Transactions of the Kansas Academy of Science
Vol. 118, no. 3-4
p. 230-236 (2015)

Tardigrades of the canopy: Evidence of stratification

Lauren Chang[1], Devon Powell[2], William R. Miller[3] and Margaret Lowman[4]

1. Department of Biology, Wesleyan University, Middletown, Connecticut 06459
2. Department of Environmental Studies, New College of Florida, Sarasota, Florida 34243
3. Department of Biology, Baker University, Baldwin City, Kansas 66006
4. California Academy of Sciences, San Francisco, California 94118
Corresponding author: William.Miller@BakerU.edu

Mosses and lichens growing on trees and rocks are a common habitat for limno-terrestrial tardigrades all over the world. However, worldwide tardigrade research has been conducted primarily with ground level samples. In order to determine the presence, absence, diversity, distribution and size of tardigrade populations in the canopy, researchers ascended 135 trees of 17 species at nine northeastern Kansas forest sites, and collected tardigrade habitat from varying heights. Analysis of 822 samples clearly evidenced the presence of tardigrade populations in the forest canopy, as well as documented stratification with a statistically significant increase in density of the tardigrades per sample from 6.4 to 11.9 with increased height. This third dimension discovery must now be factored into theories of tardigrade dispersal. Further investigations may hold new, arboreal species.

Keywords: Canopy, Stratification, Tardigrades

Introduction

Tardigrades are little studied, aquatic, microscopic invertebrates. First viewed by Leeuwenhoek in 1674 (Kinchin 1994), tardigrades inhabit a variety of aquatic environments, including oceans, rivers, and lakes. In addition, limno-terrestrial tardigrades are able to live in terrestrial habitats that periodically retain water like a sponge (mosses, lichens, leaf litter, soil, etc.). They exhibit cryptobiosis, the process of desiccating with their habitat and reconstituting with the return of moisture. This allows them to survive in dry areas of the world (Kinchin 1994). Recently, cryptobiotic tardigrades became the first multi-celled animal to survive exposure to the vacuum, temperature, and radiation of space (Jönsson et al. 2008). Cryptobiotic tardigrades are probably dispersed in the winds (Kinchin 1994; Miller 1997) and thus are rained onto the canopies of the world, where they must find acceptable habitat to survive (Miller 2004). Despite our knowledge of tardigrade physiology, we know almost nothing about their ecology or basic requirements for living (Ramazzotti and Maucci 1983; Miller 1997).

Tardigrades are typically 200-700µm long, with five body segments. The anterior or head segment includes the brain, mouth, and eyes. The four body segments each have a pair of legs ending in claws which allow tardigrades to crawl through their aquatic environment. The limno-terrestrial tardigrades are divided into two classes. Heterotardigrada have armored plates on their segments, colored bodies, and four parallel claws on each foot (Fig. 1). Eutardigrada do not have body plates, are transparent, and have a pair of claws on each foot (Fig. 2).

Forest canopies have been called the “eighth continent of planet Earth” (Wilson 2005) due to their complexity and enormous biodiversity. With the explosion of canopy exploration over the last two decades (Lowman 1999, Lowman and Rinker 2004), canopy researchers have confirmed that a large portion of global biodiversity inhabits the treetops (Erwin 1982; Lowman and Rinker 2004).

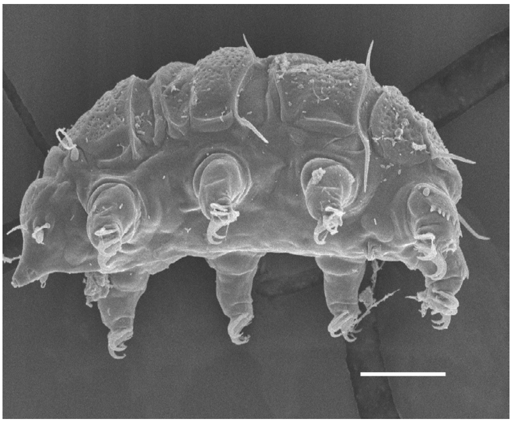

Figure 1. Class Heterotardigrada, showing parallel claws, plated dorsal cuticle and rounded body shape. Scale bar = 50 μm.

