

Geographic Patterns of Crayfish Symbiont Diversity Persist Over Half a Century Despite Seasonal Fluctuations

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ABSTRACT

We analyzed historical data from 1961 to 1965 for species occurrences of branchiobdellidans and their crayfish hosts throughout the Mountain Lake Region of southwestern Virginia, USA to quantify geographic variability in species composition and identify patterns in host and symbiont diversity. We collected contemporary census data of branchiobdellidan assemblages from the same region in 2011 to 2014 for intra-annual variation in symbiont abundance and species composition. We compared historical and contemporary records to assess the stability of geographic patterns in symbiont diversity over decadal timescales. Branchiobdellidan assemblages followed a hump-shaped relationship with Strahler stream order. Much of the geographic variation in symbiont species composition was explained by host species composition, despite low host specificity in branchiobdellidans. There were strong seasonal cycles in branchiobdellidan abundance and species composition. A comparison of historical and contemporary records revealed little change in species distributions over 50 years. Thus, branchiobdellidan species composition changes predictably along habitat gradients, tracks variation in host composition, is repeatable across decadal timescales, and follow strong cyclic seasonal changes in total and relative abundances. These results suggest that complex but deterministic ecological processes drive symbiont population dynamics at multiple spatial and temporal scales.

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INTRODUCTION

Crayfish bodies are homes to a great diversity of interacting microbial and metazoan ectosymbionts including bacteria, fungi, protozoans, rotifers, annelids, platyhelminths, and arthropods, and their associations range from obligate symbionts to incidental commensals (Edgerton et al. 2002; Skelton et al. 2016b). Of all crayfish symbionts, none are more conspicuous in nature or the scientific literature than the branchiobdellidans (Annelida: Branchiobdellida). Branchiobdellidans are a monophyletic clade of more than 150 species found throughout North and Central America, Europe, and Asia (Gelder 2010). Though they can survive for long periods without a host, branchiobdellidans appear to be reproductively dependent on freshwater crustacean hosts, particularly crayfish (Penn 1959; Young 1966; Creed et al. 2015). In some regions, such as the southern Appalachian Mountains of the

American Southeast, branchiobdellidan abundance and diversity may be remarkably high where individual crayfish host hundreds of worms belonging to many species and several genera (Hobbs et al. 1967; Gelder and Williams 2011; Skelton et al. 2016a).

Historically, the study of branchiobdellidans was limited to descriptive and taxonomic work, with a few notable exceptions including studies of their ecology (Berry and Holt 1959; Mc Manus 1960; Young 1966; Koeppe and Schlueter 1977) and host/symbiont interactions (Penn 1959; Grabda and Wierzbicka 1969; Koeppe 1975). More recently, a growing number of descriptive studies have illuminated interesting patterns in branchiobdellidan diversity at multiple spatial scales. Both broad-scale (e.g., Gelder 1999b; Füreder et al. 2009; Williams et al. 2009; Larson and Williams 2015) and finer scale (e.g., Goodnight 1941; Hobbs et al. 1967; Gelder and Smith 1987; Gelder and Williams 2011) studies

depict high rates of species turnover within the ranges of their hosts. Simultaneously, recent experimental studies have related fine-scale processes to variation in symbiont abundance and composition among individual hosts. Experimental approaches have examined the effects of branchiobdellidans on their hosts (Brown et al. 2002), variation in those effects under different ecological contexts (Lee et al. 2009; Brown et al. 2012; Ames et al. 2015; Thomas et al. 2016), the effects of variation in host behavior on branchiobdellidan abundance and species composition (Thomas et al. 2013; Farrell et al. 2014b; Farrell et al. 2014a; Skelton et al. 2014), host preferences of branchiobdellidans (Brown and Creed 2004), and the effect of host size on branchiobdellidan species composition (Skelton et al. 2016a; Thomas et al. 2016). While there is a large and growing body of broad-scale descriptive studies of branchiobdellidan diversity, as well as small scale experimental work teasing apart ecological processes, there remains a dearth of explicit quantitative links between patterns of symbiont diversity at larger scales and their causal processes at smaller scales. Moreover, there is a need for long-term assessments to evaluate the stability of geographic patterns in symbiont diversity and the strength of the underlying processes that maintain them.

