TARDIGRADES IN TEXAS: FIFTH GRADERS COLLABORATE TO ADD THREE NEW RECORDS TO THE STATE

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Abstract.—Tardigrades, or water bears, are resilient microscopic animals found in terrestrial and aquatic habitats. Because tardigrades can be easily found and yet are understudied, fifth graders from Hill Elementary in the Austin Independent School District in Texas collaborated with their teacher and an expert to conduct a biodiversity survey of microscopic animals found in lichens and mosses on their school campus. These ten-year-olds learned to differentiate between tardigrades, rotifers, and nematodes as they collected samples from different habitats. In their first year of study, they observed 520 microscopic animals and found differences in their diversity and distribution patterns. A second study of 14 samples revealed 68 tardigrades from eight identified species, representing two classes, four orders, and five genera. Three of these species have never been recorded from the state of Texas and represent the first records for the state: *Viridiscus perviridis* (Ramazzotti, 1959), *Milnesium* cf. *alpigenum* (Ehrenberg, 1853), and *Milnesium* cf. *brachyungue* (Binda & Pilato, 1990). Furthermore, *Mil. alpigenum* and *Mil. brachyungue* were previously unknown in North America, thus representing the first records for both species on the continent. This experience demonstrates that students challenged with basic research questions can learn to conduct field research, carry out scientific surveys, collaborate with scientists and discover new knowledge. In this case, students learned to find tardigrades and contributed to our overall understanding of this phylum.

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Tardigrades are members of the phylum Tardigrada and are found across all continents. Their cosmopolitan distribution is tied to their small size (0.2–0.5 mm in length) and their ability to survive long periods of desiccation through a process called cryptobiosis, where the tardigrade shrivels into a flake of "dust" called a tun (Kinchin 1994). In this state, they have survived extreme conditions such as high pressures

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(Seki & Toyoshima 1998), ultraviolet rays (May et al. 1964), vacuums (Horikawa 2012), high temperatures (Baumann 1922), temperatures near absolute zero (Becquerel 1950) and being frozen for periods as long as 30 years (Tsujimoto et al. 2016). These abilities led to speculation about tardigrades' ability to survive space travel (Miller 1997). Then, in 2008, they became the first multi-celled organism to survive exposure to the vacuum and radiation of space (Jönsson et al. 2008, 2016). When in cryptobiosis, tardigrades could be considered the toughest animal on earth (Copley 1999).

Despite what we know about the survivability of water bears, we know little about their diversity, distribution, and ecology (Ramazzotti & Maucci 1983, Miller 1997). More than 1,300 species (approximately 200 marine, 200 freshwater and 900 terrestrial) have been described (Degma et al. 2020), yet little distribution and diversity data exists for most of the world.

Originally described in 1776 (Spallanzani 1776), it was not until 1873 that the phylum was documented in North America (Packard 1873). It was another 65 years before Mathews (1938) reported the first tardigrade from Texas. Over the next 75 years, only 12 additional species of tardigrades were found in Texas (Meyer 2013). Then in 2014, the All Taxa Biological Inventory (ATBI) in the Big Thicket National Preserve in southeast Texas increased the number of known species for the state to 37 (Hinton et al. 2014). Currently, water bears have been reported in only nine of 254 counties in Texas suggesting that state diversity may be much greater (Figure 1).

While the states neighboring Texas report similar numbers (Louisiana: 30 species; New Mexico: 21 species; Oklahoma: 20 species), the Texas diversity is only about half that of states where designed biodiversity studies have been conducted (Alaska: 70 species; California: 56 species; Tennessee: 66 species) (Kaczmarek et al. 2016) (Figure 2). Bartels et al. (2016) estimate that there may be more than 4,000 species yet to be discovered worldwide. Thus, there should be far more diversity in Texas.