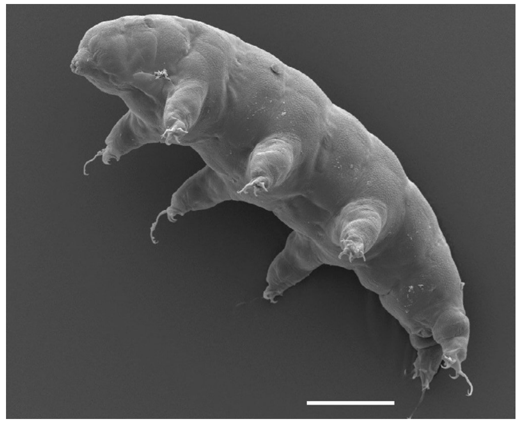

Figure 2. Class Eutardigrada showing paired claws, smooth dorsal cuticle and elongated body shape. Scale bar = 50 μm.

Historically, limno-terrestrial tardigrades have been collected within reach at ground level. Kimmel and Meglitsch (1969) presented the first hint of arboreal tardigrades, finding them three meters above the ground in Iowa trees. Voegtlin (1982) reported observing water bears in washings of Douglas-fir canopy needles in Oregon. Counts et al. (2001) reported tardigrades as "Jewels" in the canopy of Great Smoky Mountains National Park during the beginnings of the All Taxa Biodiversity Survey project (Sharkley 2001). Mitchell et al. (2009) documented substrate (tree) selection and provided evidence of vertical distribution. Recently, Miller et al. (2013) reported actual stratification with greater diversity and density of tardigrades at higher heights in the canopy (10 meters) of a white pine on the Baker University campus in Kansas.

This project was designed to demonstrate and document tardigrade canopy usage. It was hypothesized that tardigrade populations in the upper levels would have the same density, diversity and distribution as the population found on the base level of the tree. It follows that there should be no significant differences between locations, substrates (tree species), or habitats (epiphytic moss or lichen) relative to tardigrade populations.

Methods

Tardigrade habitat samples (moss and lichen) were collected from different substrates (tree species) at nine locations in northeastern Kansas. Utilizing the double-rope climbing technique (Haefke et al. 2013), researchers ascended as high as 20 meters (60 ft) into trees and collected samples (Fig. 3). Four levels, increasing in height at three-meter increments, were recorded (0-3, 3-6, 6-9, >9 meters). The samples from levels above nine meters were pooled because of the limitations of sampling. Habitat samples were scraped from the bark of the substrate using a pocketknife into a labeled paper bag.

In the lab, habitat samples were soaked in bottled water in small plastic cups for about 12 hours. After soaking, a 1 ml pipette was used to extract water and debris from the soaking cup to fill a 25 x 50 mm tray. The tray was examined with a dissecting microscope and reflected light. Tardigrades were extracted using an Irwin loop (Schram and Davison 2012). Tardigrades were mounted on slides in PVA (Salmon 1951) and labeled with a slide number and sample code. The extraction process was performed three times per sample: twice by the original processor and once by

Figure 3. Lauren Chang using the double-rope climbing technique while collecting moss and lichen habitat at the Kansas State University, Konza Long Term Environmental Research (LTER) site near Manhattan, Kansas.

a different researcher. Several days after the tardigrades were mounted on slides, the edges of the slide covers were sealed with nail polish to reduce drying.

The density or tardigrades per sample was used as the measure of presence and habitat acceptability. Because for 250 years tardigrades have been collected at the base of trees, the hypotheses of uniformity was tested by the comparison of the observed tardigrade density at upper levels to the expected density at the base level. The Chi Square test for the difference between expected and observed tardigrade density was used to judge significant differences. Significance was defined as a calculated X^2 value greater than the critical value of 3.84 for one degree of freedom or a P value less than 0.05 (X^2>3.85, 1df, p<0.05) (Zar 2010).

