and *T. pearsei*, whilst solar influence, food

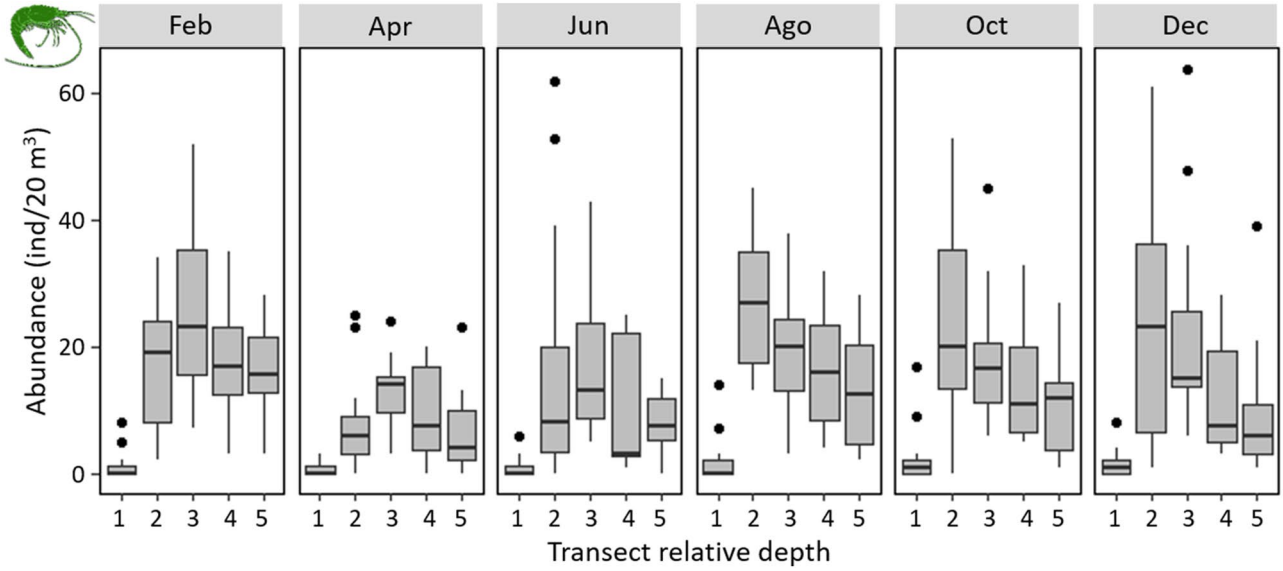

Figure 6. Depth distribution patterns of *T. mitchelli* throughout the year in Tza Itza and Kankirixche. Transect depth is shown as relative to each other and not as actual depth: transects 1 were at the cenote pool, transects 2–5 were distributed in an increasing depth gradient throughout the caverns of Tza Itza and Kankirixche systems. Boxplots indicate the first and third quartile of the data, the black line is the median and the whiskers extend to the most extreme data which is no further than 2 standard deviations, black dots represent counts which were beyond 2 standard deviations. Artwork by Alberto Guerra.

selection, space, and diel behavior would partitions the later species´ ecological niche. The upcoming discussion focuses on the biological processes that could explain the patterns identified in the present study.

One of the most outstanding characteristics of the karstic anchialine ecosystems in Yucatan is the vertical stratification of water masses, where the marine intrusion from the coast infiltrates under the meteoric water that infiltrates underground, resulting in clearly defined layers with marked salinity changes. Salinity gradients in aquatic habitats are considered one of the most important limiting factors of species distributions[22]. Changes in environmental salinity may impose severe physiological stress[23]. Therefore, salinity commonly governs geographical distributions, adaptive radiations, speciation and physiology[22,24]. Whilst crustaceans are known to have originated in the ocean, atyid shrimps have a long history since the colonization of freshwater environments and their systematics have revealed frequent cave invasions[4,25,26]. Studies on *Halocaridina rubra* from anchialine ponds in Hawaii, which are subject to marked daily fluctuations in environmental salinity, have shown these shrimp maintain constitutively activated mechanisms of ion regulation and high cellular osmoregulation in the gills regardless of salinity[24]. *Typhlatya*, as an anchialine cave restricted genus[25,] must have developed a series of adaptations enabling their survival in coastal caves. Furthermore, speciation in Yucatan's *Typhlatya* must have resulted in physiological, biochemical and genetic adaptations that derived in a different tolerance to salinity between closely related species and the colonization of subterranean habitats which are heterogeneous in depth and distance from the coast. Our results show that these shrimps are distributed according to salinity: *T. mitchelli* was found in all sites exclusively in the freshwater layer, *T. pearsei* was only observed in the fresh water at Nohmozon, and *T. dzilamensis* was only found in saline water at Ponderosa. The distribution patterns of *Typhlatya* species observed in this study constitute initial evidence in support of a physiological differentiation among species. Future research in adaptive physiology in response to salinity is key to reveal the osmoregulatory mechanisms and bioenergetics that will further explain the habitat selection (or limits) of these anchialine species (Chávez-Solís et al. *in prep*.).

It is noteworthy that *T. pearsei* was most abundant in the system with the highest dissolved oxygen in our study (Fig. 1). Perhaps, *T. pearsei* is more sensitive to hypoxia than the rest of its congeners. If this hypothesis is true, future studies should demonstrate a reduced metabolic capacity of *T. pearsei* under hypoxia, whereas *T. mitchelli* and *T. dzilamensis* should be comparatively less affected. Moreover, *T. mitchelli* should present a higher physiological performance, enabling to outcompete *T. pearsei* in hypoxic freshwater environments. Correspondingly *T. pearsei* should outcompete *T. mitchelli* under normoxia. Research on tolerance to hypoxia in stygobionts could test these predictions and provide a deeper understanding of the distribution patterns and adaptation mechanisms to dissolved oxygen variations in caves.

Despite the importance of oxygen and salinity determining the distribution patterns of many crustacean populations[22,27], it does not explain the vertical and horizontal distribution of *Typhlatya* species in systems without a halocline. Abundance differences of *T. mitchelli* with depth in both Tza Itza and Kankirixche (Fig. 5) suggest that other explanatory variables are involved. Whilst light would appear to be a poor candidate explaining the distribution of blind stygobionts, negative phototaxis, as suggested by the results in the present study, has also been observed in anophthalmic stygobiont beetles *Paroster macrosturtensis* from Australian calcrete aquifers[28]. Evidence of this behavior is supported by observations of *T. pearsei* in the cenote pool at Nohmozon occurring only at night. In addition, transects nearest to the surface in Tza Itza and Kankirixche (i.e. those where sunlight

had its greatest influence) consistently had low occurrence of *T. mitchelli*, particularly during day observations (Fig. 5). Furthermore, day/night differences in the abundance of *T. mitchelli* in Tza Itza and Nohmozon were limited to the shallow transects, were light influence was strongest. Differences in the way direct sunlight enters and reaches the water surface at Kankirixche compared to Tza Itza, together with the negative phototaxis, could account for the variations in the daily patterns of *T. mitchelli* observed between these two systems. Measuring traces of light in a cavern with commercial instruments can be challenging. Mejía-Ortiz et al.[29] implemented an elegant solution by using long exposure photographs to show trace light at different depths in a dry cavern. Automated light quantification, however, is needed to determine whether light intensity triggers diel behavior in *Typhlatya*.

Diel migrations and nocturnal activity as that observed in this study have been previously reported in other blind stygobitic crustacean species, such as *Creaseria morleyi*[30,31], *Halocaridina rubra*[32], and *Hadenoecus subterraneous*[33]. Species restricted to caves are generally characterized by the reduction of visual structures and are part of the common troglomorphic features observed in stygobionts. Although some vestigial eye structures are observable in *Typhlatya*, no sign of visual function or pigments are evident, suggesting these species could be grouped as microphthalmic, or even anophthalmic (sensu Friedrich[33]). Whilst the assumption of anophthalmy—defined as "the lack of eyes at any stage of the life cycle and across populations"—is based on the absence of peripherally observable eyes, it may overlook vestiges of internalized visual organs and does not exclude the existence of other extra-retinal photoreceptors[33]. The negative phototactic behavior as a probable cause for the diel migrations described in the present study must find support in a mechanism of light detection amongst *Typhlatya* or closely related species. If these shrimps are still capable of perceiving light despite their visual reduction, then the way light reaches the water column will have a relevant role in keeping the circadian clock tuned, hence activity confined exclusively to dark hours night. Our observations also suggest that populations inhabiting the aphotic cave hydro-regions are present regardless of the time of day or night, as in *T. dzilamensis*. The constancy of biotic and abiotic parameters in this region may prevent the synchronization of the biological clocks of stygobionts[33]. The lack of a synchronizer in a cave population could result in a shift of their circadian rhythm producing an unsynchronized circadian rhythm among the population, an arrhythmic biological clock[33,] or a reduction of sleep duration[34]. Our recurrent observations of *T. dzilamensis* in caves at any given time of day or night is consistent with any of these scenarios. Research on the anatomy of the eye, the nervous system, photoreceptors, biological clocks and genetic expression in *Typhlatya* is needed to further explain the differences in behavior and activity patterns observed both among and within these species.

A possible contributing factor to *Typhlatya* diel behavior in the cenote pools and light influenced caverns could be related to the presence of epigean and stygobitic predators that may also influence the distribution and size of prey populations. Predation has been shown to modify prey behavior by inducing vertical migration patterns or forcing prey to retrieve to refuges during light periods[32,35]. Predators in cenote pools include a diverse array of freshwater fish[36] and other stygobionts, such as *Ophisternon infernale*, *Typhlias pearsei*, *Creaseria morleyi* and the stygofile *Rhamdia guatemalensis*, all of which were recorded during night observations in the present study.

Habitat preference in the underground ecosystems is certainly linked to a number of ecological tradeoffs. A balancing component for blind prey living in the sun influenced hydro regions (thus an easy prey) could be the access to recently deposited plant debris rich in nutrients, or algae which are high in nitrogen content and easier to assimilate than plants[10,37]. This could be selecting *T. mitchelli* and *T. pearsei* to remain close to the cenote pools.

Advantages of shallow waters could also be a greater amount of dissolved oxygen and other organic inputs that are unlikely to reach the cave passages. If cenote pools are the only place in anchialine systems where photosynthesis takes place and constitute sinkholes for allochthonous input, then these hydro regions represent a nutrient attraction for cave primary consumers. Stygobionts in this trophic level would increase in density at cenote pools, further attracting epigean and hypogean predators. Results in this direction would suggest that cenote pools are "feeding hotspots" for all species in the heterotrophic anchialine ecosystem. If, on the other hand, photosynthetic and allochthonous nutrient input is scarce or absent in the cenote pool or represents a decimating risk due to visual predators, then *Typhlatya* must find food in the oligotrophic caves. If the aphotic dwelling *T. dzilamensis* deep inside caves has developed a strategy to incorporate in situ production sources (such as chemosynthesis or methane derived biomass) to their diet, then most individuals would keep away from the busy photosynthetic *hot-spots*.

Caves are considered oligotrophic because of a severe and almost constant scarcity of food. Additionally, bacterial mats have been suggested to yield lower energy transfer than that of photosynthesis[38]. Even so, a trade-off in energy transfer versus the risk of predation can be recognized. In either scenario, the anchialine ecosystems would appear to have a bottom-up control trophic structure, where the availability of autotrophs governs the abundance and distribution of the community. Our results show a greater abundance in hydro-regions linked to the surface (namely cenote pools and caverns), while cave populations were the least abundant throughout this study. Stable isotopes and radiocarbon analysis would link the distribution patterns herein observed with the available feeding sources and the importance of these sources to each of the species.

Metabolic pathways in autotrophs have different isotopic fractionation rates—a differential uptake of isotopes—which create specific carbon-isotope fingerprints[11]. $\delta^{13}C$ values of consumers reflect those of their feeding sources and will be passed on to higher trophic levels, enabling the reconstruction of food webs[9–11]. The wide range of $\delta^{13}C$ and $\Delta^{14}C$ values observed in *Typhlatya* species collected from fresh groundwater (FGW) and saline groundwater (SGW) environments suggests a mixed contribution of photosynthetic and chemosynthetic derived matter, as well as modern and ancient carbon contributing to their biomass. Nevertheless, our results show each species has a specific carbon composition indicating a differential food proportion from each of the available sources.

The range of C/N ratios of < 10 indicates that these species primarily obtain their carbon from algae derived organic matter (OM), rather than terrestrial plant derived OM[39]. To our knowledge, this ratio has not been evaluated for bacterial mats or methane oxidizing bacteria. C/N ratios indicate *Typhlatya* feeds mainly on algae or aquatic derived matter, but to further understand the differences and precise nature of their feeding sources, stable isotopes and radiocarbon analysis were implemented.

Bulk $\delta^{13}C$ in *T. mitchelli* distributed in the cavern show values similar to those of photosynthetically derived OM (i.e. algae, phytoplankton and C3 plants). The old apparent age of dissolved inorganic carbon (DIC) in fresh groundwater (FGW) in Tza Itza, contrasts with the modern carbon age of *T. mitchelli* collected from this system. This suggests that the carbon source of *T. mitchelli* in Tza Itza is not using ancient DIC from the dissolution of the limestone, but rather a modern source of carbon, most likely obtained through the assimilation of modern CO_2 by freshwater algae. Precipitation and gas diffusion at the water surface could fuel the algae and phytoplankton production with modern CO_2 and result in a modern-aged carbon assimilation by the *T. mitchelli* which fed on such sources. Furthermore, the mixing model based on stable isotopes and radiocarbon suggests that *T. mitchelli* incorporates the vast majority of its carbon from modern OM (Fig. 4).

Seasonal variations in the abundance and vertical distribution of *T. mitchelli* in Tza Itza and Kankirixche (Fig. 6) are in accordance with the rainy seasons, suggesting a strong dependence on external OM inputs. Overall, the vertical distribution of *T. mitchelli*, decreases in depth as probably allochthonous input of OM is not carried into the deepest region of the system. Modern OM is a carbon source that is most available during the rainy season, when modern CO_2 and allochthonous sediment inputs increase in the cenote pools. Seasonal shifts of allochthonous inputs have been suggested to modify dietary selection in stygobiont beetles which showed a marked difference between dry and rainy seasons[40]. Considering that more than 90% of *T. mitchelli* diet derives from a photosynthetic origin, a reduction in allochthonous input or photosynthetic in situ production could represent a reduction of food availability, resulting in the decline of its population size or a shift in its distribution, as observed in Kankirixche and Tza Itza, where the population was smallest and mainly distributed at deeper transects during dry months (Fig. 6). These hypotheses are in accordance with our stable- and radiocarbon isotope analyses, which show this species is predominantly feeding from modern and photosynthetically derived sources associated to the surface and, their distribution is predominantly higher in the caverns, closest to the cenote pools.