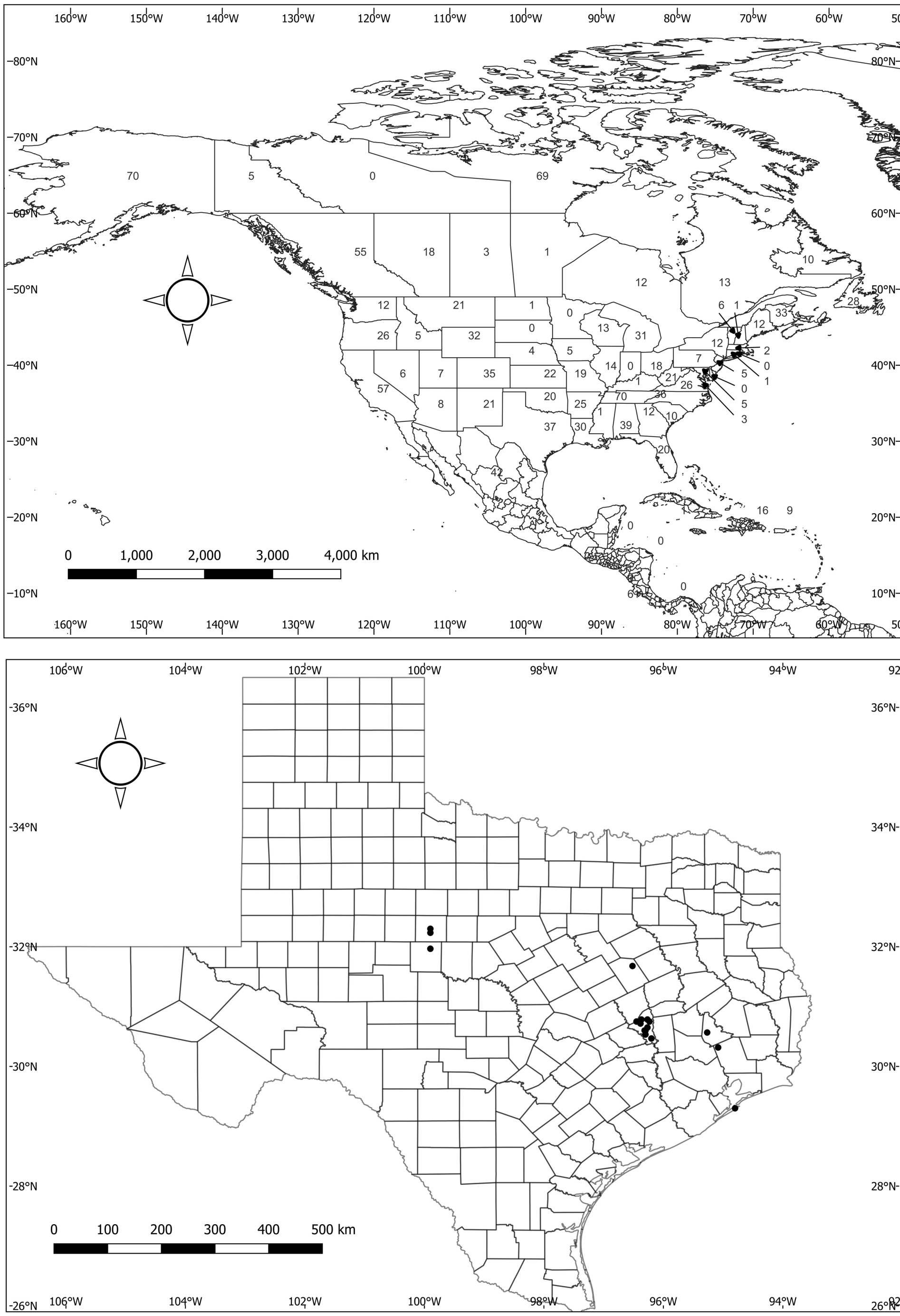

Figure 1. Distribution of tardigrades in North and Central America (top; numbers indicate the number of species reported per state, province, or country), and in Texas (bottom; circles indicate counties from which tardigrades have been reported).

Tardigrades are assumed to be distributed by the wind (Kinchin 1994), but recent reports suggest that migratory birds (Mogle et al. 2018) and small mammals (Villella et al. 2020) may also be contributors in tardigrade dispersal. Little is known about tardigrade ecology or how they positively or negatively affect life around them. While tardigrades do not cause human diseases, they can harbor and transport pathogenic bacteria that can damage crops (Kranz et al. 1999, Benoit et al. 2000). Recent data suggests tardigrades may be decisive in maintaining the balance of harmful, parasitic nematodes in the soil around the roots of trees (Sanchez-Moreno et al. 2008). Additional research is needed to further our understanding of the tardigrade role in ecosystems, and Hill Elementary students intend to be a part of that research.