Results

During the summer of 2014, a Research Experiences for Undergraduates (REU) was conducted at Baker University in northeastern Kansas. One hundred thirty-five trees (substrates) of 17 different species were climbed at nine different locations within a two-hour radius of Baldwin City. Tardigrade habitats (moss and lichen) were collected at four different levels at approximately three meters apart. Eight hundred twenty-two samples were taken (250 mosses and 572 lichens) (Table 1). Sample distribution by level was reasonably uniform between 23% and 26% (Table 1). Eight thousand sixty-four tardigrades were extracted. The two upper levels yielded 51% (419) of the samples but 62% (4,987) of the tardigrades vs. 49% (403) of the samples and only 38% (3,077) of the tardigrades (Table 1). The two upper

Table 1. Stratification of tardigrades in the canopy. Showing balance of sampling, increasing numbers of tardigrades at higher levels, and differences between habitats (moss and Lichen).

		Sample Counts				Tardigrade Counts			
Level	Height meters	Moss	Lichen	Total	%	Moss	Lichen	Total	Tardigrades /sample
4	>9	53	158	211	26%	437	2,004	2,506	11.9+
3	6-9	51	157	208	25%	287	2,148	2,481	11.9+
2	3-6	53	138	191	23%	297	1,428	1,727	9.0
1	0-3	93	119	212	26%	558	789	1,350	6.4
		250	572	822		1,579	6,369	8,064	

+ = P<0.05 for tardigrades/sample significantly different from base Level 1.

levels (3 and 4) each exhibited significantly (X^2>3.85, 1df, p<0.05) more tardigrades per sample (11.9) when compared to the expected number (6.4) found at the base level (Table 1).

At eight of the nine collection sites, there was significantly greater tardigrade density in the canopy samples than the base samples (Table 2). But at one, the Black Jack Battlefield site, two miles east of the Baker campus, there was no difference in tardigrade density indicated between the three upper levels and the base at this location (Table 2).

Seventeen substrates (tree species) were climbed and sampled. Fourteen substrate species exhibited at least one level that was significantly different in tardigrade density from the expected base level (Table 3). Three species of substrate, American elm, chinquapin oak and sugar maple, exhibited a tardigrade density at upper levels approximately equal to the expected base level while two other substrate species, sycamore and red mulberry, exhibited at least one level with a significantly lower species density per sample than expected (Table 3). On seven substrate species, hackberry, shag bark hickory, honey locust, red oak, Virginia creeper, burr oak, and ponderosa pine, all three upper levels exhibited significantly greater tardigrade density than expected (Table 3).

Discussion

This project aimed to clarify tardigrade canopy usage. Based on the data, tardigrades are present in the canopy in great numbers and variety. However, it is necessary to reject the hypothesis that canopy tardigrade populations are uniform and not significantly different from those at the base level. Finally, the hypotheses that the population density would not vary depending on substrate or location are also rejected.

The location of the sampled trees did not seem to be a factor as eight of nine locations presented significantly more tardigrades in the

Table 2. Stratification of tardigrades in the canopy. Showing a significantly greater number of tardigrades per sample at higher levels at all but one of nine locations. Lack of differences suggest regional uniformity of suitable habitat for tardigrades higher in the canopy.

Level	BUP	BJB	BUC	BUW	KUR	BUL	OPA	KUFS	*Konza*	Total
4	30.7+	4.3	18.2+	11.4+	8.4+	12.1+	1.3+	21.1+	14.9+	11.9+
3	24.9+	4.6	9.8	11.1+	7.7+	10.8+	1.0+	20.5+	8.8+	11.9+
2	17.9+	4.9	14.0	6.1+	2.8	7.5+	0.4	14.0	6.5	9.0
1	9.4	7.9	9.3	2.3	3.0	3.2	0.1	10.3	4.2	6.4

+ = P<0.05 for tardigrades/sample significantly different from base Level 1.
BUP = Baker University Prairie, BJB = Black Jack Battlefield, BUC = Baker University Campus, BUW = Baker University Woods, KUR = University of Kansas Rice Woods, BUL = Baker University Wet Land, OPA = Overland Park, KS, KUFS = University of Kansas Field Station, Konza = Konza Prairie Field Station at Kansas State University.

Table 3. Stratification of tardigrades in the canopy. Showing a significantly greater number of tardigrades per sample at higher levels when compared to the base level on different substrate tree species. Note: differences suggest significant substrate influence on suitable habitat for tardigrades higher into the canopy.