Bulk $\delta^{13}C$ of *T. dzilamensis* (collected from the cave in saline groundwater of Ponderosa) and that of *T. pearsei* (collected in FGW from the cenote pool of Nohmozon), reflect extremely negative values which cannot be derived alone from pathways such as photosynthesis, ammonium or sulfur oxidation, and thus, are most likely derived from methane oxidization[41]. Furthermore, chemoautotrophic bacteria from caves in Yucatan and Quintana Roo have been reported to have a $\delta^{13}C$ trace ranging from − 25 to − 46‰[8,10], which is in accordance with our $\delta^{13}C$ results from *T. pearsei* and *T. dzilamensis*. Observing a similar $\delta^{13}C$ trace in both *T. pearsei* and *T. dzilamensis* suggests they are incorporating a portion of their carbon from a non-photosynthetic microbial source that is present both in the exclusively freshwater system of Nohmozon and in the anchialine system of Ponderosa (Fig. 3). Our $\delta^{13}C$ results indicate both *T. pearsei* and *T. dzilamensis* are not exclusively feeding from methane derived sources, but rather a combination of the lighter source, such as methane ($\delta^{13}C \approx -66$‰) and a heavier photosynthetic derived carbon source, such as algae or plant derived.

The combined determination of ^{13}C and ^{14}C from *Typhlatya* tissue reflects that each species obtains carbon from a different source or from the same source but in different proportions. Adding the use of radiocarbon analysis in each species allowed the identification of the modern and ancient carbon sources from which they are feeding. The first of our mixing model scenarios suggests that all *Typhlatya* species preferentially consume carbon derived from organic matter (OM). The isotopic composition in *T. mitchelli* is intimately related with a modern derived photosynthetic source (C3 plants or algae) and only a marginal proportion is assimilated from other sources (Fig. 4). *Typhlatya pearsei* and *T. dzilamensis*, under this model, incorporate greater proportions of MM and AM in addition to OM, as compared to *T. mitchelli*. In addition to OM and MM, there is a partial contribution to the biomass of all *Typhlatya* from AM, derived most likely from ^{14}C-dead carbon. It is noteworthy that *T. pearsei* and *T. dzilamensis* show similar carbon source incorporation of both $\delta^{13}C$ and $\Delta^{14}C$, despite the fact they inhabit systems with different salinity and are several hundred kilometers apart. These results are in accordance with the hypothesis that biogeochemical processes in groundwater ecosystems are likely to be universal, as observed in other systems around the world[8,42–44].

Our second mixing model scenario considers an alternative carbon source for the ancient methane provided by the bacterial reduction of the available DIC. This is an alternative source of carbon in anchialine systems, such as Ponderosa. The carbonate reduction pathway may not be important for *T. mitchelli* in FGW environments, since the DIC isotopic values of the freshwater in Tza Itza are strikingly different to the bulk biomass isotopic readings. Nevertheless, this pathway would increase its importance in fresh groundwater if other substrates were limited[41], as suggested by the isotopic readings of *T. pearsei* in Nohmozon. The carbonate reduction pathway, by contrast, should be dominant in saline environments, where organic carbon is scarce[41]. This second scenario shows how important DIC incorporation can be to *T. pearsei* and *T. dzilamensis* given that the incorporation of AM derived carbon markedly increases as OM incorporation decreases when compared to the first scenario. This reduction could suggest a reduced availability of OM in the cenote pool of Nohmozon and in the saline layer of the Ponderosa cave hydro region, driving *T. pearsei* and *T. dzilamensis* into feeding from alternative sources.

Given that the analyzed *T. pearsei* were collected from the cenote pool of a FGW system, it seems counter-intuitive that it would be feeding on similar sources than *T. dzilamensis* which inhabit the saline region under the halocline. A higher pH, as observed in Nohmozon, facilitates the production of carbonates and reduces the dissolution of CO_2, possibly contributing to a greater availability for older DIC for primary producers to assimilate. Nevertheless, further research on the available sources is necessary to determine the genetic identity of these chemosynthetic or photosynthetic ancient-carbon feeding sources.

Combined results from the mixing models show *Typhlatya* species are feeding from shared sources in different proportions, thus identifying a niche partitioning among these closely related congeners. In freshwater environments, *T. mitchelli* feeds almost exclusively on photosynthetic derived sources while *T. pearsei*, under the first scenario, complements its diet with roughly a third of methane derived matter (MM and AM). In the saline groundwater *T. dzilamensis* has a similar dietary proportion uptake as *T. pearsei*, with approximately a third of its diet coming from methane derived sources. Identifying their feeding sources helps to explain the observed specific distribution patterns within the anchialine systems and accounts for the widespread observation of *Typhlatya* throughout the Yucatan underground.

A different selection of food sources by *Typhlatya* species indicates an ecological niche separation. It has been suggested that the degree of trophic specialization is expected to decrease in low productivity environments resulting in omnivorous or generalist species[45]. Our results are in accordance with these assumptions as *T. mitchelli*, which is distributed in the vicinity of sinkholes and would most likely have the greatest feeding diversity, is feeding almost exclusively on OM input. On the contrary, *T. dzilamensis* distributed in the aphotic passages which have the least OM input, compliments its diet with modern and ancient methane derived sources (Fig. 4). *Typhlatya pearsei* in Nohmozon is an extraordinary case, as it would be expected to have abundant availability of OM input and production, yet it is also complimenting its diet with modern and ancient methane derived carbon. The broader range in feeding sources of *T. pearsei* could be linked to a competition strategy that enables it to coexist with *T. mitchelli* in freshwater environments.

Such apparent differentiation on food sources must of course be accompanied by potential differences in foraging and feeding behavior. Although these are difficult to record and observe in situ, it could also be that selective forces related to the access to slightly different food sources has selected for different morphologies in mouth parts, which are much simpler to observe and compare. With the findings of the present work we hypothesize that a quantitative comparison of morphological variables associated with the filtering and scraping of bacterial mats and biofilms will discover significant specialization on the setae and dactyls between these 3 species.

Closing remarks

What we observe today in these anchialine caves is, indeed, the result of co-evolution of these species with the geologic history, environmental change and biologic interactions within these underground habitats. Overall, combined results of species distribution, stable isotopes and radiocarbon analysis, and diel behavior obtained in this study have shown how *Typhlatya* species in Yucatan have partitioned their niche to coexist throughout evolution. Niche partition between these sister species shows their specific role and importance in energy transfer to higher trophic levels and highlights the exclusion distribution patterns within groundwater ecosystems. These results help understand the wide distribution of *Typhlatya* species throughout the Yucatan Peninsula. The salinity preference of *Typhlatya* species, nevertheless, impels further research in the physiology of *Typhlatya* and raise questions as to whether the halocline is such a barrier that would drive allopatric speciation. It now becomes crucial to understand the physiological traits and adaptations that separate these species into different habitats and ecological niches.

Methods

Surveys were performed in Tza Itza (20.730311° N, 89.46608° W), Nohmozon (20.623266° N, 89.384207° W), Ponderosa (entering from cenote Xtabay 20.499183° N, 87.260842° W) and Kankirixche (20.637306° N, 89.632892° W). Selection of these four systems was based on their contrasting hydrological, morphological and biological features that make each of them singular, thus, systems are not considered random replicates of each other. These systems represent two distinct hydrographic basins. Ponderosa lies in the Quintana Roo basin, separated from the rest of the Yucatan Peninsula by the Holbox fracture, whereas, Tza Itza, Nohmozon and Kankirixche are located in the southern section of the ring of cenotes in the Yucatan basin[46]. Vertical profiles of salinity, temperature, pH, redox potential and dissolved oxygen in Tza Itza, Nohmozon and Ponderosa were obtained using a multiprobe Hydrolab DS5X. Because diving may itself influence environmental conditions in caves[47], only measurements recorded by the multiprobe in its way into the cave were considered, whereas those taken returning towards the surface were discarded. Previous studies revealed that these physical and chemical properties are markedly constant through time[48], hence measures were only recorded once during our survey. Trials using Hobo data loggers were unable to measure light in the cavern even when it was noticeable by the naked eye. Consequently, light was recorded by observers as present or absent where transects were installed.

In order to identify patterns in the distribution of *Typhlatya*, we divided each system into three "hydro-regions" considering light penetration and the external influence of runoff water and organic matter from the surroundings. The *cenote pool* was defined as the region under the water surface that receives direct influence from the surroundings i.e. the air interface, organic debris, interaction with terrestrial animals and, in some cases, direct or indirect sun light. The *cavern* was defined as the transition zone from the cenote pool to the cave: a region where sunlight is still perceivable but has no direct incidence, there is less external influence and has no vertical access to the surface. The *cave* was defined as the aphotic region, with minimum or no influence from external factors and no vertical access to the surface (Fig. 7).

Spatial distribution of *Typhlatya* species throughout the anchialine system. To characterize the distribution of *T. mitchelli, T. pearsei* and *T. dzilamensis* at Tza Itza, Nohmozon and Ponderosa, two underwater transects (sensu Sutherland[49]) in each of the three hydro-regions (cenote pool, cavern and cave) were used (Fig. 7A). Each transect was temporarily deployed installing a 10 m rope close to the ground and traversed simultaneously by two divers, one on each side at one meter above the ground. The number of individuals of *Typhlatya* species encountered as far as 2 m away from each side of the rope were registered. Only those

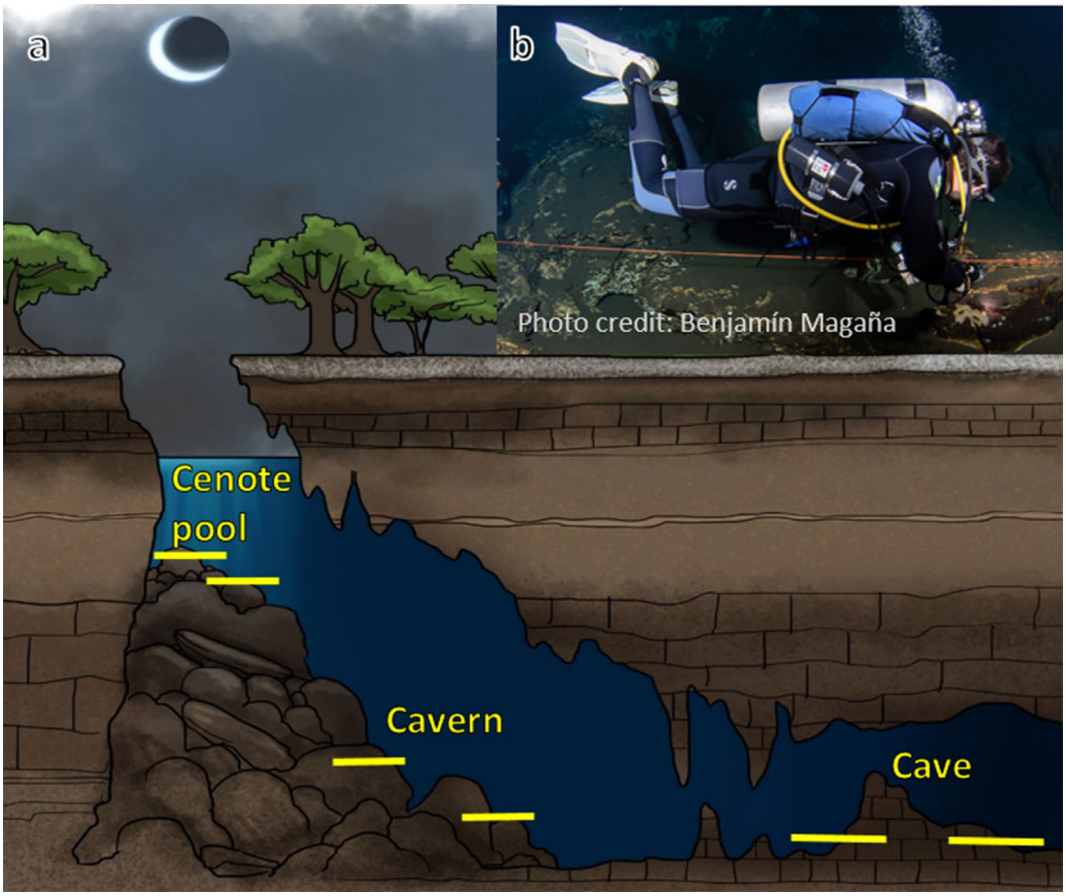

Figure 7. (**A**) Conceptual model of groundwater hydro regions depicting the general profiles of transects used to monitor *Typhlatya* abundance and distribution within the systems: cenote pool, cavern and cave. Two transects (yellow lines) were installed in each hydro-region and traversed simultaneously by two divers, twice during each of three seasons (dry, rainy and winter) throughout 1 year. (**B**) Diver approaching a transect in the cavern of Kankirixche. Artwork by Alberto Guerra.

individuals that were unequivocally identifiable underwater as pertaining to any of the species examined were considered. This resulted in 20 m^3 surveyed by each diver in each transect. Sampling procedures were repeated twice in each of the three meteorological seasons previously described for limnologic systems in the Yucatan Peninsula[48]: warm–dry (March–May), winter-storms with occasional short showers (November–February) and rainy seasons (June–October). All observations were performed after dark.

A multivariate approach was used to describe changes in *Typhlatya* species composition through the systems, hydro-regions and seasons examined. A non-metric multi-dimensional scaling (nMDS) procedure was used to order (n = 402) samples in a 2-dimensional configuration. The Bray–Curtis similarity index was used to obtain the resemblance matrix of previously log-transformed count data (log (x + 1)). Differences in species composition were then analyzed using a multivariate analysis of variance (followed by pairwise t-test when relevant) with the following underlying factorial model: System, fixed factor with 3 levels (Tza Itza, Nohmozon and Ponderosa); Season, fixed factor with 3 levels (dry, rainy and winter); and Hydro-region, fixed factor with 3 levels (cenote pool, cavern and cave). The observer was taken as a random factor (with 2 levels) nested within each three-way combination of system, hydro-region and season. A total of 9,999 permutations of residuals under the reduced model were used to obtain empirical distributions of F values. Statistical analyses were performed using PRIMER software and PERMANOVA add in[50].