The authors embarked on this project to explore the use of tardigrade studies to introduce the basic research process to grade school students as a tool for discovery (Miller & Case 1998, Case & Miller 1999). In doing so, fifth graders conducted a biodiversity survey on their campus to answer the question, "Are there more tardigrades in lichen or moss?" The idea of a null hypothesis was introduced, and students predicted they would find the same numbers of animals and the same mix of species between lichen and moss and at different heights from the ground. This report documents the design, process and results of their first year of study and adds three species to the biodiversity list for the state of Texas.

MATERIALS & METHODS

Moss and lichen samples were taken from substrate trees and man-made structures on the grounds of Hill Elementary in Austin, Travis County, Texas (30.3764°N, -97.7486°W), by students in the senior author's fifth grade class, as well as members of the senior author's new after school club, known as the "Water Bear Club." Google Maps and iPads were used to find the GPS coordinates of substrate trees. The species of tree (if applicable) and the height above ground (base of tree (0–1.5 m) and canopy level (4.5–15 m)) were recorded. Samples were removed by hand, placed in small plastic or paper bags and labeled with location, habitat data, and a unique sample code.

To characterize the animals in each sample, one gram of lichen or moss was randomly separated and weighed then submerged in 50 mL of tap water in marked beakers for 1–24 hours. Because tardigrades are denser than water, they sink to the bottom of a dish. Thus, one mL of water and debris was vacuumed from the bottom of each aqueous preparation with a disposable pipette and placed in 25 by 50 by 15 mm petri dishes for examination. Students used dissecting microscopes with a magnification of 20x to scan for and count tardigrades, rotifers, and nematodes.

Two separate exercises were conducted by the students based on their interest and increased skills through the school year. In the first exercise, students counted and compared the meiofaunal animals (nematodes, rotifers, and tardigrades) in their habitat samples. Students recorded the data on a large sheet mounted on the wall above their microscopes. Students then transferred this data to an Excel spreadsheet in order to classify, compare and analyze the population numbers based on habitats, substrates and sample height.

In the second exercise, students examined the species diversity of tardigrades in their habitat samples. Tardigrades were collected with an Irwin loop (Schram & Davidson 2012) or micropipette and placed on microscope slides with polyvinyl alcohol (PVA) media under a cover slip. Slides were assigned a unique number and labeled with a sample code with a permanent felt pen. The location of the tardigrade on the slide was marked with dots on the cover slip. Some students learned to identify tardigrades to family and genus with their student-grade compound microscopes, using a dichotomous visual chart (Miller, unpublished data) and the keys from Ramazzotti & Maucci (1983) and Pilato & Binda (2010).

To verify their identifications, the students sent a set of habitat samples to the second author (Miller) who processed them the same way and mounted the specimens on slides. Miller imaged the specimens with an Olympus DX60 compound microscope using DIC (Differential Interference Contrast) microscopy and a DX72 8-megapixel camera and returned an illustrated PowerPoint report identifying the species for the students. Nomenclature follows Guidetti & Bertolani (2005), Degma & Guidetti (2007), and Degma et al. (2020).

Significance between expected and sampled diversity and density by locations and habitat were tested using chi square (Zar 2010). A 0.05 confidence level of chi square for two degrees of freedom $\chi^2 > 3.84$ was used to accept or reject a hypothesis of similarity.

RESULTS

For the first exercise, students collected 64 habitat samples (21 moss, 29 lichen, and 14 moss-lichen) from two height levels (base and canopy) from 15 trees and structures at Hill Elementary from 23 January to 11 April 2018. Fifty-eight of the 64 samples (89%) contained 520 microscopic meiofaunal animals. Students found 49.4% were rotifers, 35.2% were nematodes, and 12.1% were tardigrades (Table 1). From a hypothesis of uniformity among the three phyla (520/3=173), rotifers occurred more frequently and tardigrades occurred less frequently than expected. Both rotifers and nematodes were found more frequently in the canopy samples than the base samples while the tardigrades were about evenly distributed between the levels.

Sixty-five tardigrades were found in the 64 samples for an average density of 1.0 per sample. Yet, only 24 of the 64 samples (37%) yielded tardigrades for an average density of 3.69 per positive sample. Fifty-seven percent (37 of 65) of tardigrades were found in lichens compared to 31% (20 of 65) found in moss and just over 12% (8 of 65) found in moss-lichen mixtures (Table 1). From a hypothesis of uniformity (65/3=22.6), tardigrades were found in lichen significantly more often

Table 1. Meiofaunal population survey for rotifers, nematodes, and tardigrades in moss and lichen samples from tree trunks (0-1.5 m) and canopies (4.5-15 m) at Hill Elementary January-April 2018.