To the branchiobdellidans, crayfish assemblages are dynamic and heterogeneous archipelagos embedded in a sea of uninhabitable benthos. As each new island in the crayfish archipelago is colonized by a community of interacting symbionts, layers of ecological processes impart their influences. This metaphor is not just aesthetically pleasing, but is also an emerging and critical change in the way we understand the lives of individuals and the evolution of populations. Waves of discoveries have linked host and symbiont biology and blurred the lines that define individuals (Gilbert et al. 2012). Simultaneously, modern theoretical and empirical work unites community ecology with parasitology and symbiosis ecology to disentangle networks of complex interactions among diverse symbiont assemblages and unravel the processes that operate at scales from continental to cellular to assemble symbiont communities (e.g., Pedersen and Fenton 2007; Graham 2008; Mihaljevic 2012; Skelton et al. 2016a). Thus, there is a recent paradigm shift away from the historical perspective of symbioses as pair-wise species interactions, and towards a framework reminiscent of island biogeography. While this change in perspective promises a new and profound understanding of organismal ecology and evolution, it requires extensive empirical observations to assess the underlying concepts. Because of their diversity, ubiquity, and conspicuous nature, the branchiobdellidans offer a remarkably amenable study system for garnering observational and experimental evidence to develop such frameworks.

Considerable variation among local branchiobdellidan assemblages occurs at various spatial scales from relatively small (e.g., Goodnight 1941; Berry and Holt 1959; Hobbs et al. 1967; Gelder and Smith 1987; Gelder 1999a; Gelder and Williams 2011), to expansive or even global (Holt 1969, 1973; Gelder 1999b; Gelder et al. 2002; Füreder et al. 2009; Williams et al. 2009; Larson and Williams 2015). While variation in branchiobdellidan assemblage at large spatial scales is best explained by historical barriers to dispersal, host colonization events, local extinctions and allopatric

speciation events (e.g., Holt 1969; Füreder et al. 2009), variation at finer scales, such as within a single mountain stream, is likely to be mostly driven by ongoing ecological processes. A recent flurry of studies explored how ongoing ecological processes effect single branchiobdellidan species at the scale of the individual host. Examples include studies of host preferences (Brown and Creed 2004; Tanaka et al. 2016), variation in host resistance among crayfish of different species (Farrell et al. 2014a), size (Dewitt et al. 2012; Skelton et al. 2014), or reproductive stage (Farrell et al. 2014b). Others have discussed the possible effects the physical environment has on branchiobdellidan populations, such the effects of siltation (Bishop 1968), temperature (Berry and Holt 1959; Dewitt et al. 2012), and salinity (Tanaka et al. 2016). While each of these processes has been shown to have a significant influence on branchiobdellidan populations, the relative importance of each process, their emergent effects on branchiobdellidan assemblages, and the persistence of their effects are yet unknown. These same questions stand at the forefront of parasitology and symbiosis ecology, and continued study of the branchiobdellidans promises a better understanding of the basic principles that define symbiotic systems in general.

In this study of crayfish and their branchiobdellidans of the mountain streams in southwestern Virginia, USA, we combined a re-analysis of historical symbiont distributional data with recent multi-year sampling to quantify geographic patterns of symbiont diversity and correlate those patterns with host species composition and geographic position within stream networks. Specifically, we asked if branchiobdellidan assemblages are spatially structured and if they are, is spatial structure correlated with host species composition and/or position within stream networks? We also assessed the temporal stability of branchiobdellidan abundance and species composition at intra-annual and decadal time-scales. We asked if branchiobdellidan assemblages vary in abundance and relative abundance of species with season using contemporary quantitative survey data, and assessed long-term symbiont assemblage stability by comparing historical and contemporary records of species occurrences across the study region.

METHODS

Data Collection

During the years 1960 to 1965, Hobbs et al. (1967) recorded species occurrences of 1,600 branchiobdellidan specimens from 2,700 crayfish collected at 126 sampling sites across three major watersheds in southwestern Virginia, USA; the New River, James River, and Roanoke River watersheds, hereafter referred to as the Mountain Lake Region. These historical collections were made by placing multiple crayfish from each site in large containers of preservative and later separating and identifying symbiotic taxa. Consequently the data for symbionts were reported as species occurrences, i.e. presence/absence only (Hobbs et al. 1967). For quantitative re-analysis of descriptive results of Hobbs et al. (1967), we transcribed species occurrence records into a binary species by sampling site matrix. We also inferred the Strahler stream order of each sampling site from the map of sampling sites presented in Hobbs et al. (1967). Subsequently, while working in the same region, the first author (JS) collected and identified