Carbon tracing in *Typhlatya* tissue. In order to study the potential contribution and relative age of different carbon sources to the biomass of *Typhlatya* species, accelerator mass spectrometry (AMS) $\delta^{13}C$ and $\Delta^{14}C$ from individuals of *T. mitchelli*, *T. pearsei* and *T. dzilamensis* (obtained respectively from Tza Itza, Nohmozon and Ponderosa, under collection permit SEMARNAT/SGPA/DGVS/004471/18) were analyzed in the Accelerator Mass Spectrometry Laboratory (LEMA) at the Instituto de Física of the Universidad Nacional Autónoma de Mexico (UNAM). $\delta^{13}C$ and $\Delta^{14}C$ from dissolved inorganic carbon (DIC)—carbon dioxide (CO_2) and carbonates (CO_3)—were measured in additional samples of fresh groundwater (FGW) from Tza Itza, Nohmozon and Ponderosa, and saline groundwater (SGW) from Ponderosa. Values were then related to carbon isotopic ratios

from known sources that have been measured in cenotes and caves in Yucatan, and which could be potential feeding sources for *Typhlatya*.

The *Typhlatya* samples were cleaned with ultrapure water, treated with HCl 0.5 M to remove inorganic carbon, salts and other adhered contaminants, rinsed again with ultrapure water and then freeze-dried. For ^{13}C and ^{14}C analyses, samples were processed in an automated graphitization equipment (AGE III from Ion Plus) where the carbon was first converted to CO_2 and then to pure graphite. Carbon and Nitrogen content was estimated in the AE (Elementar vario MICRO cube) from the AGE III, as weight percent of the total sample mass. Oxalic acid II (NIST SRM 4990C) was used as a primary standard and Phthalic acid (with no ^{14}C) was used as a blank. Analysis of ^{14}C, ^{13}C and ^{12}C of the obtained graphite was performed in a 1 MV Accelerator Mass Spectrometry (AMS) High Voltage Europe Engineering (HVEE) at LEMA[51].

The content of ^{13}C, reported as $\delta^{13}C$ is the difference of a sample $^{13}C/^{12}C$ relative to Vienna Pee Dee Belemnite (PDB) carbonate standard and is expressed in parts per thousand (‰)[52].

$$\delta^{13}C = (^{13}C/^{12}C_{sample} - ^{13}C/^{12}C_{standard})/^{13}C/^{12}C_{standard} \times 1000$$

The content of ^{14}C is reported as $\Delta^{14}C$; the deviation of a sample $^{14}C/^{12}C$ ratio from its atmospheric preindustrial level expressed in parts per thousand (‰)[13,53], and the precision of ^{14}C measurements was ± 3‰ for modern sample. Measured $^{14}C/^{12}C$ isotopic ratios were corrected for isotopic fractionation using the $^{13}C/^{12}C$ isotopic ratios measured in the accelerator. Corresponding radiocarbon contents (apparent age) were calculated using computer codes developed at LEMA[51]. When measuring $\delta^{13}C$ by AMS, some isotopic fractionation can be introduced through the sample preparation and AMS measurement. Therefore, a subsample of *Typhlatya* individuals was halved to compare $\delta^{13}C$ by AMS and by isotope-ratio mass spectrometry (IRMS) which is the standard technique for $\delta^{13}C$ analysis. Two replicates of $\delta^{13}C$ IRMS analyses were performed in a Delta V Plus Thermo Scientific equipment at the Laboratorio de Análisis de Isotópos Estables at the Unidad Académica de Ciencias y Tecnología in Yucatan of the UNAM.

Mixing models. In order to explain the range of $\delta^{13}C$ and $\Delta^{14}C$ values found in *Typhlatya* from Yucatan and considering that carbon fixation through methanotrophy has been suggested in similar sites[8,16], we built a mixing model considering three potential carbon sources that could contribute to the food of *Typhlatya* species:

a. Organic matter (OM), represents a modern carbon source that is fixed through photosynthesis by both aquatic algae and phytoplankton located in the cenote pool or by terrestrial plants in the immediate surroundings.
b. Modern methanogenic carbon (MM) represents terrestrial methane produced by fermentation of methylated substrates.
c. Ancient methanogenic carbon (AM) results in ^{14}C depleted sources due to either the methanogenic decomposition of old organic matter by methane oxidizing bacteria (MOB), or the assimilation of ancient dissolved inorganic carbon (DIC) made available through the dissolution of limestone.

To assess the contribution of these carbon sources to *Typhlatya* species, biomass values of $\delta^{13}C$ and $\Delta^{14}C$, as well as values published for potential sources were incorporated into the IsoSource mixing model software[54]. We considered two possible scenarios in which the supply of ancient methane could be fueled by either (1) organic ^{14}C-dead carbon, or (2) the oldest ^{14}C measured from DIC in monitored systems. Values of $\delta^{13}C$ were fixed for both scenarios: $\delta^{13}C_{OM}$ represents a mix of photosynthetic organic matter from terrestrial and aquatic sources, $\delta^{13}C_{MM}$ corresponds to a fresh water bacterial modern methane measured in anchialine caves[8], and $\delta^{13}C_{AM}$ corresponds to bacterial methane produced by carbonate reduction in a saline environment (taken from measurements in Quintana Roo caves[8]) (Table 3). OM was assigned the average $\Delta^{14}C$ from the most modern biomass measured in this study, which reflects the modern signature of the terrestrial organic matter in this freshwater layer. Carbon derived from modern methane was considered as $\Delta^{14}C_{MM} = 0‰$. For the first scenario, a fossil value of $\Delta^{14}C_{AM1} = -1{,}000‰$ was assigned for ancient methane derived carbon, representing a ^{14}C depleted source which would be older than 50,000 yBP (Table 3). The second scenario, maintained above described values except for the ancient methane derived carbon which was established as $\Delta^{14}C_{AM2} = -260‰$ corresponding to the average DIC measured in the saline layer of Ponderosa cave (Table 3).

Diel vertical distribution of *T. mitchelli*. To explore possible variations due to daily and seasonal behavior-related migrations, we used *T. mitchelli* as a biological model in two systems that had a particularly high abundance and depicted different light regimes: Tza Itza and Kankirixche. Tza Itza is a cave-type cenote with a horizontal entrance and dry cave access, in which light only reaches the water surface indirectly. Kankirixche is a jug-shaped cenote with an opening directly above the water surface, through which direct sunlight reaches the water surface during part of the day.

The number of *T. mitchelli* individuals was recorded using five transects which were located one at the cenote pool directly under the water surface, followed by four transects distributed in a depth gradient throughout the cavern (Fig. 8). Due to morphological differences between sampling sites, the deepest transect reached 19 m and 27 m at Tza Itza and Kankirixche, respectively. Each transect was monitored by two divers traversing simultaneously as described earlier. Sampling took place at approximately midday and 30 min after sunset, and was repeated twice every 2 months throughout a year. Differences in *Typhlatya* counts related to between-observer variation were first examined by introducing a random factor (observer) with two levels within each transect and testing its significance.

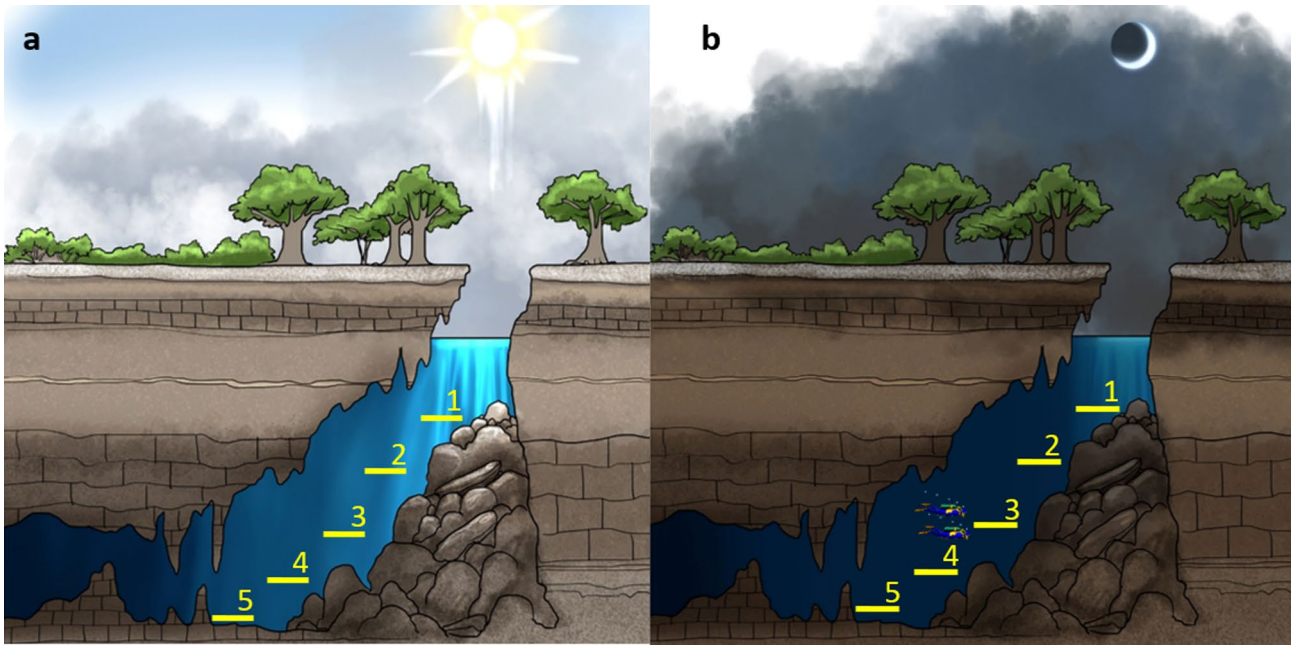

Figure 8. Diagram showing the location of sampling transects installed in Kankirixché and Tza Itza where the number of *Typhlatya mitchelli* were counted during day (**A**) and night (**B**) observations performed by two divers every 2 months throughout 1 year. Artwork by Alberto Guerra.

Changes in *T. mitchelli* distribution with depth were statistically analyzed using generalized linear models (GLM). The underlying experimental design was a three-factor model with "depth" (5 levels), "light" at the time of observation (2 levels: day and night), "system" (2 levels: Tza Itza and Kankirixche) and the "month" of the year (6 levels: February, April, June, August, October and December). Models were adjusted using a *Poisson* distribution with a log link function, and letting the over-dispersion parameter (rho) to be estimated by the model (i.e. using the "glm" R function with "family = quasipoisson"). The model with the best set of explanatory variables was obtained using a backward step procedure starting with the full model and testing the inclusion of each variable one by one[55].

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Author contributions

E.M.C.S., N.S. and M.M. designed the study; E.M.C.S. wrote the original draft and prepared figures; C.S., N.S. and M.M. wrote, reviewed and edited the manuscript, E.M.C.S. and N.S. conducted field work; E.M.C.S. and M.M. conducted statistical analysis; C.S. conducted stable isotopes and radiocarbon analysis; N.S., C.S. and M.M. acquired funding; M.M. supervised this investigation. All authors reviewed the final manuscript.

Competing interests

The authors declare no competing interests.

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Artículo 2: Adaptive physiology of Typhlatya cave shrimps: linking habitat with aerobic metabolism.

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Adaptive physiology of Typhlatya cave shrimps: linking habitat with aerobic metabolism

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Author contribution statement

EC, CR and MM contributed to conception and design of the study. EC and MM performed the statistical analysis and prepared the figures. EC and GR obtained and analysed the biochemical indicators of all individuals. EC, FD, DR and CR performed field experiments and analysed metabolic response and thermal tolerance. EC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Keywords

Anchialine, Groundwater, Stygobionts, metabolic rates, thermal tolerance, Antioxidants, phenotypic plasticity

Abstract

Word count: 285

The anchialine environment is characterized by a vertical stratification of water masses with different salinities. Cave shrimps of the genus Typhlatya are widespread inhabitants of the aquifer in fresh, brackish, and marine groundwater. Here we describe physiological aspects of three of the most abundant and widespread Typhlatya species that thrive in the fresh and marine groundwater habitats of the anchialine ecosystem of the Yucatan Peninsula.

The aerobic scope (AS) of Typhlatya mitchelli, Typhlatya pearsei and Typhlatya dzilamensis were estimated through induced physical activity, whilst monitoring protein carbonylation and lipid peroxidation (as indicators of cellular damage), lactate accumulation (as an indicator of anaerobic metabolism) and the antioxidant system response. The critical thermal limits (CTL) of all three species as an additional measure of physiological plasticity were also determined. Our results showed that metabolic rates, AS and CTL were similar amongst the two species that inhabit fresh groundwater habitats and differed markedly from T. dzilamensis, a species typically found in marine groundwater. The antioxidant system response in all three Typhlatya species accompanied the levels of aerobic metabolism following physical activity. However, the great amount of GSH observed in T. dzilamensis may be indicative of an adaptive trait to a more heterogeneous environment.

The differences observed among Typhlatya species reflect differential physiological adaptations that correspond to the environmental heterogeneity of their natural habitats. Our results suggest that the marine groundwater species, T. dzilamensis, could be permanently prepared to respond to a naturally more heterogeneous environment, in contrast to Typhlatya mitchelli and Typhlatya pearsei which rarely face environmental clines in the fresh groundwater habitat. Our findings contribute to a better understanding of the consequences of environmental change on ecologically important species that are restricted to living in the aquifer.

Contribution to the field

This study explores the physiology of three species of cave shrimp endemic to the aquifers of the Yucatan Peninsula, Mexico, that are distributed according to differences in groundwater salinity. Considering that these Typhlatya species are phylogenetically close and are strictly restricted to the underground habitat, comparisons of their thermal tolerance, respiratory metabolism and biochemical response to induced physical activity is an opportunity to evaluate their ecophysiology from an adaptive perspective. Our results show that Typhlatya dzilamensis has a wider thermal tolerance, greater aerobic scope and an antioxidant system that responds effectively to the increase of reactive oxygen species produced by increased respiration. It is also the only species found in saline environments. The significance of these findings are discussed in the context of the micro-habitat in which species occur, their physiological plasticity and its adaptive implications. We believe this contribution will inspire future work on these delicate groundwater communities and will enable a novel approach for the conservation of groundwater environments.