	Rotifer			Nematode			Tardigrade		
	Moss	Lichen	Both	Moss	Lichen	Both	Moss	Lichen	Both
Trunk	72	35	4	22	30	3	9	21	1
Canopy	41	63	45	44	82	14	11	16	7
Total	113	98	49	66	112	17	20	37	8

than expected and in mixed habitats significantly less than expected.

In terms of collection height, 47.7% (31 of 65) of the tardigrades were found on the base of the trees at heights ranging from 0–1.5 m while 52.3% (34 of 65) of tardigrades were found in the canopy at heights above 4.5 m. There were no significant differences in the number of water bears found at different heights (Table 1).

Students attempted to identify tardigrades to species but found their depth of knowledge in tardigrade taxonomy forced them to settle for genus level identifications. They were able to distinguish among *Milnesium, Macrobiotus, Ramazzottius*, and *Minibiotus* (Table 2.)

In order to identify the species on their campus, the students sent 14 additional habitat samples collected on the 2nd and 3rd of March 2018 to the second author. These samples included nine mosses and five lichens from three different substrates: trees (8), roofs (2), and ground samples (4). Eight of the 14 samples (57.1%) were positive for tardigrades (three lichens and five mosses). Sixty-eight tardigrades were recovered, 51 (75%) from moss samples and 17 (25%) from lichens. Of the 68 tardigrades found, 4 (6%) were from ground substrate, 21 (31%) from tree substrates and 43 (63%) from habitat growing on a roof. Tardigrade density averaged 4.8 individuals per collected sample and 8.5 individuals per positive sample with a range from 0 to 31 specimens per sample.

With 68 tardigrades from 14 samples with hypothesized uniformity, we expected 4.85 tardigrades per sample. With 51 specimens from eight

Table 2. Hill Elementary tardigrade survey data collected March 2-3, 2018. Abbreviations for level category are canopy (C), trunk (T) , roof top (RT), roof lower (RL), and ground (G). Abbreviations for tardigrade genera are *Macrobiotus* (*Mac*), *Minibiotus* (*Min*), *Milnesium* (*Mil*), *Ramazzottius* (*Ram*), and *Echiniscus* (*Ech*).

Sample	Substrate	Level	Habitat	*Mac*	*Min*	*Mil*	*Ram*	*Ech*
TX1002	*Q. virginiana*	C	Moss				2	
TX1003	*Q. virginiana*	T	Lichen			1	3	1
TX1005	*Q. virginiana*	C	Moss				2	
TX1006	*Q. virginiana*	T	Lichen				2	
TX1007	Gazebo	RT	Moss	17		13	1	
TX1008	Gazebo	RL	Moss	4	4	3	1	
TX2001	Ground	G	Moss	4				
TX2002	*Q. virginiana*	C	Lichen			3	7	

moss samples and 17 specimens from six lichen samples, no significant departure from expected was suggested. Tardigrades were distributed randomly within the habitat samples. The same was true for diversity, since tardigrade diversity averaged 2.0 species per sample (range: 1 to 4). With an expected value of two species per sample, the chi square values did not exceed the critical value so we accept the null hypothesis that the diversity measured is not unusual.

The tardigrades found in the Hill Elementary samples represented two classes, three orders, four families, five genera and eight species (Table 2). Four of the five genera were identified in the students' samples. The identified species are: *Viridiscus perviridis* (Ramazzotti, 1959) (n=1), *Milnesium* cf. *alpigenum* (Ehrenberg, 1853) (n=5), *Milnesium* cf. *brachyungue* (Binda & Pilato, 1990) (n=6), *Milnesium* cf. *tardigradum* (Doyere 1840) (n=4), *Ramazzottius baumanni* (Doyere, 1840) (n=17), *Macrobiotus hufelandi* (C.S.A. Schulttze, 1843) (n=25), *Mesobiotus harmsworthi* (Murray, 1907) (n=4) and *Minibiotus intermedius* (Platte, 1888) (n=3) (Table 3). Three of the species found at Hill Elementary had not previously been recorded from the state of Texas: *Viridiscus perviridis*, *Milnesium* cf. *alpigenum*, and *Milnesium* cf. *brachyungue* (Kaczmarek et. al. 2016) (Table 3).