25,688 branchiobdellidan specimens from 658 crayfish at 24 sampling sites within the Mountain Lake Region throughout the years of 2011 to 2014. For all contemporary records, live crayfish were placed individually in plastic bags filled with 70% ethanol for transport from field sites to the laboratory. In the laboratory, crayfish were identified, sexed, weighed, and measured for total carapace length (TCL in mm). Branchiobdellidans were recovered by vigorous agitation of crayfish within the sample bags and dissection of the crayfish gill chamber, yielding complete quantitative branchiobdellidan census data for each crayfish. Thus, this new dataset consisted of quantitative measures of branchiobdellidan species composition for each crayfish sampled (i.e., the multivariate variable of counts of individual worms belonging to each worm species, on each crayfish individual).

Hobbs et al. Re-analysis

We used the extensive species occurrence records from Hobbs et al. (1967) to identify geographic patterns in branchiobdellidan species composition and correlations with potential underlying drivers. Hobbs et al. (1967) reported branchiobdellidan distributions as occurrence only (i.e., the presence or absence of each species for each location). Thus, species composition in this dataset consisted of the multivariate occurrences (present or absent) of each branchiobdellidan species at each sampling site, but did not contain information regarding species abundances or relative abundances. We visualized the correlation of branchiobdellidan species composition with the species composition of crayfish hosts and Strahler stream order of the sampling sites using non-metric multidimensional scaling (NMDS), implemented by the metaMDS() function in the vegan package for R (Oksanen et al. 2013). Ordination was conducted on a Jaccard dissimilarity matrix for branchiobdellidan species occurrences. To visualize correlations between branchiobdellidan species occurrences and crayfish species occurrences, we plotted the species scores from the ordination. We calculated the correlation coefficient for site scores with crayfish species occurrences, scaled them to the plot area of our ordination, and used the scaled correlation coefficients as coordinates to plot crayfish species names. We also scaled the size of symbols representing site scores to reflect Strahler order of the stream.

We tested for statistically significant relationships between branchiobdellidan species composition and the stream identity and Strahler order of the sampling sites using permutations multivariate analysis of variance (PERMANOVA; Anderson 2001) implemented by the adonis() function in the vegan package for R (Oksanen et al. 2013), with stream order as a continuous predictor and stream identity as factor, and an interaction term. Because we were interested in variation at the within-watershed scale, we constrained permutations to within watersheds to avoid conflating the high degree of cross-watershed variation with variation among sampling sites within watersheds. Once a significant effect of stream order was determined, we used variation partitioning to estimate the amount of variation in branchiobdellidan species composition that could be explained by crayfish species composition versus strictly stream order. This *post-hoc* analysis was conducted to disentangle the effects of physical stream characteristics from those arising from variation in the available host species. Variation

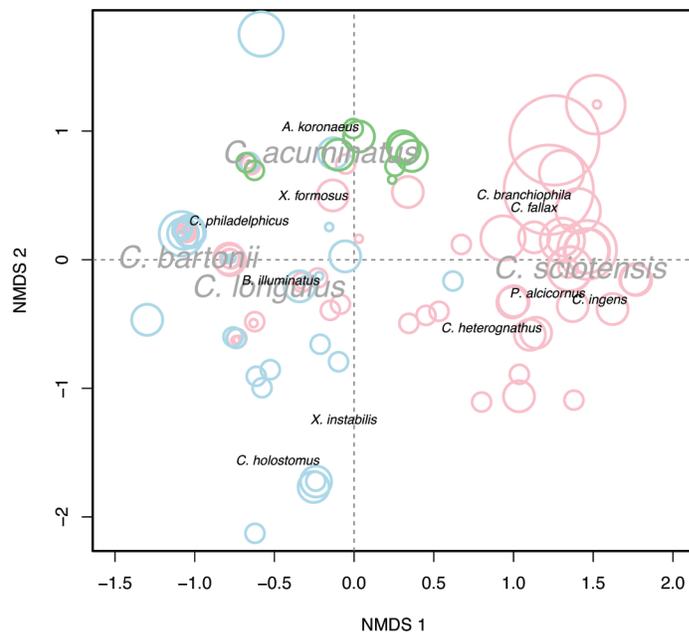


Figure 1. Non-metric multidimensional scaling (NMDS) ordination of branchiobdellidan composition observed throughout the Mountain Lake Region of southwestern Virginia, USA, showing correlation between branchiobdellidan and crayfish species composition. Data taken from Hobbs et al. 1967. Presence/absence data for 11 species of branchiobdellidan (species scores shown in small text) from 123 sites (circles) were ordinated against presence/absence data for 4 host crayfish species (correlation with site scores shown in large text). Circle size is scaled to Strahler order of sampling site. Colors represent watershed; light red = New River, light blue = James River, and light green = Roanoke River. Convergent solution found after 14 tries, final stress = 0.118.

partitioning was implemented using the varpart() function in the vegan package for R (Oksanen et al. 2013).