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Ethics statements

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Studies involving human subjects
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Inclusion of identifiable human data
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Data availability statement

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Adaptive physiology of *Typhlatya* cave shrimps: linking habitat with aerobic metabolism

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Keywords: Anchialine, groundwater, stygobionts, metabolic rates, thermal tolerance, antioxidants, phenotypic plasticity.

Contribution to the field

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1 Introduction

Species restricted to living in flooded caves (stygofauna) possess adaptations, known as troglomorphisms, that distinguish them from aquatic species living in surface water bodies (Christiansen, 1962; Pipan and Culver, 2012). Morphological differences, such as the reduction or loss of eyes and pigments, and extension of appendages are some of the most obvious. However, physiological traits of stygobionts in response to the distinct conditions of anchialine caves are determinant for the persistence of their populations. In the adaptation process, individuals in a population respond with a set of behavioral, physiological, biochemical, and metabolic mechanisms to environmental change. This responsiveness to different stimuli can be considered phenotypic plasticity, which can be fixed in a population through natural selection over evolutionary time scales, ultimately resulting in a population with greater fitness to the novel environment (Gorban et al., 2016). The study of physiological plasticity and its relation to the adaptive capacity of species to their environment has allowed to establish tolerance limits to different environmental factors, such as temperature, oxygen or salinity (Guderley and Pörtner, 2010; Pörtner, 2010; Sokolova et al., 2012).

The response to environmental change, whether of biotic or abiotic origin, necessarily involves changes in energy production and allocation (Sokolova et al., 2012). Metabolism is the process that produces energy (ATP) at the cellular level, and the metabolic rate (the speed at which it is produced) measures the amount of energy required (and supplied) by the organism. The energy required for the maintenance of essential functions, such as respiration, blood circulation, and excretion, is known as the basal metabolic rate (BMR). Due to methodological difficulties in measuring BMR, the standard metabolic rate (SMR) has been used as the practical measure of BMR (Chabot et al., 2016a).

Once the energy requirements for the maintenance of homeostasis have been met, organisms can generate a surplus of energy for growth, reproduction, food digestion, physical activity, and response to environmental variations (Pörtner and Farrell, 2008; Sokolova et al., 2012). One way to assess that capacity is by measuring the maximum metabolic rate (MMR), which has been recognized as the physiological limit at which an individual can produce ATP via the aerobic pathway. The difference between the MMR and SMR was first defined as the aerobic scope of metabolic activity (Fry, 1947) and is now called the aerobic scope (AS). The AS is the energy that an individual can produce beyond the metabolic cost of basal processes and which is intended for performing ecological functions (Sokolova et al., 2012; Norin and Metcalfe, 2019). Since basal maintenance always prioritizes energy resource allocation over any other process, when energy production is limited or the cost of basal maintenance increases, AS is reduced (Sokolova et al., 2012). Therefore, higher AS has been linked to greater fitness, both in long-term processes, such as adaptive potential, growth and reproduction (Meza-Buendía et al., 2021), as well as in the short-term processes, such as predator evasion, foraging and response to immediate environmental disturbances (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Clark et al., 2013). Likewise, changes in AS may distinguish an optimal situation from one of moderate or even critical stress (Pörtner, 2002, 2010; Sokolova et al., 2012; Pörtner et al., 2017).

One way to quantify stress is by assessing the energetic-metabolic costs that environmental conditions produce in an organism (Pörtner, 2010; Lushchak, 2011; Sokolova et al., 2012; Paschke et al., 2018). It is possible to establish how environmental changes modulate the physiological mechanisms that enable the response of individuals to the environment (Sokolova et al., 2012). This is how the study of metabolism, and its response to environmental change allows the evaluation of the capacity to obtain energy under a variety of stress conditions (Sokolova et al., 2012; Paschke et al., 2018). Moreover, physiological response to environmental heterogeneity contributes to the definition of some dimensions of the fundamental ecological niche (Hutchinson, 1957; Elton, 2001; Soberón and Peterson, 2005; Ángeles-González et al., 2020, 2021).

Temperature is one of the environmental factors that determine the metabolic rate of an individual (Gillooly et al., 2001; Clarke and Fraser, 2004). Since the early work of Fry (1947) and Reynolds and Casterlin (1979), a body of theoretical and methodological knowledge on the thermoregulatory capacity of ectotherms has accumulated. These studies have provided insight into how temperature imposes limits on organisms exposed to a wide diversity of environments (e.g. (Lutterschmidt and Hutchison, 1997b; Gillooly et al., 2001; Clarke and Fraser, 2004; Pörtner, 2010; Rezende et al., 2011; Tepolt and Somero, 2014; Ern et al., 2015; Magozzi and Calosi, 2015; Cumillaf et al., 2016; Mascaró et al., 2016; Rodríguez-Fuentes et al., 2017; Pallarés et al., 2019).

The immediate biochemical effect of increasing the metabolic rate to produce energy is the massive influx of oxygen into the mitochondria. During this process, the monovalent reduction of oxygen generates a series of free radicals, called reactive oxygen species (ROS), which can be harmful to organisms as they oxidize other molecules to reach ionic equilibrium (Martínez-Álvarez et al., 2005). Oxidative stress occurs when the production of ROS is greater than the capacity of the antioxidant system to eliminate them (Martínez-Álvarez et al., 2005; Díaz-Acosta and Membrillo-Hernández, 2006). Organisms rely on a series of oxidation-reduction reactions that continuously maintain cells in a highly dynamic equilibrium to prevent cellular damage from ROS, known as the antioxidant system (AOS). Antioxidants yield electrons to ROS, transforming compounds into other less reactive or detoxified molecules (Regoli et al., 2011; Gumulec et al., 2013). The elevation of antioxidants such as superoxide dismutase (SOD), Catalase (CAT), Glutathione (GSH) and Glutathione S-transferase (GST) indicate an increase in ROS production, whereas lipid peroxidation (LPO) and protein carbonylation (PO) are biomarkers of oxidative stress showing cellular damage.

Typhlatya mitchelli, *Typhlatya pearsei* and *Typhlatya dzilamensis* occupy distinct habitats in the cenotes (local term for dolines/sinkholes) and caves of the Yucatan Peninsula (Chávez-Solís et al., 2020). These anchialine habitats are characterized by vertically stratified water bodies, where temperature exhibits slight variations (~1°C) across this gradient, but salinity may change abruptly through depth, frequently accompanied by changes in dissolved oxygen, oxidation-reduction (redox) potential and pH (Pohlman, 2011; Brankovits et al., 2017; Benítez et al., 2019; Chávez-Solís et al., 2020). Such systems, therefore, present unique abiotic characteristics that generally keep fresh and marine groundwater species spatially and physiologically separated. Here, *T. mitchelli*, and *T. pearsei* inhabit strictly freshwater environments, while *T. dzilamensis* inhabit environments with high salinity levels (Chávez-Solís et al., 2020).

If there is a strong association between the physiology of an organism and its habitat (Huey, 1991), it is reasonable to hypothesize that differences in the distribution of these *Typhlatya* species correspond to their ability to cope with the environmental conditions acquired through evolution. In aquatic organisms, the thermal window, the metabolic capacities and the antioxidant mechanism have been used to understand how the environment can influence the adaptive capacity of organisms (Vinagre et al., 2016, 2021). These findings have been key to understanding species' adaptation from an ecophysiological point of view (Niklitschek and Secor, 2009; Paschke et al., 2013; Sumaila et al., 2020; Eddy et al., 2021) and predict changes in future warming scenarios (Cheung et al., 2008, 2010; Maharaj et al., 2018). If this is true, then the distribution patterns of *T. mitchelli*, *T. pearsei* and *T. dzilamensis* could be partially explained in terms of the metabolic capacities of each species to respond to environmental constraints. Thus, the comparison of metabolic, behavioral, and biochemical indicators among these closely related species presents a sublime opportunity to evaluate their physiology from an adaptive perspective.

This work aimed to characterize the physiological plasticity of three *Typhlatya* species through its thermal window, respiratory metabolism and biochemical indicators that reflect their aerobic capacity under the conditions of their natural habitats within the anchialine ecosystems of the Yucatan Peninsula. For this purpose, *in situ* experiments to measure thermal window and oxygen consumption rates under routine conditions and in response to the swimming activity were carried out under the natural conditions of each species. In addition, samples were taken to examine the response of the antioxidant system (enzymes and metabolites indicators of oxidative damage) and anaerobic metabolism derived from the swimming activity at different times throughout the experiments.

2 Materials and Methods

2.1 Origin of experimental individuals

Individuals of *T. mitchelli*, *T. pearsei* and *T. dzilamensis* were collected manually using SCUBA cave diving techniques in the Tza Itza (20.730311° N, 89.46608° W), Nohmozón (20.623266° N, 89.384207° W) and Ponderosa systems (entering through cenote Xtabay 20.499183° N, 87.260842° W). Tza Itza and Nohmozón are characterized by being totally freshwater (0.64 ± 0.02 ups), while the Ponderosa system is an anchialine, vertically stratified system with freshwater body (3 ups) floating above a marine intrusion (36 ups). The experiments performed in the present study considered that *T. mitchelli* and *T. pearsei* are native to the fresh groundwater, while *T. dzilamensis* is native to the marine groundwater portion of the anchialine ecosystems. Samples were collected in compliance with the permits granted by the Dirección General de Vida Silvestre of the SEMARNAT: SGPA/DGVS/02068/17, /004471/18 and /05996/19. A total of 46, 41, and 45 individuals of *T.*

mitchelli, *T. pearsei* and *T. dzilamensis* were captured, respectively, and used in the *in situ* experiments described herein.

2.2 Metabolic rates and aerobic scope.

The AS of *T. mitchelli*, *T. pearsei* and *T. dzilamensis* was calculated as the difference between the routine metabolic rate (RMR) and the MMR. The RMR was obtained from individuals maintained in a resting state and without external perturbations, while the MMR was measured in the same individuals after being submitted to physical activity for 5 minutes (Figure 1). In order to maintain the water temperature similar to that of the collection site, the respirometer chambers were placed at a depth of 50 cm in the cenote from which the experimental individuals were collected. The respiratory rate of each individual was measured directly in each respirometer chamber by means of an oxygen sensor connected through an external optic fiber to an amplifier (Witrox 4; Loligo systems, Denmark) that sent the signal to a computer. This allowed continuous monitoring at a speed of one record of oxygen concentration per second.

The RMR of eight individuals of each species was measured for a period of 150 minutes, during which, shrimp remained undisturbed (Figure 1). Following this time, the water in the chambers was totally renewed, and the chambers were placed in a system that rotated the chambers on their longitudinal axis at 25 rpm for 5 min, thereby inducing animals to physical activity that simulated swimming. The swimming activity of shrimps during this period was confirmed by direct observation. Immediately after exercise, respirometer chambers were returned to the cenote, connected to the optic fiber for an additional 120 min measure of oxygen consumption after physical activity (Figure 1). Oxygen consumption was also measured in control chambers containing only ambient water in order to subtract oxygen consumption of microorganisms contained in the water and the experimental device.

The Witrox sensors and computer were installed near the entrance of the cenote and connected to a GoalZero Yeti 1250 portable battery or a Honda gasoline electric generator (Inverter EU 2000i), located outside the cenote gallery at each study site. Once measurements were completed, all individuals were placed in Eppendorf tubes and frozen in liquid nitrogen for further biochemical analysis in the laboratory (Figure 1).

The RMR was calculated as the slope of the oxygen concentration change over a period of time of at least 500 seconds. Special care was taken to select intervals where reduction was constant, excluding variations attributed to the manipulation of the chamber and sensor adjustments during the first moments measuring. The MMR was obtained from the slope of the decrease in oxygen concentration during 3 minutes of stable reading within the first 5 minutes immediately after the induced physical activity. Similarly, initial variations caused by sensor adjustment or manipulation of the respirometer chamber were excluded. The metabolic rate of each individual was calculated using the following equation:

$$VO_2 = \frac{\delta PO_2 * V_r - C}{T * W}$$

Where VO2 is the oxygen consumption of each individual (expressed in $mgO_2\ hr^{-1}\ g^{-1}$); δPO_2 is the slope of oxygen concentration change over time (expressed in $mgO_2\ seg^{-1}$); Vr is the volume in the chamber, calculated as the total volume of the chamber (L) minus the weight of each individual (g) (assuming its density was equal to that of water); C is the slope of the reduction in oxygen concentration within the control chamber ($mgO_2\ seg^{-1}$); T is the time (hour); and W is the weight of the organism (g).

In order to assess differences in metabolic rates (RMR, MMR) and the magnitude of the AS between species, a MANOVA with permutations was used (Anderson, 2017). Data were log-transformed (log(x+1)) and normalized prior to obtaining an Euclidean distance matrix between all pairs of samples. A fixed factor (species with three levels) was used as the underlying model, and 9999 unrestricted permutations were used to obtain the empirical distribution of *pseudo*-F values under the null hypothesis.

2.3 Biochemical indicators

The response of the antioxidant system, oxidative damage, and lactate accumulation in the tissues of individuals (referred in this work as biochemical indicators) of the three species of *Typhlatya* was characterized at different moments through a period of recovery after physical activity. Nine individuals of each species were placed in 50 ml Falcon tubes and subjected to the physical activity described previously (Figure 1). Three individuals of each species at each of three post-exercise moments were sampled: 0 (immediately), 30, and 60 minutes after physical activity. These samples, together with individuals that were kept 120 minutes in respirometer chambers after exercise, constituted the four groups respectively referred to as T0, T1, T2, and T3. An additional group of 15 individuals of *T. mitchelli*, 12 of *T. pearsei* and 16 of *T. dzilamensis* was placed in liquid nitrogen immediately after collection to establish a baseline (BL) as a reference for all the indicators quantified. Samples of all individuals were frozen in liquid nitrogen and further stored at -80°C until analysis.