Viridiscus perviridis is a green, armored, terrestrial tardigrade in the class Heterotardigrada. Defining features that separate this species

Table 3. New records of occurrence for tardigrades in North America, Texas. Table format follows Kaczmarek *et al*. (2016).

Genus Species Author, Year [Aquatic or Terrestrial].
Country (State):
Latitude, longitude, altitude; location, City, County, State; Habitat on substrate, other information.

Viridiscus perviridis Ramazzotti, 1959 [T]

USA (Texas):
30.37703°N, 97.74796°W, 260 m asl; Hill Elementary, Austin, Travis Co, TX; Lichen on lateral branch of a southern live oak, *Quercus virginiana*, Tag#206917, DBH=5.79, at 1.5 m.

Milnesium cf. *alpigenum* (Ehrenberg, 1853) [T]

USA (Texas):
30.376882°N, 97.747767°W. 264 m asl; Hill Elementary, Austin, Travis Co, TX; Moss on roof of gazebo, 2 m above ground.

Milnesium cf. *brachyungue* (Binda & Pilato, 1990) [T]

USA (Texas):
30.376882°N, 97.747767°W. 264 m asl; Hill Elementary, Austin, Travis Co, TX; Moss on roof of gazebo, 2 m above ground.

Milnesium cf. *tardigradum* (Doyere 1840) [T]

USA (Texas):
30.376138°N, 97.748242°W, 267 m asl; Hill Elementary, Austin, Travis Co, TX; Lichen on trunk of a southern live oak, *Quercus virginiana*, DBH=52 cm,

Ramazzottius bermanni (Ramazzotti, 1962) [T]

USA (Texas):
30.376212°N, 97.748411°W, 262 m asl; Hill Elementary, Austin, Travis Co, TX; Moss in canopy of a southern live oak, *Quercus virginiana*, DBH=7.2 cm

30.376138°N, 97.748242°W, 267 m asl; Hill Elementary, Austin, Travis Co, TX; Lichen on trunk of a southern live oak, *Quercus virginiana*, DBH=5.2 cm

30.376004°, 97.748703°W, 261 m asl; Hill Elementary, Austin, Travis Co, TX; Lichen on trunk of a southern live oak, *Quercus virginiana*, 30.375888°N, 97.748695°W, 260 m asl; Hill Elementary, Austin, Travis Co, TX; Lichen on trunk of a southern live oak, *Quercus virginiana.*

30.376882°N, 97.747767°W, 264 m asl; Hill Elementary, Austin, Travis Co, TX; Moss on roof of gazebo, 2 m above ground.

Macrobiotus hufelandi (C.S.A. Schulttze, 1843) [T]

USA (Texas):
30.376882°N, 97.747767°W, 264 m asl; Hill Elementary, Austin, Travis Co, TX; Moss on roof of gazebo, 2 m above ground.

Mesobiotus harmsorthi (Murray, 1907) [T]

USA (Texas):
30.376882°N, 97.747767°W, 264 m asl; Hill Elementary, Austin, Travis Co, TX; Moss on roof of gazebo, 2 m above ground.

Minibiotus intermedius (Platte, 1888) [T]

USA (Texas):
30.376882°N, 97.747767°W. 264 m asl; Hill Elementary, Austin, Travis Co, TX; Moss on roof of gazebo, 2 m above ground.

from other tardigrades include its green color, length of cirrus A, lack of lateral cirri, the presence of a dentated collar, the few pores, uniform large granulation and sculpture on the plates (Figure 2, Table 2). *Viridiscus perviridis* was originally described from Italy by Ramazzotti (1959) and had only been reported three times in North America from Tennessee and North Carolina (Kaczmarek et al. 2016). Recently, two new papers have expanded the known range of *Vir. perviridis.* Miller et al. (2020) added new records from Kansas, West Virginia, New Jersey and Maine and Nelson et al. (2020) expanded the records in Tennessee. All locations are further east and north than Austin, Texas. Both *Mil. alpigenum* and *Mil. brachyungue* are transparent, unarmored and terrestrial Eutardigrades (Figures 3, 4). These species have a broad buccal tube, 6 peribuccal lamellae and a smooth cuticle. The genus is recognized by a lack of placoids in the pharynx, peribuccal papilla, and paired but separated claws. The species are separated by the nature of