We used generalized linear models to assess species-specific responses and total species richness as a function of Strahler stream order. For each species, we modeled the binary response of presence/absence as a function of stream order, as well as a quadratic term to capture hump-shaped responses. A similar model assuming a Poisson error distribution was used to model species richness. Models were fit using the glm() function of the R stats package v. 3.1.1. For each model we used an automated AIC model selection to find the best model of all possible nested models (step() function or R stats package 3.1.1; Venables and Ripley 2002). One species (*Xironodrilus formosus* Ellis 1919) was omitted from analysis because it had a very low frequency of occurrence (7 out of 126 sites).

Seasonal Variation

We assessed annual variation in branchiobdellidan abundance and species composition using the branchiobdellidan census data collected from 2011 to 2014. The seasonal analysis was restricted to streams for which there was sufficient sampling effort throughout the year; Sinking Creek and Big Stoney. We used trigonometric regression to fit an intra-annual periodic regression function for branchiobdellidan abundance and species composition. This approach accounts for the cyclic non-linearity of seasonal effects

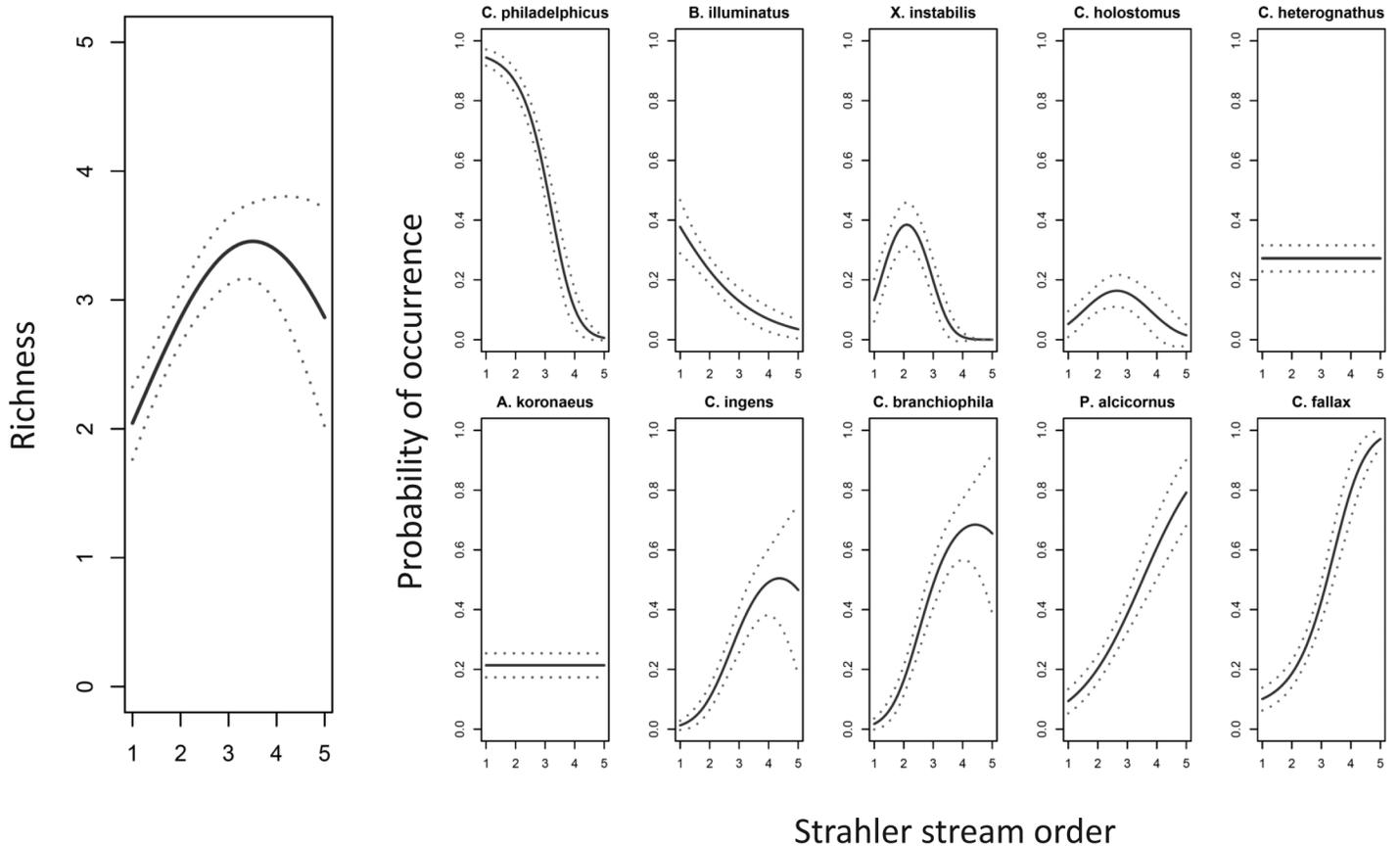


Figure 2. Species turnover of the branchiobdellidans in mountain streams of southwestern Virginia, USA. (left) Model fit (dotted lines = ± 1 SE) for relationship between stream order and branchiobdellidan richness observed by Hobbs et al. (1967). (right) Individual responses of ten branchiobdellidan species to stream order shown as model fits (dotted lines = ± 1 SE) for relationship between presence/absence of each species and stream order. Flat lines indicate intercept-only model as the best model recovered from AIC-based model selection; i.e. no relationship with stream order.

(Galbraith 2005). For each observation, we converted centered Julian day to radians by:

$$Radians_i = 2\pi \times \frac{J_i - 0.5}{365}$$