The following components of the antioxidant system (AOS) were quantified in each sampled individual: catalase (CAT), glutathione S-transferase (GST), total glutathione (GSH), and superoxide dismutase (SOD). Oxidative damage by reactive oxygen species (ROS) was estimated by quantifying protein carbonylation (PO) and lipid peroxidation (LPO). Finally, lactate (LAC) as a residue of anaerobic metabolism was quantified. For the purposes of this study, the set of indicators of the antioxidant system, oxidative damage, and anaerobic metabolism will be referred to as biochemical indicators (BI).

Each sample was homogenized in a Potter-Elvehjem homogenizer with a PTFE pistil immersed in ice. According to the procedures described in Rodríguez-Fuentes *et al*. (2008), and according to the weight of each individual, Tris buffer pH 7.4 with a concentration of 0.05M (pre-set crystals pH7.4 of Tris [hydroxymethyl] aminomethane and Tris HCL) was added at a proportion of 1 ml of buffer per 50 mg of tissue. From the resulting homogenate, 200 µl were used for PO determination, 40 µl for LPO, and 40 µl for GSH. The rest of the homogenate was centrifuged at 10,000 rpm for 5 minutes at 4°C. From the supernatant, we extracted 40 µl for the determination of GST and total protein concentration, 50 µl for CAT, 20 µl for SOD, and 40 µl for LAC.

A Sigma CA0260 kit was used to determine GSH levels. GST activity was obtained following the methods of Ellman *et al*. (1961) and Habig *et al*. (1974) using a Sigma CS04 kit with CDNB as the substrate for spectrophotometric measurement at 412 nm every 15 seconds for 5 minutes. CAT activity was determined, using the molybdate method (Góth, 1991) as modified by Hadwan and Abed (2016), by measuring the reduction rate of hydrogen peroxide at 405nm upon reaction with ammonium molybdate. SOD was determined using a Sigma 19160 kit. Total protein was determined using the method of Bradford (1976) adapted to microplate with the protocol developed by personnel from the Ecotoxicology laboratory of the Faculty of Chemistry, UNAM, in Sisal, Yucatan, Mexico. PO quantification was based on the methods of Mesquita (2014), while for LPO quantification, the FOX method was followed using the peroxidetect kit (PD1) from Sigma. Finally, LAC was quantified using TRINITY and Pointe Scientific reagents following the procedures indicated by the manufacturer.

All determinations were performed with duplicate subsamples. This allowed negative absorbances to be treated according to the following criteria: 1) when both replicates resulted in negative absorbances, the value was considered as zero, i.e., not detected. 2) When one of the two replicates was negative, only the positive value was considered.

To analyze changes in the AOS and oxidative damage of the three species during the recovery period, a principal coordinate analysis (PCoA) was applied to a triangular matrix of Euclidean distances between samples. Data were square-root transformed and normalized prior to analysis. A permutational MANOVA with 9999 random permutations of the residuals under the reduced model (Anderson, 2001) was applied on the matrix. A bifactorial model was used with species (3 levels: *T. mitchelli, T. pearsei* and *T. dzilamensis*) and time (5 levels: T0, T1, T2, T3 and BL) as fixed factors. A significant interaction would imply that changes in AOS and oxidative damage over time, and relative to BL were different depending on the species of *Typhlatya*. Likewise, paired comparisons between species centroids for each time would establish whether species returned to a baseline condition at different times.

To compare the amount of LAC in the three species before and at different moments after physical activity, a two-factor ANOVA with the same underlying model was applied to the data, followed by post hoc comparisons using Tukey's Honest Significant Difference (Tukey´s HSM) (Hothorn et al., 2008).

2.4 Critical thermal limits

The procedure for determining critical thermal limits requires exposing individuals to a sufficiently rapid increase in temperature so the physiological mechanisms of thermoregulation cannot be expressed (Lutterschmidt and Hutchison, 1997b). Following a discussion on critical temperatures in the past decade (Rezende et al., 2011; Terblanche et al., 2011; Mermillod-Blondin et al., 2013; Mascaró et al., 2016; Díaz et al., 2017; Hernández-Sandoval et al., 2018), there is consensus that increasing the temperature at the rate of 1 °C per minute is appropriate to meet the stipulations of the method (Hutchison, 1961). The critical thermal limit is "the arithmetic means of the collective thermal points at which locomotor activity becomes disorganized and the animals lose their ability to escape from conditions that will promptly lead to their death, when heated at a constant rate that allows deep body temperatures to follow environmental test temperatures without a significant time lag" (Hutchison, 1961), and it is defined in a manner that no damage at the neuronal, physiological or biochemical levels should remain after a brief period at this temperature. The thermal window is the temperature range between the upper and lower critical temperatures (Thyrring et al., 2019), and it indicates the physiological temperature limits that an organism can tolerate and survive, hence delimitating its thermal niche.

The CTmax and CTmin were estimated following the method described by Lutterschmidt and Hutchison (1997a). To obtain the CTmax, specimens of *T. mitchelli* (n=8), *T. pearsei* (n=6) and *T. dzilamensis* (n=5) were placed individually in 25 ml Falcon tubes filled with water from the cenote where each species came from. The tubes were placed in a 20 L glass container filled with water, a CheckTemp®1 digital thermometer (Hanna Instruments), a 1000 W heater, an aerator and a water pump connected to a Honda EU2000i gasoline generator. Animals were exposed to an increase of 1°C min^{-1} (Hutchison, 1961; Lutterschmidt and Hutchison, 1997a), and their behavior was continuously recorded until the critical temperature was reached. This was defined as the temperature at which individuals showed onset of spasms or tail flip, followed by loss of movement or constant loss of equilibrium (Lutterschmidt and Hutchison, 1997a; Díaz et al., 2013). Once CTmax was reached,

animals were immediately placed individually in Eppendorf tubes, and frozen in liquid nitrogen for subsequent biochemical characterization.

To obtain the CTmin, specimens of *T. mitchelli* (n=6), *T. pearsei* (n=6) and *T. dzilamensis* (n=5) were also placed individually in 25 ml Falcon tubes within a 12 L glass container similar to the one used previously. The glass container was now placed inside an aluminum box containing a mixture of crushed ice, salt, and alcohol (96°). Temperature in the glass container was measured with the same digital thermometer and calibrated to decrease at a rate of 1°C min^{-1}. The behavior of each individual was again recorded until CTmin was identified as a loss of equilibrium without eminent recovery, spasms or the total absence of movement.

The comparison of the critical thermal maximum and minimum between species was evaluated using two separate one-way ANOVAs followed by post hoc comparisons using Tukey's HSM. Biochemical indicators to characterize the response of the AOS and assess cell damage as a response to the critical temperature procedure were evaluated using a PCoA. Data were square-root transformed and normalized prior to analysis. In this case, the bifactorial model had two fixed factors: species (3 levels: *T. mitchelli, T. pearsei* and *T. dzilamensis*) and treatment (3 levels: CTmax, CTmin and BL). Results were statistically evaluated with a permutational MANOVA with 9999 random permutations of the residuals under the reduced model (Anderson, 2001). A significant interaction would imply that cell damage and AOS response to instantaneous exposure to critical thermal limits vary depending on the species. The amount of LAC resulting from the critical thermal limits elicitation was evaluated with an ANOVA, followed by post hoc comparisons using Tukey's HSM.

All statistical analyses and graphic representations of results were performed using the ggplot2 library (Wickham, 2016) of the R platform (R Core Team, 2019) and the PRIMER 7 program (Clarke and Gorley, 2015) with PERMANOVA (Anderson et al., 2008).

3 Results

3.1 Aerobic Scope

While the RMR of the three species was markedly similar (Figure 2A), the MMR of *T. dzilamensis*, was 9.9 and 6.2 times greater than in *T. mitchelli* and *T. pearsei*, respectively. This resulted in an AS of *T. dzilamensis* 12.4 and 7.5 times greater than *T. mitchelli* and *T. pearsei*, respectively (Figure 2A). It should be noted that the high mean values in *T. dzilamensis* were associated with greater variability, indicating considerable heterogeneity in activity metabolism in this species.

The PCoA applied to the physiological indicators of aerobic metabolism (Figure 2B) showed a clear separation of *T. dzilamensis* with high values of MMR and AS, but not RMR. These differences were confirmed by the MANOVA, which showed significant differences in these indicators between at least two species (pseudo-F = 3.99; $p < 0.01$; 9951 unique permutations). Further post hoc showed significant differences between *T. dzilamensis* and both *T. pearsei* (pseudo-t = 2.24; $p < 0.01$; 5052 unique permutations) and *T. mitchelli* (pseudo-t = 1.188; $p < 0.05$; 330 unique permutations), although the latter were marginal. By contrast, these indicators in *T. pearsei* and *T. mitchelli* were statistically similar (pseudo-t = 1.051; $p > 0.05$; 495 unique permutations).

PCoA also showed a markedly large dispersion among individuals of *T. dzilamensis* compared to the other two species (Figure 2B). In the context of a confirmed heterogeneity of variance (*pseudo-F* = 15.43; $p < 0.001$), it is not clear whether statistical differences occurred among the centroids representing these species or they are a product of large and unequal multivariate dispersions.

Nevertheless, several individuals of *T. dzilamensis* showed higher indicators of aerobic metabolism, particularly MMR and AS than any of the other two species (Figure 2B).

3.1.1 Biochemical indicators of aerobic scope

The PCoA applied to indicators of the AOS, and oxidative damage in *Typhlatya* species at rest and post-activity explained 50% of the total variation in the first two principal coordinates (Figure 3). The eigenvectors that contributed most to sample separation in the first coordinate were SOD (0.8637), GST (0.8236) and, to a lesser extent, PO (0.4365). Data points corresponding to BL in *T. dzilamensis* were clearly separated from the rest on the horizontal axis (Figure 3). For the second principal coordinate, LPO (0.7807), CAT (-0.6409), and GSH (0.3935) were the eigenvectors that contributed most to the separation of samples. However, samples with high values of these indicators did not correspond to any species or experimental treatment in particular.

The MANOVA showed a significant interaction between species and time (pseudo-F = 1.46; $p < 0.05$; 9887 unique permutations; Table **1**A), indicating that the response of the AOS and oxidative damage to physical exercise over time varied between *Typhlatya* species. Post hoc paired comparisons (Table **1**B) indicated that physical activity significantly impacted AOS in *T. dzilamensis* through differences between BL and samples taken at all times after exercise. Only marginal differences were found between BL and T0 in *T. mitchelli,* possibly due to an individual with anomalous cell damage (PO and LPO). No significant differences in AOS indicators and oxidative damage derived from exercise were found in *T. pearsei*. Overall, these results suggest that exercise-derived metabolic activation in *T. mitchelli* and *T. pearsei* was not associated with oxidative damage or alterations in AOS indicators.

When comparing AOS indicators between species at each sampling time, differences were only found between the BL of *T. dzilamensis* and that of the other two species (pseudo-t between 1.63 and 2.76; $p < 0.05$; 9953 and 9801 unique permutations). These results indicate that AOS enzymes under routine metabolism conditions vary among species and suggest that *T. dzilamensis* could be constitutively primed to respond to environmental changes.

The ANOVA on LAC quantity only showed significant differences between species (Table **2**A), implying that there was no accumulation of lactate derived from exercise at any time. Post hoc comparisons confirmed that *T. dzilamensis* had a higher mean lactate concentration than *T. mitchelli* and *T. pearsei* (Table **2**B), while the two latter had similar concentrations. In general, these results suggest that physical activity did not involve lactate accumulation in any of the *Typhlatya* species and that *T. dzilamensis* has more LAC than any of the two other species studied.

3.2 Critical thermal limits

One-way ANOVAs, used to compare the critical thermal limits of the three species, showed significant differences in CTmax between species ($F = 10.32$; $p < 0.01$). Multiple comparisons showed that the CTmax of *T. dzilamensis* (36.5 ± 0.8 °C) was significantly higher than *T. mitchelli* (34.9 ± 0.9 °C; mean difference = 1.55, adjusted $p < 0.01$) and *T. pearsei* (34.7± 0.3 °C; mean difference = 1.77, adjusted $p < 0.01$), whilst no difference was found between the latter (mean difference = 0.22, adjusted $p = 0.88$) (Figure 4). In contrast, the CTmin ($F = 1.13$; $p = 0.35$) of *T. mitchelli* (13.8 ± 0.7 °C), *T. pearsei* (13.9 ± 0.5 °C) and *T. dzilamensis* (14.3 ± 0.7 °C) was statistically similar (Figure 4).

The thermal window for the three species was 20.8 °C for *T. mitchelli*, 21.1 °C for *T. pearsei* and 22.2 °C for *T. dzilamensis*. The net difference in the thermal window of *T. dzilamensis* compared to that of *T. pearsei* and *T. mitchelli* was 1.4 and 1.1 °C, respectively.

3.2.1 Biochemical indicators of critical thermal limits

PCoA applied to indicators of AOS and oxidative damage in individuals subject to experimental procedures to determine CTmax and CTmin explained 51% of the total variation in the first two principal coordinates (Figure 5). The indicators that contributed most to the variation in the first principal coordinate (horizontal) were SOD (0.8763) and GST (0.7911), followed by PO (0.4698) and GSH (-0.4246). The indicators with the highest contribution to the second coordinate (vertical) were CAT (0.7539) and LPO (-0.6233). The ordination showed that samples corresponding to *T. dzilamensis* were all associated with high GSH and low SOD, GST, and PO values, with no marked distinctions between BL individuals and those used to determine critical thermal limits (Figure 5). Samples corresponding to *T. pearsei* and *T. mitchelli* were located to the left of the ordination map, associated with relatively high SOD, GST, and PO values. Whereas *T. mitchelli* samples presented a higher concentration of CAT, *T. pearsei* samples had relatively higher values of LPO. Here again, no significant differences were observed between BL individuals and those used to determine critical thermal limits (Figure 5).

The MANOVA showed a significant interaction between treatment and species (Table **3**A). However, further paired comparisons showed that BL, CTmax and CTmin samples within each species were generally similar, with only two cases having marginal differences (Table 3B). These results indicate that the procedures to determine critical thermal limits, did not have a significant effect on oxidative damage at the cellular level or on the activity of AOS enzymes compared to a baseline condition. The MANOVA also showed significant differences between species, suggesting that the overall AOS enzyme activity differed between *T. dzilamensis*, *T. pearsei* and *T. mitchelli* irrespective of having been subject to critical thermal determinations.