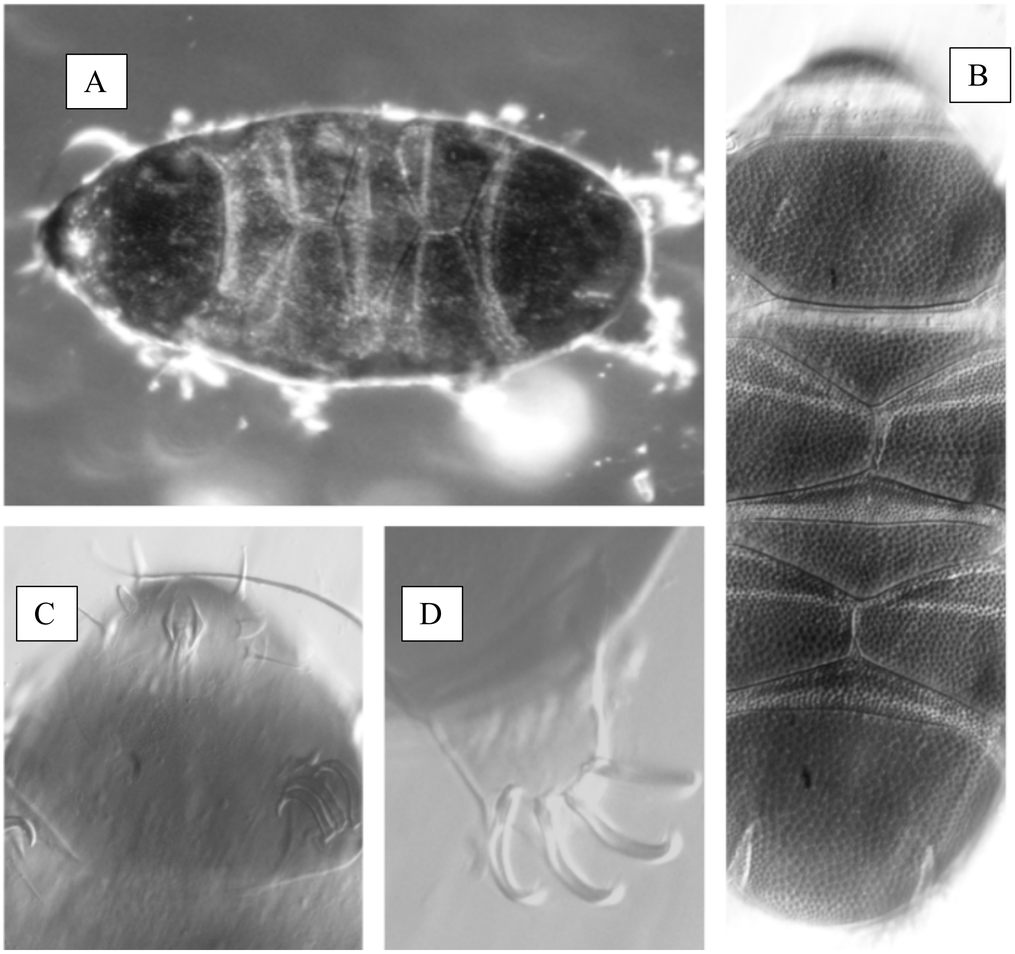

Figure 2. *Viridiscus perviridis*, a new Record for the State of Texas. A. body; B. dorsal cuticle; C. mouth and buccal cirri; D. claws of leg IV.

their cuticular sculpture, presence of pseudo plates, the number of peribuccal lamellae, and the arrangement of the points on the secondary branches of the claws. The differences between *Mil. alpigenum* and *Mil. brachyungue* are not in the visible attributes but in the measured variables expressed as proportions relative to the buccal tube length. *Milnesium alpigenum* is known from many locations in Europe but *Mil. brachyungue* was known only from its type location in Chile, South America. Both present questions of intercontinental dispersion for these first records from North America. The identification of these three species expands the number of tardigrade species found in Texas from 37 to 40 species.