		Tardigrades / Sample			
Level	n	1	2	3	4
Hackberry	19	4.2	10.6+	10.1+	9.6+
Black Walnut	17	3.5	6.1	11.1+	15.3+
Sugar Maple	14	8.8	4.9	5.4	4.7
E. Cottonwood	14	3.1	6.4	12.7+	9.9+
SB Hickory	11	3.3	9.9+	14.4+	16.1+
Green Ash	11	10.2	12.4	22.9+	15.4
Am. Elm	9	5.8	4.8	6.7	4.3
Honey Locust	7	7.1	14.5+	20.2+	21.6+
Post Oak	7	2.4	2.2	2.2	15.0+
Sycamore	6	14.1	17.8	16.0	3.4-
Red Oak	5	10.1	19.5+	23.0+	33.7+
Chinquapin Oak	5	3.0	2.8	4.7	3.2
Red Mulberry	3	10.6	9.5	7.8	3.5-
Virginia Creeper	3	0.0	1.0+	7.8+	15.0+
Bur Oak	2	0.7	4.8+	3.4+	7.7+
E. Red Cedar	1	1.0	11.3+	26.5+	0.0
Ponderosa Pine	1	13.0	44.0+	31.0+	55.0+

+ = $P<0.05$ for tardigrades/sample significantly different from base Level 1.

canopy than at their bases. The one location (Black Jack) that presented no significant differences was dominated by sugar maple trees, one of the trees demonstrating no differences among the levels.

Three substrate tree species exhibited no significant difference in the tardigrade populations in their canopy (sugar maple, American elm, and chinquapin oak), two substrate species were identified with significantly fewer tardigrades at the upper most level (red mulberry and sycamore), and 12 substrate species exhibited significantly more water bears higher in the trees. These data clearly reject the argument of uniformity and lead to the conclusion that substrate is more important than location within a small region.

Spiers et al. (2013) identified a species of tardigrade, *Doryphoribius dawkinsi* Michalczyk and Kaczmarek, 2010 in the canopy of a Kansas forest that occurred only above 3 meters and that had been previously only known from Costa Rica, Likewise, Haefke et al. (2014) described a second member of the same genus, *Doryphoribius elleneddiei* Haefke, Spiers, Miller and Lowman 2014 from the same area and also found only in the canopy. A third member of the genus, *Doryphoribius gibber* Pilato and Lisi 2006 has also been reported from the canopy of the same area (Chappell et al. 2015). None of these species would have been collected with base level only sampling, suggesting the diversity of tardigrades species may be different in the canopy.

The unanswered question is how did the tardigrades get into the canopy of so many different species of trees at so many locations? They certainly did not walk, nor do they actively select their habitats. Tardigrades are believed to be passively distributed, most likely carried on the winds and/or in the fur/feathers of animals (Ramazzotti and Maucci 1983; Kinchin 1994; Miller 1997). While continental drift may explain global foundation stock, the occupation of the moss or lichen habitat in the canopy of a tree in eastern Kansas is limited by the age of the tree (seldom more than 75 years), the availability of suitable habitat which must also colonize the substrate at each location, and the frequency of dispersal events. These data suggest the process is sufficient to predict that tardigrades are a common taxon in the canopy of eastern Kansas.

Our consistent result of greater density of tardigrades higher in the trees suggests one of two things: either tardigrades are arriving frequently at the top of the tree (tardigrade rain) and filtering down through the branches and leaves due to the flow of water and gravity, or they arrive with equal frequency at all parts of the tree (horizontal wind) but are more protected in habitats in the canopy, and thus able to develop more productive populations than at the more exposed base of the trees.

The increasing evidence of canopy usage by tardigrades brings up other three dimensional questions. Are the patterns the same for angiosperm vs. gymnosperms? Which tardigrade species are arboreal? Is the density and diversity of tardigrades in the canopy the same in different regions of the country or in different forest types? Is this pattern the same in the tropics or arctic? Are tardigrades in the tallest of trees? Do they exist in the same ratios in the deep forest, or is there an edge effect? Answering these questions will add to our knowledge of water bear requirements for living.

Acknowledgments

This project was funded by the National Science Foundation through DEB: REU grant #1156550 to Baker University. A special thank you is expressed to the Kansas Biological Survey for fostering access to the forests of the University of Kansas Field Station, and to the Konza Prairie Biological Station at Kansas State University for collecting Permit 2014:269.