In close correspondence, LAC content also only differed between species (Table 4A), indicating that the procedures to determine critical thermal limits did not involve anaerobic metabolism in these species. Post hoc comparisons showed a higher concentration of lactate in *T. dzilamensis* compared to *T. mitchelli* and *T. pearsei*, while the latter did not differ significantly from each other (Table **4**B).

4 Discussion

The present study for the first time compares the AS, AOS, LAC content, and thermal tolerance of three closely related species of *Typhlatya*, and shows striking differences related to the characteristics of the subterranean systems of the Yucatan Peninsula in which they coexist. Results indicate that *T. dzilamensis*, the only marine groundwater inhabitant, has a higher AS and thermal tolerance than *T. mitchelli* and *T. pearsei*, which are common in fresh groundwater habitats. This difference is noteworthy because all three species showed a similar RMR, indicating that the energetic cost of routine activities of the three species is independent of the environment where they inhabit. It also shows that the difference in AS between species is mainly due to variations in the MMR attained after the same type, intensity, and duration of physical exercise had been induced in all individuals. Considering the strong effect of environmental conditions on the metabolism of aquatic animals (Pörtner, 2002, 2010; Levin, 2003; Lushchak, 2012; Fang et al., 2019), results herein are discussed in the light of the pressure exerted by the abiotic characteristics of the anchialine systems of the Yucatan Peninsula and how such pressure may have promoted the colonization of the marine groundwater environment.

According to several authors (Sokolova et al., 2012; Farrell, 2016; Pörtner et al., 2017; Rodríguez-Fuentes et al., 2017) the AS is an indicator of the metabolic energy available once the basal physiological requirements have been satisfied. Therefore, differences in the ability to adjust

metabolism in the face of the same stressor – in this case induced physical activity – can be interpreted as differences in physiological plasticity amongst species. The greater AS as a result of MMR in *T. dzilamensis* suggests that the metabolic increase involves physical capacities to deliver oxygen to the cells, as well as the onset of biochemical mechanisms for energy production and pathways to control ROS, among others. Physiological plasticity has been linked to an increased resilience (Tepolt and Somero, 2014; Magozzi and Calosi, 2015; Seebacher et al., 2015; Vinagre et al., 2016; Norin and Metcalfe, 2019) and greater adaptive capacity (Gorban et al., 2016; Norin and Metcalfe, 2019). Furthermore, it has been proposed that a limited antioxidant capacity implies a lower acclimation potential, leading to reduced fitness and, ultimately, an inability to respond to environmental stress (Madeira et al., 2013; Pallarés et al., 2020). In our study, *T. dzilamensis* was the species with the greatest metabolic response to physical activity, coupled with a primed and responsive AOS and a greater CTmax. It is possible that the greater physiological plasticity and resilience found in *T. dzilamensis* could have increased its potential to colonize new saline under-ground environments beyond the limits of those where *T. mitchelli* and *T. pearsei* could endure.

Considering that osmoregulation in euryhaline crustaceans requires, among other things, an increase of metabolic rate (Ramaglia et al., 2018), it is likely that only those individuals with a broad AS can meet the energy expenditure that the change in environmental salinity demands. Based on our results and given that *T. dzilamensis* is the only species recorded in both fresh and marine groundwater bodies of the Yucatan Peninsula (Benítez et al., 2019), it may be the only one of the three studied species with the physiological plasticity required to cope with such osmotic challenge. In the hypothetical case that an individual of *T. dzilamensis* could cross the halocline, it would face an abrupt and extreme environmental change that requires an instantaneous increase in energy production to cope with the demand. Physiological adjustments such as these have been previously described in *Halocaridina rubra*, an Atyid from Hawaiian anchialine systems where salinity fluctuates abruptly and frequently (~20‰ in 24-hour periods). This species presents structural modifications, an increase in mitochondria-rich cells (involved in osmoregulation) in the gills (Havird et al., 2014a), and maintains active its physiological mechanisms for ion regulation as well as the expression of genes involved in osmoregulation (Henry, 2001; Jayasundara et al., 2007; Rahi et al., 2018; Havird et al., 2019). Although the presence of these adaptations has not been studied in any *Typhlatya* species, similar adaptations in *T. dzilamensis*, additional to an increased physiological plasticity, may be the key for explaining how this species can be present in both fresh and marine groundwater.

Food availability and primary production in anchialine systems are heterogeneous. Cenotes receive a greater input of allochthonous organic matter and, in some instances, there can be photosynthesis (Pohlman et al., 1997; Chávez-Solís et al., 2020). In contrast, caves are considered oligotrophic due to a severe and continuous lack of food availability (Brankovits et al., 2017; Hershey and Barton, 2018). In environments where food is scarce, the survival of a population may be determined by extreme physiological modifications. Such is the case of cavefish *Astyanax mexicanus* that have developed insulin resistance, allowing them to survive for long periods without feeding (Riddle et al., 2018). Auer *et al*. (2018) observed that juvenile wild salmons with greater MMR had higher survival rates in environments with lower food availability, suggesting that higher MMR confers greater competitive ability in an environment where food is the limiting resource. Along with the differences in the dietary carbon apportionment amongst the studied *Typhlatya* species, (Chávez-Solís et al., 2020), it seems that the higher MMR observed in *T. dzilamensis* could enhance its fitness in the oligotrophic cave environment below the halocline. Under the strong selective pressure exerted by food scarcity, a higher AS could represent an expansion in the availability of potential energy for foraging.

One noteworthy aspect of the exercise-derived metabolic responses measured herein, is the high intraspecific heterogeneity observed in *T. dzilamensis*. In addition to the inherent variability of measuring physiological indicators in the laboratory, it is necessary to keep in mind that the experiments were conducted *in situ*, with wild organisms that had not been subject to previous treatments that could standardize their immediate previous experiences. Although we do not have enough information now to explain these differences, sources of variation in metabolic descriptors of individuals of the same species are usually related to size, reproductive state, feeding, sex, and molting stage in the case of crustaceans (Alter et al., 2015; Chabot et al., 2016b). While it can be assumed that these sources of variation were common to the three studied species, individuals of *T. mitchelli* and *T. pearsei* were more homogeneous in their responses compared to *T. dzilamensis*. These differences may be explained if physiological responses to stress that are smaller in magnitude tend to be more homogeneous than those larger in magnitude.

Aerobic metabolism in the mitochondria generates about 90% of ROS (Balaban et al., 2005), even during basal metabolism (Sies, 1993), and ROS generation increases with increasing metabolic rate (Sies, 1993; Storey, 1996; Rosa et al., 2008; Rodríguez-Fuentes et al., 2017). An increased production of reactive molecules can damage proteins such as DNA and lipid membranes and, if the propagation of these ROS is not prevented, the organism may undergo severe cell stress, lose functionality, or ultimately die. The AOS response in all three *Typhlatya* species accompanied the levels of aerobic metabolism following physical activity. As *T. dzilamensis* increased oxygen consumption to a higher extent, the antioxidant system was activated accordingly to the increase in ROS production, thus preventing cellular damage. In contrast, *T. mitchelli* and *T. pearsei* did not significantly increase oxygen consumption and consequently did not show an increase in AOS indicators or cell damage. As expected, *T. mitchelli* and *T. pearsei* are display an efficient regulation of ROS even under induced metabolic activity, demonstrating that in the caves where they inhabit, the energetic and biochemical mechanisms triggered by maximum activity are highly efficient.

A striking difference from the antioxidant analysis was the high quantity of GSH in BL individuals of *T. dzilamensis* compared to *T. mitchelli* and *T. pearsei*. Storey (1996) was the first to propose that animals that frequently face oxidative stress maintain the AOS constitutively activated. This strategy has been termed "defensive priming" and was first postulated for species with high thermal tolerance (Dong et al., 2008). A similar mechanism has been postulated for sulfur-tolerant populations (Joyner-Matos and Julian, 2012). An alternative strategy to defensive priming is a quick activation of the AOS when ROS generation increases and the activation of oxidative stress repair mechanisms is needed (Joyner-Matos and Julian, 2012). The great amount of GSH observed in *T. dzilamensis* may be indicative of an ongoing defensive priming strategy, as an adaptive trait to a more heterogeneous environment associated with frequent interactions with the halocline and hypoxia conditions surrounding it. In contrast, *T. mitchelli* and *T. pearsei* may have a more conventional stress response strategy, as they exclusively inhabit freshwater environments, such as Tza Itza and Nohmozon where there is little or no interactions with haloclines (Chávez-Solís et al., 2020).

The metabolic response to temperature change has proven to be an indicator of the physiological plasticity of organisms in the face of environmental variability (Fry, 1947; Pörtner, 2010). Given that temperature is remarkably constant in these anchialine systems (compared to surface systems), both on daily and annual time scales (Chávez-Solís, 2015; Mejía-Ortíz et al., 2020), it was expected that the three species would exhibit similar thermal tolerance. However, *T. dzilamensis* presented a higher CTmax than *T. mitchelli* and *T. pearsei*, further supporting the idea of a differential physiological plasticity even amongst these closely related species. Likewise, a greater physiological plasticity has been linked to higher thermal tolerance and the potential to invade and colonize environmentally

diverse habitats (Sokolova and Pörtner, 2003; Lande, 2009; Mascaró et al., 2016). Tepolt and Somero (2014) suggested that due to its thermal tolerance (measured as critical thermal maxima and cardiac function), *Carcinus maenas* could colonize large subpolar areas of the European continent, as well as temperate and subtropical areas of the American continent. Vinagre *et al*. (2016) and Cumillaf et al., (2016) found a direct relationship between the size of the thermal window width and tolerance to warming in 20 species of crustaceans and fish, showing that species from temperate environments and wider thermal windows have greater tolerance to warming than the tropical species. A wider thermal window in *T. dzilamensis* further emphasizes the greater tolerance of this species to environmental heterogeneity.

The critical temperature is a metric that integrates cellular, biochemical and tissue scales which allows a direct comparison of temperature sensitivity among populations, species and communities (Bates and Morley, 2020). The similarity of the biochemical indicators from BL individuals of all species and those who underwent the critical thermal limits procedures is evidence that the method complies with the theoretical expectations when applied to *Typhlatya*, and that there is a difference in thermal tolerance among these species.

None of the species studied showed LAC accumulation derived either from exercise or short-term exposure to critical temperatures, which indicates that these conditions or procedures did not require resorting to anaerobic metabolism to satisfy the energy demand. It is noteworthy, however, the significantly higher lactate concentration in *T. dzilamensis* under base line conditions compared to *T. mitchelli* and *T. pearsei*. Records of *Typhlatya* swimming in sulfur clouds (Pakes et al., 2014), hypoxic zones (Brankovits et al., 2017; Chávez-Solís et al., 2020), as well as lower levels of dissolved oxygen below the halocline in Ponderosa (Chávez-Solís et al., 2020), suggest that *T. dzilamensis* may have to resort to anaerobic metabolism frequently. It is possible that these behaviors together with habitat conditions common to *T. dzilamensis* result in chronic lactate accumulation. Such an effect has been reported in *Niphargus virei*, a hypoxia-resistant amphipod (Hervant et al., 1999), where increased lactate during periods of hypoxia can be *de novo* synthesized to glycogen once re-oxygenation is possible. Similarly, experiments with *H. rubra* (Atyidae) have revealed their capacity to survive up to 7 days under anoxia conditions, without any oxygen consumption (Havird et al., 2014b). Although lactate concentration was not measured, the authors concluded that this species must rely on anaerobic metabolism to meet metabolic energy needs. The rise of these and other questions on lactate fluctuations and the role of anaerobiosis in supplying energy makes it necessary to further investigate hypotheses about unexpected physiological capacities in species that inhabit environments where hypoxic or anoxic conditions are common.

According to Pigliucci *et al*. (2006), phenotypic plasticity can be defined as the attribute of an individual's genotype to produce different phenotypes in response to environmental stimuli. Physiological plasticity, therefore, is a component of the response repertoire of the expressed phenotypes and is related to the organic functioning of systems at various levels of organization. It is also postulated that physiological plasticity allows the emergence of a new environmentally induced phenotype, the expression of which can be fixed in a new environment through a process of natural selection (Pigliucci et al., 2006). Recent ancestral reconstructions support the hypothesis of a low salinity last common ancestor for the Yucatan *Typhlatya* (Ballou et al., In Press), which in turn suggests that the salinity tolerance trait observed in *T. dzilamensis* is an independent transition. Results herein provide evidence to support that the physiological plasticity observed in *T. dzilamensis* was naturally selected by the heterogeneous environment they inhabit.

5 Concluding remarks

The species of the genus *Typhlatya* in the present study possess contrasting physiological traits that correspond to the environmental characteristics of the microhabitat in which each species is distributed. *T. dzilamensis*, commonly found in the subterranean saltwater environment, displayed the greatest physiological plasticity compared to *T. mitchelli* and *T. pearsei*, hence has a greater resilience to environmental fluctuations. The activity of GSH and lactate concentration in *T. dzilamensis* further suggest that this species has a permanent "defensive priming" strategy that keeps the antioxidant system constitutively prepared for the eventuality of physiological stress, a recurrent condition in the relatively heterogeneous environment they inhabit.

In contrast to *T. dzilamensis*, the freshwater species *T. mitchelli* and *T. pearsei*, were very similar in exhibiting low aerobic scope, low lactate and an overall low reactivity to imposed physical activity. This suggests that *T. mitchelli* and *T. pearsei* have less physiological capacity for an immediate response to intensive exercise, which indicates a reduced plasticity, and thus expected to be more vulnerable to abrupt environmental fluctuations.

Current anthropogenic pressures on the aquifer may pose an increased risk to these species, all the more knowing that freshwater species may be particularly susceptible to environmental shifts. Future research should focus on evaluating physiological limits and resilience of species to changes in salinity, dissolved oxygen, and the presence of contaminants in the aquifer for the purpose of prevention and restauration of subterranean aquatic ecosystems.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

EC, CR and MM contributed to conception and design of the study. EC and MM performed the statistical analysis and prepared the figures. EC and GR obtained and analysed the biochemical indicators of all individuals. EC, FD, DR and CR performed field experiments and analysed metabolic response and thermal tolerance. EC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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908

909 **11 Tables**

910 Table 1 Results of the permutational MANOVA on the biochemical indicators of individuals of *T.*
911 *mitchelli*, *T. pearsei* and *T. dzilamensis* subjected to five minutes of exercise and sampled at four
912 time intervals: 0 (immediately), 30, 60 and 120 minutes after physical activity (T0 – T3 respectively),
913 which were compared to baseline individuals (BL). A) Overall MANOVA; B) Post hoc comparisons
914 between post-exercise and BL individuals within each species.