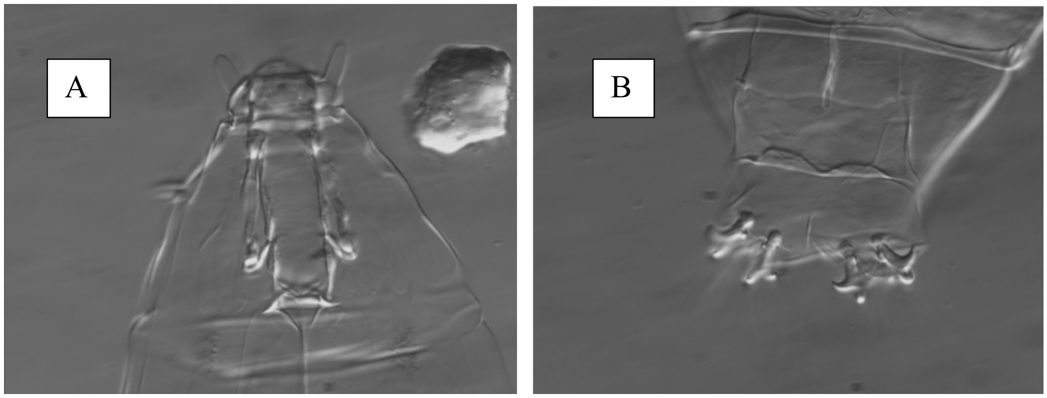

Figure 3. *Milnesium alpigenum*, New Record for the state of Texas and North America. A. buccal apparatus, B. claws of leg IV.

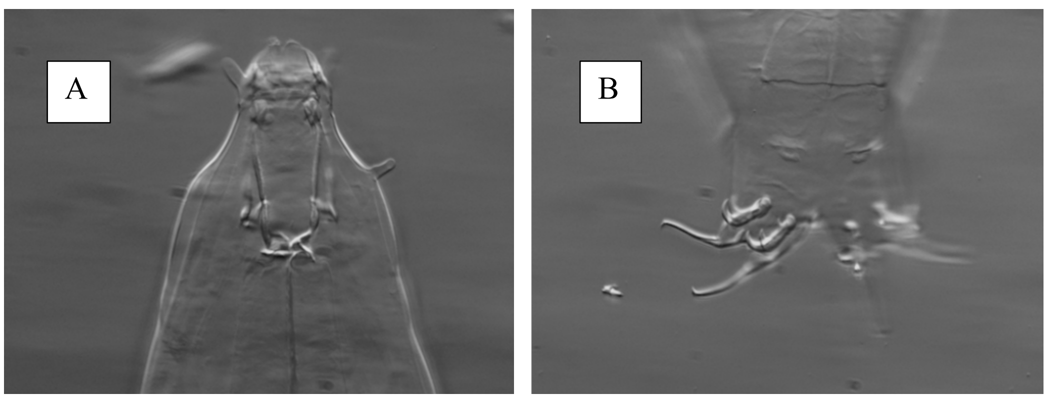

Figure 4. *Milnesium brachyungue*, New Record for the state of Texas and North America. A. buccal apparatus, B. claws of leg IV.

Discussion

The fifth graders of Hill Elementary discovered that in their collection the number of tardigrades was greater in lichen than moss habitats (57% in lichen). However, this was the reverse of the result the junior author recorded (47% in lichen). This was a teachable moment as to the impact of small sized collections and the influence of the components in the collections on results. While the individual records of occurrence and the records of each collection are valid, no broad conclusion about habitat suitability in Texas can be drawn based on these small assemblages.

Furthermore, at Hill Elementary, tardigrades were found in 57% of the lichen samples compared to 63% in Oklahoma (Beasley 1978), 67% in Illinois (Puglia 1964), and 71% in Kansas (Lehmann et al. 2007). By comparison, water bears occurred in 65% of moss samples in Arkansas (Land et al. 2012).

It has been suggested there is no relationship between species of tardigrade and species of moss or lichen habitat in which they are found (Nelson 1975, Kathman & Cross 1991, Kinchin 1994, Hinton & Meyer 2007). Miller et al. (1996) observed tardigrades associated with mosses at the generic level in Antarctica. Several tardigrade species were reported to be highly associated with the lichen genera *Physca* and *Xanthoria* by Puglia (1964) and Lehmann et al. (2007). Meyer (2008) found only weak habitat preference among the tardigrades of Florida and Mitchell et al. (2009) observed substrate (tree species) relationships. Chang et al. (2015) found increased tardigrade diversity and density at higher levels in the canopy.