where J_i represents the Julian day of each observation. The sine and cosine of the transformed data were then used as model terms. Branchiobdellidan abundance (total number of worms per crayfish) was modeled using a negative binomial generalized linear model, implemented using the `glm.nb()` function of the MASS package for R (Venables and Ripley 2002). In our original model, we included terms for crayfish size (TCL in mm), stream identity (Sinking vs. Big Stony), sine and cosine terms for transformed Julian day, and interaction terms for trigonometric Julian day terms and stream identity. To model the effects of season on branchiobdellidan composition, we used principle coordinates analysis to create a composite response variable to describe variation in branchiobdellidan species composition. We then used a generalized linear model assuming a Gaussian error distribution to model the first principal coordinate of branchiobdellidan species composition as a function of crayfish size (TCL in mm), stream identity (Sinking vs. Big Stony), sine and cosine terms for transformed Julian day, and interactions between transformed Julian day and stream. To avoid over fitting, we used the `step()`

function in the R stats package for automated AIC based model selection (Venables and Ripley 2002). Model fit was assessed visually by Q-Q plots and plots of fit versus residuals (Zuur et al. 2009).

Historical Versus Present Composition

We created binary matrices of sampling area by species occurrence for the observations of Hobbs et al. (1967), and our observations from recent years. Because our choice of sampling sites either did not perfectly match those of Hobbs et al. (1967), or the exact location of sampling sites from Hobbs et al. (1967) could not always be reliably determined, we aggregated sites from both data sets into comparable “sampling areas”. For smaller streams and those with few sampling sites, sites were aggregated within the stream. For streams with extensive sampling in both data sets (Sinking Creek, Big Stony Creek, Little Stony Creek, Spruce Run), sites were aggregated into upstream and downstream sampling areas. Sampling sites that occurred on first order feeder streams connected to downstream reaches were not included because they typically did not harbor branchiobdellidan or crayfish species typical of lower reaches, yet were too far removed from upstream sections to be reasonably aggregated with upstream sampling areas. We used a Mantel test to assess the similarity between historical and contemporary records. We converted each