A) Overall MANOVA						
Variation source	df	SS	MS	*pseudo-F*	P	Unique perms
Species (S)	2	36.385	18.193	3.5801	< 0.001	9924
Time (T)	4	49.909	12.477	2.4554	< 0.001	9907
S x T	8	59.24	7.405	1.4572	< 0.05	9887
Residuals	67	340.47	5.0816			
Total	81	486				
B) Paired post hoc comparisons						
Compared groups				*pseudo -t*	P	Unique perms
Within level "*T. dzilamensis*" of factor "species"						
T0, T1				0.81517	0.704	10
T0, T2				1.0954	0.4001	10

T0, T3	1.1829	0.316	56
T0, BL	2.1528	< 0.05	1140
T1, T2	0.58873	0.9009	10
T1, T3	0.42492	0.9819	56
T1, BL	2.2105	< 0.01	1140
T2, T3	0.66481	0.7898	56
T2, BL	1.6311	0.0538	1139
T3, BL	2.6353	< 0.001	8345
Within level "*T. mitchelli*" of factor "species"			
T0, T1	0.55682	1	10
T0, T2	0.75895	0.9043	10
T0, T3	1.2782	0.1741	120
T0, BL	1.678	< 0.05	364
T1, T2	0.46368	0.9037	10
T1, T3	0.9655	0.4358	120
T1, BL	1.4068	0.0693	364
T2, T3	0.6044	0.8831	120
T2, BL	1.1359	0.2543	364
T3, BL	1.2173	0.182	8556
Within level "*T. pearsei"* of factor "species"			
T0, T1	0.57311	0.8006	10
T0, T2	0.83583	0.4947	10
T0, T3	0.97637	0.4439	165
T0, BL	0.97867	0.4435	120
T1, T2	0.87674	0.9001	10
T1, T3	1.1917	0.231	165
T1, BL	0.9194	0.5538	120
T2, T3	0.99714	0.4047	165
T2, BL	0.43791	0.9376	120
T3, BL	1.1502	0.2594	5059

915 Table 2 Lactate content (LAC) in BL and post-exercise individuals of *T. mitchelli*, *T. pearsei* and *T.*
916 *dzilamensis*. a) Overall ANOVA. b) post hoc contrasts, Tukey's multiple mean comparison.

A) Overall ANOVA (LAC)

Variation source	df	SS	MS	*F*	*p*
Species	2	0.67	0.33	7.78	<0.001
Residual	72	3.08	0.04		
Total	74	3.75			

B) Tukey's multiple mean comparison (LAC)

Compared groups	Mean difference	Lower limit	Upper limit	adjusted *p*
T. pearsei vs. T. mitchelli	0.01	-0.13	0.15	0.99
T. dzilamensis vs. T. mitchelli	0.20	0.06	0.34	<0.01

T. dzilamensis vs. T. pearsei	0.20	0.05	0.34	<0.01

Table 3 Results of the permutational MANOVA on the biochemical indicators of individuals of *T. mitchelli*, *T. pearsei* and *T. dzilamensis* subjected to procedures to obtain the critical thermal maximum and minimum (CTmax and CTmin) which were compared to baseline individuals (BL). A) Overall MANOVA; B) Post hoc comparisons between pairs of species for each treatment level.

A) Overall MANOVA						
Variation source	df	SS	MS	*pseudo-F*	*P*	Unique perms.
Species (S)	2	83.2	41.6	8.93	< 0.001	9929
Treatment (T)	2	13.83	6.92	1.49	0.131	9936
S x T	4	35.68	8.92	1.92	< 0.01	9908
Residual	63	293.29	4.66			
Total	71	426				
B) Paired post hoc comparisons						
Compared groups				*pseudo-t*	*P*	Unique perms.
T. dzilamensis						
BL *vs* CTmax				1.69	0.031	9482
BL *vs* CTmin				1.37	0.105	8278
CTmax *vs* CTmin				1.04	0.426	462
T. mitchelli						
BL *vs* CTmax				0.81	0.688	9333
BL *vs* CTmin				0.63	0.862	6909
CTmax *vs* CTmin				0.84	0.656	2893
T. pearsei						
BL *vs* CTmax				1.66	0.058	1706
BL *vs* CTmin				1.21	0.222	1712
CTmax *vs* CTmin				1.75	0.021	462

Table 4 Lactate accumulation before and after attainment of critical thermal limits in individuals of *T. mitchelli*, *T. pearsei* and *T. dzilamensis*.

A) Overall ANOVA					
Variation source	df	SS	MS	*F*	*p*
Species	2	0.56	0.28	6.744	<0.01
Residual	60	2.49	0.04		
Total					

B) Tukey's multiple mean comparison				
Compared groups	Mean difference	Lower limit	Upper limit	adjusted *p*
T. pearsei vs. T. mitchelli	0.02	-0.13	0.18	0.93
T. dzilamensis vs. T. mitchelli	0.21	0.06	0.35	<0.01
T. dzilamensis vs. T. pearsei	0.18	0.03	0.34	<0.05

12 Figure legends

Figure 1 Flow chart showing the methods used for the metabolic and biochemical characterization of *T. mitchelli*, *T. pearsei* and *T. dzilamensis*. The number outside each box indicates the number of individuals (n) used for each experimental test or control. Baseline (BL) individuals are those that did not undergo an experimental treatment. The evaluation of biochemical indicators (BI) includes indicators of the antioxidant system: catalase (CAT), total glutathione (GSH), glutathione S-transferase (GST), and superoxide dismutase (SOD); indicators of oxidative damage: lipid peroxidation (LPO) and protein carbonylation (PO) and lactate (LAC) accumulation by anaerobic metabolism.

Figure 2 **(A)** Routine metabolic rate (RMR), maximum metabolic rate (MMR) and aerobic scope (AS) of *T. mitchelli*, *T. pearsei* and *T. dzilamensis*. Numbers above the columns are the mean oxygen consumption values for each species and treatment, also showing a standard deviation. **(B)** Principal coordinate analysis (PCoA) of oxygen consumption of individuals of the three species in RMR, MMR, and AS.

Figure 3 Principal coordinate analysis (PCoA) of antioxidant system indicators (CAT, GST, GSH and SOD) and cell damage (PO and LPO) from untreated (BL) *T. mitchelli*, *T. pearsei* and *T. dzilamensis* and individuals sampled 0, 30, 60 and 120 minutes (T0 to T3) after induced activity.

Figure 4 Critical thermal maximum and minimum of *T. mitchelli*, *T. pearsei* and *T. dzilamensis*. The black line in each box shows the median of each group, the white dot the mean, the whiskers extend to an extreme data not greater than two standard deviations and the black dots represent outliers.

Figure 5 Principal coordinate analysis (PCoA) of antioxidant system indicators [Catalase (CAT), Glutathione S-transferase (GST), total Glutathione (GSH) and Superoxide dismutase (SOD)] and cellular damage [protein carbonylation (PO) and lipid peroxidation (LPO)] of *T. mitchelli*, *T. pearsei* and *T. dzilamensis* individuals sampled at the end of the critical thermal limits procedures (CTmax and CTmin), and compared to baseline (BL) individuals.