The percentage of tardigrade positive samples at Hill Elementary was about average at 41% compared to Illinois at 27% (Puglia 1964), Oklahoma at 30% (Beasley 1972), Missouri at 54% and Kansas at 57% (Lehmann et al. 2007) or Montana at 58% (Miller 2006).

With the addition of three new species, the 40 species now known to occur in Texas exceed the documented tardigrade diversity of neighboring states: Louisiana (30 species), Arkansas (25), Oklahoma (20) and New Mexico (21) (Miller & Perry 2019). The diversity of tardigrades in Texas also exceeds that of several other states: Illinois (14 species), Kansas (23), Florida (20), Montana (20), and South Carolina (26). The number of reported tardigrade species from Texas is exceeded by several other states, including Alaska (70 species), Tennessee (66) and California (57) (Miller & Perry 2019).

Hill Elementary students found a maximum diversity of four species in a single sample, supporting Hinton et al. (2014) who also recorded up to four species in samples in the Big Thicket National Preserve study. Other researchers have noted a similar species diversity per sample in tardigrade collections from other Gulf Coast areas (Hinton & Meyer 2007, Hinton et al. 2010; Meyer 2001, 2008). In well-sampled mountainous collections, greater tardigrade species diversity (up to ten) has been reported (Dastych 1988 in Poland, Bartels & Nelson 2006 in the Great Smoky Mountains National Park, and Kaczmarek et al. 2011 in Costa Rica). The present study supports the idea that tardigrades appear to be less numerous and less diverse along the southern coastal plains and flatlands than in northern forests and mountains (Hinton et al. 2014).

In their first year of study, the students found only 65 tardigrades. In their second and third years of study, they have found hundreds. The numbers are increasing as the students share with each other their growing knowledge of how to find tardigrades, make slides and identify specimens.

However, student identification to species is still not immediately possible. Challenges such as their beginner skills, student equipment, and lack of a current monograph all worked against them. During the project the authors built a PowerPoint "Field Guide" of the Texas tardigrades they were finding and used that to match new specimens to previous captures. This guided the students to the important characteristics necessary to separate species within each genus.

In terms of scientific interest, the students were most excited to add three species of tardigrades to the records of the state of Texas. *Viridiscus perviridis* was unlike most of the other tardigrades the students had seen. Initially, they thought it to be leaf litter. Then with a bit of coaching, they recognized a difference in the body shape, the presence of plates, cirri and papillae near the mouth and the bright green color. When *Mil. alpigenum* and *Mil. brachyungue* were separated and identified, students began to appreciate the subtlety of speciation. They developed keen observation skills as they began looking at details like the presence of plates, the number and length of claws, the presence of cirri A, the number of rows of placoids, the presence of buccal papillae, and the mouth structure and length. Their current goal is to learn how to independently identify not only down to the family and genus, but even to the species, without assistance from an expert. They look forward to sharing more about their learning process as well as additional discoveries in their second and third year of study soon.

Texas is a large state with a wide variety of habitats including grasslands, deserts, hill country, thorn scrub, and piney woods. If three new records for the state can be found in a small, localized sampling by grade school students, there must be an even greater diversity of water bears in Texas than we are currently aware. With many more to be discovered, it is our hope that our findings will promote continued exploration of the state and will expand our knowledge of the diversity of tardigrades across the vast ecoregions of Texas.

These discoveries could not have been made without the collaboration between the authors and the diligent field work of the students. Through learning the scientific process, students developed new advanced skills and became scientists. These young students made positive contributions to science, they added new reports of occurrence and expanded the known distribution of species. Performing this biodiversity survey has highlighted the ability of citizen scientists, regardless of age, to contribute to scientific knowledge. When scientists, educators, and students work together, further exploration and discovery can occur which enhances our understanding and in turn fosters good stewardship of our world.

It is vital that we continue to bridge the gap between the scientific community and the public, and students can be a major catalyst for change and progress, if given the opportunity. The overarching goal for the students at Hill Elementary is to continue their research, share what they are doing with scientists, educators, and other students and build a wider community of tardigrade researchers. Because of the paucity of data about tardigrades, students can be put on the edge of knowledge where almost everything they find is a true scientific discovery. This paper is a testament to their successful first year. These partnerships show that our students can be scientists now and make a positive difference in the world.

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