Literature Cited

Chappell, B.M., Perry, D., Miller, W.R. and Lowman, M.D. 2015. Tardigrades of the Canopy: *Doryphoribius gibber* Beasley and Pliato, 1987 (Eutardigrada: Parachela: Hypsibiidae) new records from eastern Kansas, U.S.A. Transactions of the Kansas Academy of Science 118(1-2):48-52.

Counts, J.W., Henley, L., Skrabal, M. and Keller, H.W. 2001. Biological jewels in tree canopies. Transactions of the Missouri Academy of Science 35:69-70.

Erwin, T.L. 1982. Tropical forests: Their richness in Coleoptera and other arthropod species. *The Coleopterists Bulletin* 36:74-75.

Haefke, B.J., Spiers, A.I., Miller, W.R. and Lowman, M.D. 2013. Tardigrades in the Canopy: Using double rope techniques to conduct sampling along vertical transects. Kansas Academy of Science, Transactions 116(3-4):119-124.

Haefke, B.J., Spiers, A.I., Miller, W.R. and Lowman, M.D. 2014. Tardigrades of the Canopy: *Doryphoribius elleneddiei* nov. sp. (Eutardigrada, Parachela, Hypsibiidae), a new species from eastern Kansas, U.S.A. Kansas Academy of Science, Transactions 117(3-4):299-304.

Jönsson, K.I., Rabbow, E., Schill, R.O., Harms-Ringdahl, M. and Rettberg, P. 2008. Tardigrades survive exposure to space in low earth orbit. Current Biology 18(17):729-731.

Kimmel, R.G. and Meglitsch, P.A. 1969. Notes on Iowa Tardigrades. Iowa Academy of Science 76:454-462.

Kinchin, I.M. 1994. *The Biology of Tardigrades*. Portland Press, London, 186 pp.

Lowman, M.D. 1999. Life in the Treetops. Yale University Press.

Lowman, M.D. and Rinker H.B. 2004. (eds.) Forest Canopies. Elsevier Academic Press.

Miller, W.R. 1997. Tardigrades: Bears of the moss. The Kansas School Naturalist 43(3):1-15.

Miller, W.R. 2004. Tardigrades: Moss Bears in the Canopy. pp. 251-258 in Lowman, M.D. and Rinker, H.B (eds.), Forest Canopies, Academic Press.

Miller, W.R., Gallardo, L. and Clark, T. 2013. Chapter 30: Do water bears climb trees too? pp. 307-311 in Lowman, M.D. (ed.), Tree Tops at Risk: the challenges of global canopy ecology and conservation, Springer, New York.

Mitchell, C., Miller, W.R. and Davis, B. 2009. Tardigrades of North America: Influence of substrate on habitat selection. Journal of the Pennsylvania Academy of Science 83(1):10-16.

Ramazzotti, G. and Mauchi, W. 1983. *Il Philum Tardigrada* (3rd edition), Memorie dell'Istituto italiao id Idrobiologia Marco Marchi 41:1-1012.

Salmon, J.T. 1951. Polyvinyl Alcohol as a mounting medium in microscopy. The Microscope 8(6):139-142.

Schram, M.D. and Davidson, P.G. 2012. A history and method of constructing homemade loops. Kansas Academy of Science, Transactions 115(1-2):35-40.

Sharkey, M.J. 2001. The All Taxa Biological Inventory of the Great Smoky Mountains National Park. Florida Entomologist 84(4):556-564.

Spiers, A.I., Haefke, B.J., Miller, W.R. and Lowman, M.D. 2013. Tardigrades in the Canopy: *Doryphoribius dawkinsi* Michalczyk and Kaczmarek, 2010 – New records from eastern Kansas, U.S.A. Kansas Academy of Science, Transactions 116(3-4):125-128.

Voegtlin, D.J. 1982. Invertebrates of the H.J. Andrews Experimental Forest, western Cascade Mountains, Oregon: a survey of arthropods associated with the canopy of old-growth *Pseudotsuga Menziesii*. Forest Research Laboratory, Oregon State University, Special Publication 4:1-31.

Wilson, E.O. 2005. *The Future of Life*. Harvard University Press.

Zar, J.H. 2010. Biostatistical Analysis. Prentice Hall, 4th Edition, 663 pp.