Figure 4.JPEG

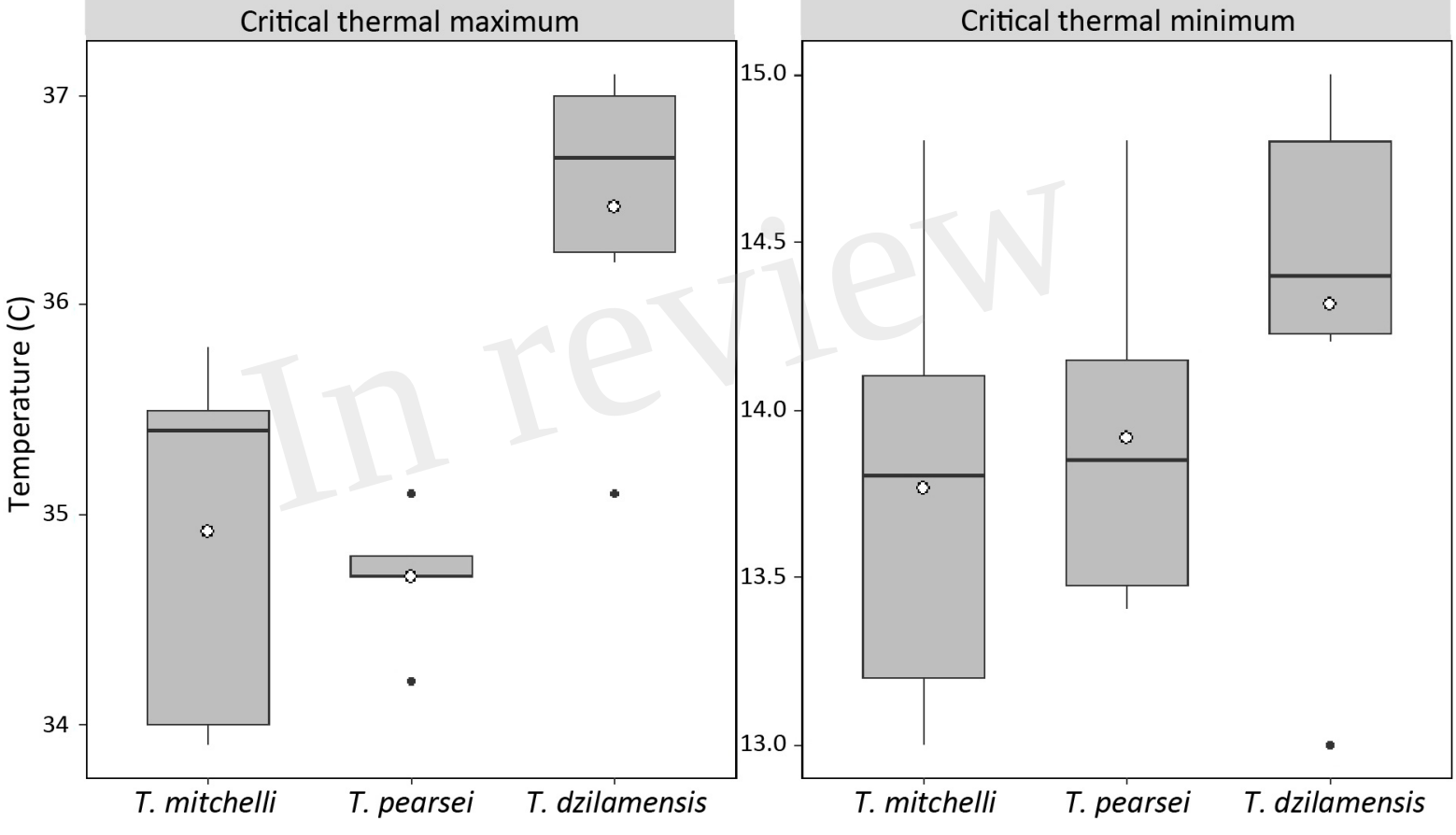


Figure 5.JPEG

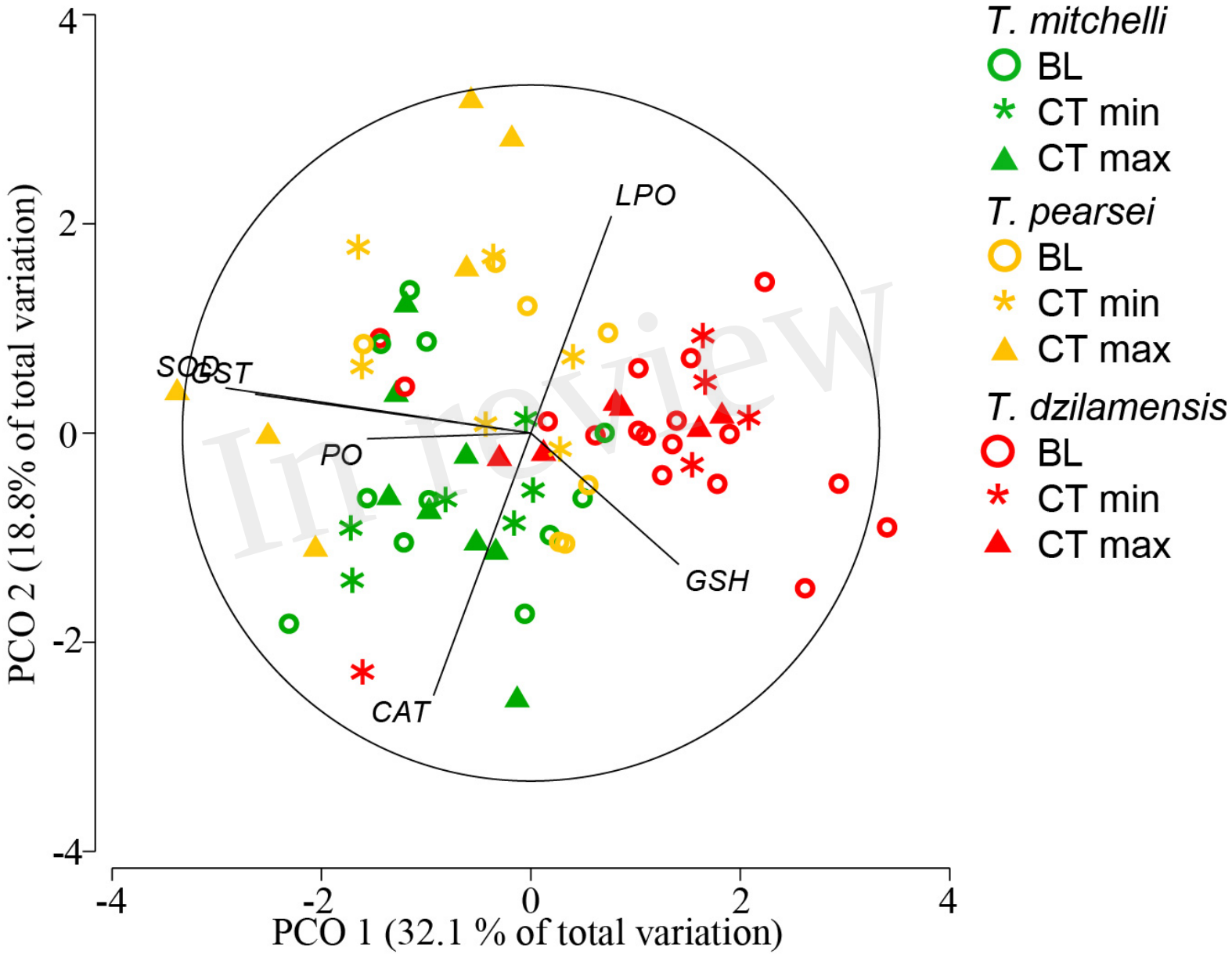


Figure 1.JPEG

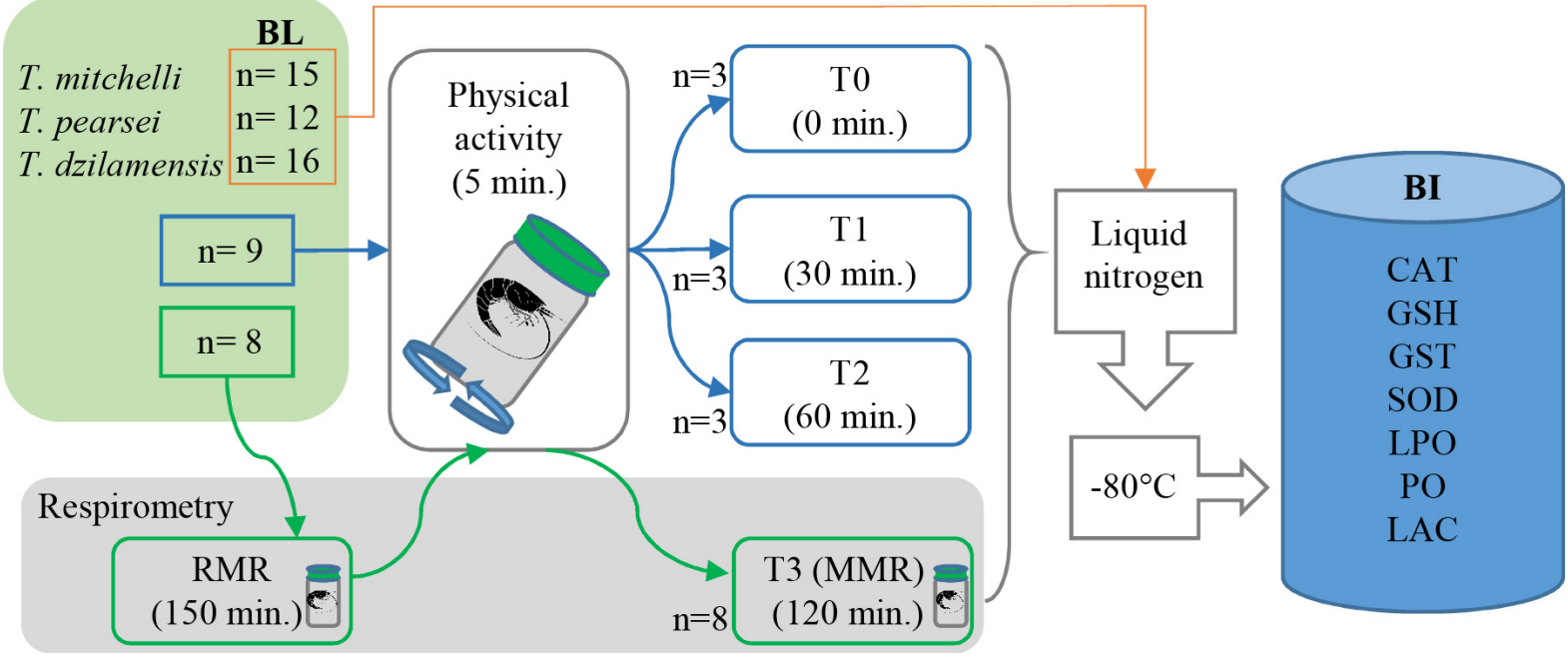
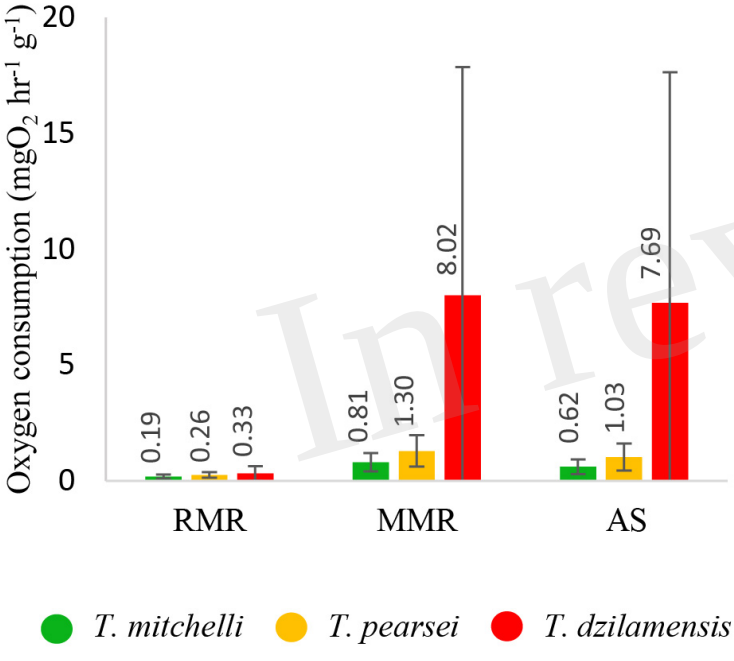


Figure 2.JPEG



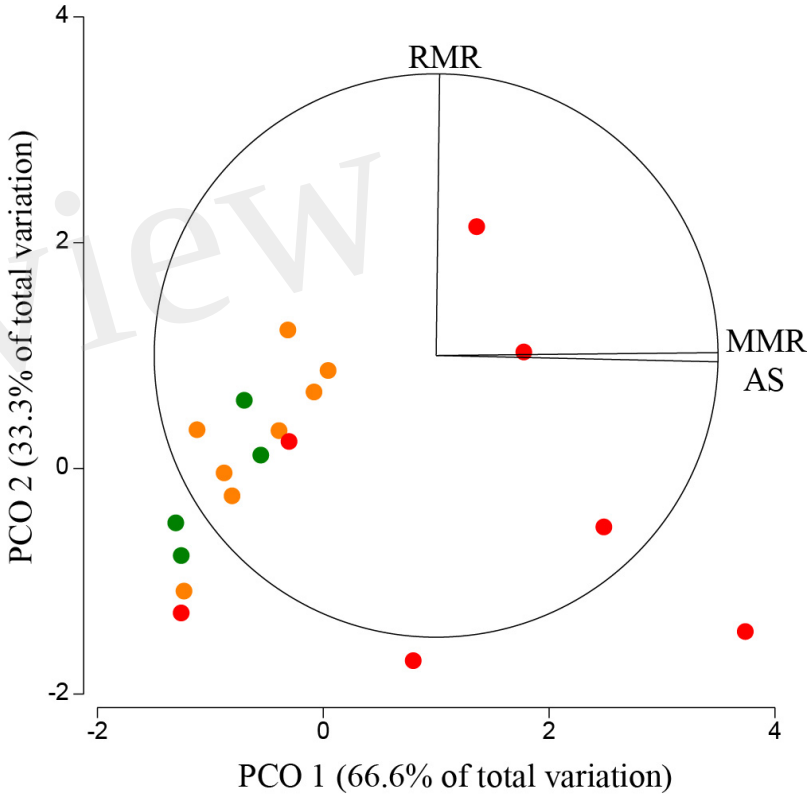
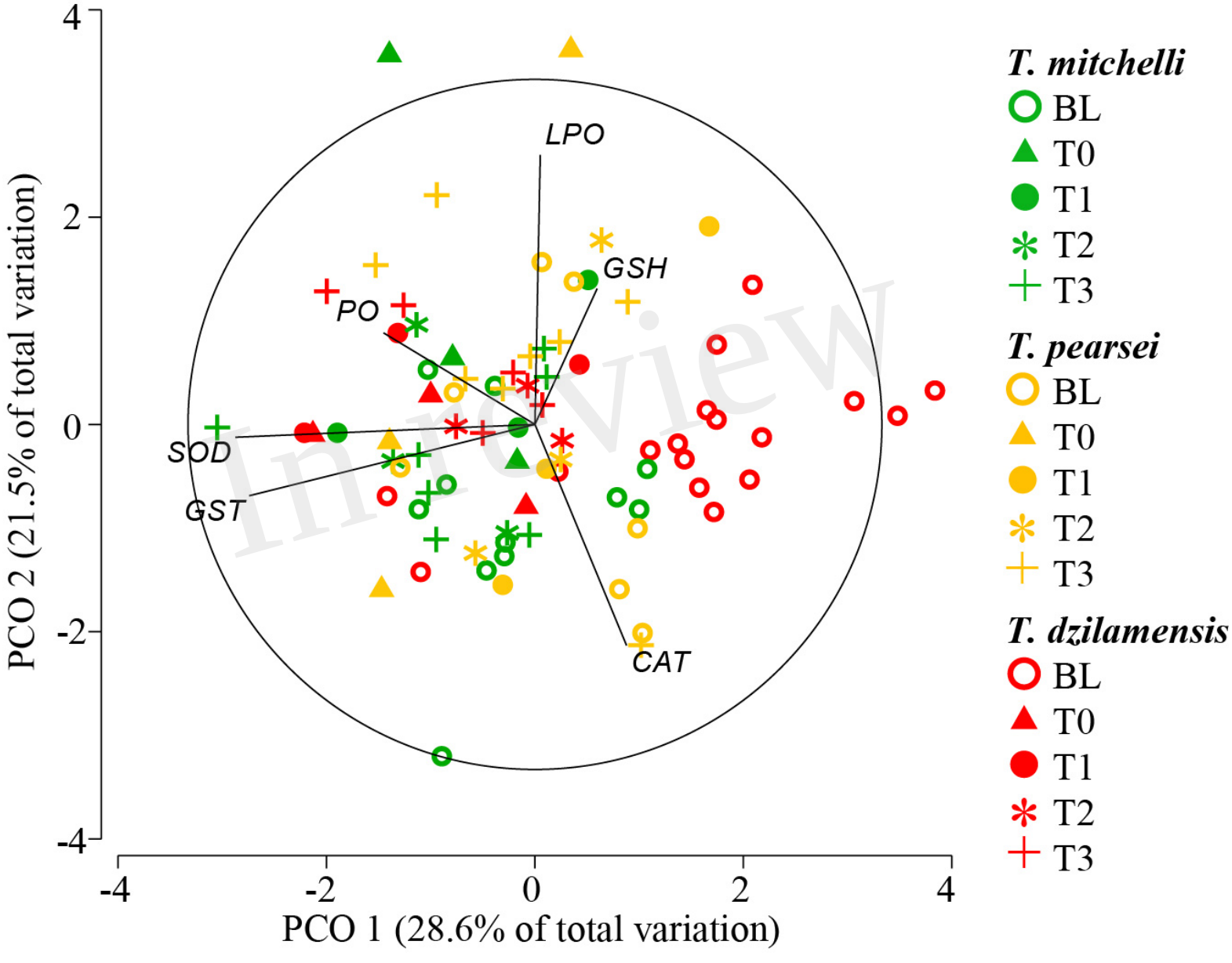


Figure 3.JPEG



Discusión y Conclusiones generales

Este es el primer trabajo sobre la ecofisiología de tres especies estigobíticas cercanamente emparentadas del género *Typhlatya*. Los resultados contribuyen a entender cómo se sostiene la biodiversidad de estigobiontes en los ecosistemas anquihalinos. Las sutiles diferencias ecológicas y fisiológicas entre estas especies explican su coexistencia y describen las estrategias fisiológicas que permiten su desarrollo en el ambiente anquihalino de la Península de Yucatán. Como uno de los representantes más abundantes y ampliamente distribuidos del subsuelo de la Península de Yucatán, el género *Typhlatya* se revela crucial en la transferencia de energía en la red trófica y el ciclo de nutrientes en el acuífero.

Este trabajo describe patrones de distribución, de comportamiento, y aporte de carbono de diferentes orígenes que evidencían una partición de nicho ecológico, la cual permite la coexistencia de estas especies en ambientes, que son principalmente oligotróficos. Además, se identificaron características y respuestas fisiológicas particulares a cada especie que coinciden con el hábitat de distribución.

Se puede concluir que cada especie tiene rasgos particulares que las distinguen ecofisiológicamente de las demás. En particular, *T. dzilamensis* se observa como la especie con mayor plasticidad fisiológica, con aporte de carbono de fuentes de origen diverso (moderno y antíguo), distribuido en la zona de cueva y en el agua salada. Estas características la distinguen como la especie con mayor capacidad de respuesta ante cambios ambientales, lo que permite postular que también podría ser la más resiliente. Con respecto a las especies evaluadas en agua dulce, la diversidad de fuentes de aporte de carbono, en conjunto con el potencial metabólico aerobio registrado en *T. pearsei*, sugieren que esta tiene mayor capacidad de respuesta ante variaciones adversas en comparación con *T. mitchelli*. Finalmente, la falta de diversidad de fuentes de carbono, la baja respuesta metabólica y del sistema antioxidante, sugieren que *T. mitchelli* pudiera ser la especie más sensible a cambios ambientales, y por ello, la más vulnerable.

El aquífero de la Península de Yucatán está siendo actualmente sometido a presiones de diversa índole y de gran envergadura. La falta de programas de prevención y medidas de mitigación de los riesgos de contaminación y sobreexplotación de esta vital fuente de agua

puede derivar en cambios ambientales drásticos a corto plazo. Escenarios de impacto ambiental como el que se prevee ponen en alto riesgo a la estigofauna y al conjunto de los ecosistemas anquihalinos, por lo que se sugiere atención especial a las actividades sanitarias y agroindustriales que se desarrollan en la plataforma cárstica de la Península de Yucatán.

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