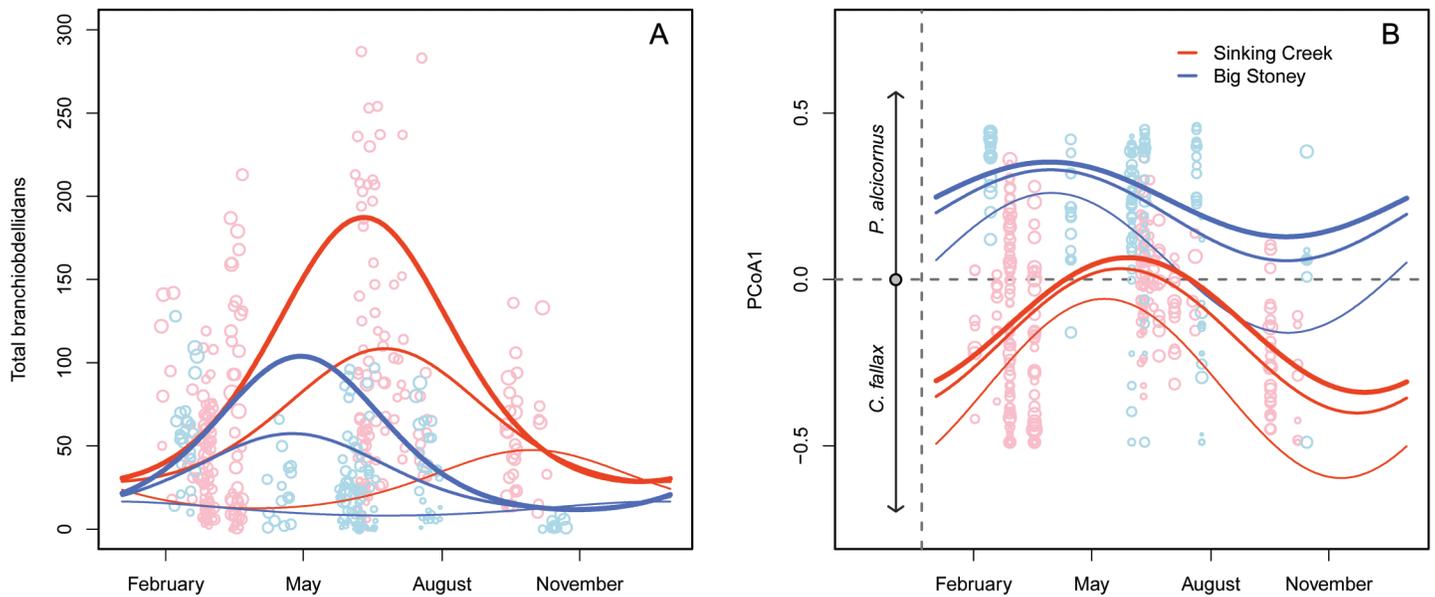


Figure 3. A, Model fit for negative binomial regression of total branchiobdellidan abundance (worms per crayfish) as a function of crayfish size (CL in mm), sampling location (Sinking Creek vs. Big Stoney), and season. B, Model fit showing variation in branchiobdellidan species composition as a function of crayfish size, sampling site, and season. Composition is represented as the first principal coordinate of a Jaccard distance matrix (PCoA1). Arrows show strength and direction of correlation of PCoA1 with relative abundances of the two most abundant branchiobdellidan species. In both panels, line thickness corresponds to crayfish size (20, 35, and 40 mm CL). Circles represent observed data and circle size is scaled to crayfish size (CL in mm). Time of year was modeled as trigonometric terms by taking the sine and cosine of Julian day after conversion to radians. See Tables 2 and 3 for model details.

matrix to a Jaccard distance matrix using the `vegdist()` function, and implemented a Mantel test using the `Mantel()` function in the `vegan` package for R (Oksanen et al. 2013). Significance of the Mantel correlation coefficient was tested using 10,000 matrix permutations.

RESULTS

Hobbs et al. re-analysis: Re-analysis of the observations made by Hobbs et al. (1967) revealed significant variation in branchiobdellidan species composition among streams, and correlations between branchiobdellidan species composition and crayfish species composition, as well as stream order (Table 1). Variation partitioning revealed that 21% of variation in branchiobdellidan species composition was explained exclusively by crayfish species composition, whereas 3% was explained exclusively by stream order, and 8% was shared by crayfish composition and stream order. Throughout the Mountain Lake Region, headwater streams were characterized by the branchiobdellidans *Bdellodrilus illuminatus* Moore 1894, *Xironogiton instabilis* Moore 1894, and *Cambarincola philadelphicus* Leidy 1851 which were typically associated with the dominant headwater crayfish species *Cambarus bartonii* Fabricius 1798 (Figure 1). While compositionally similar among headwater sites, the branchiobdellidan and crayfish assemblages of the three watersheds diverged at downstream sites. This effect was visible in the ordination visualization (Figure 1) and statistically evidenced by the significant interaction term for stream identity and stream order (Table 1). In the New River watershed, the branchiobdellidans *Cambarincola heterognathus* Hoffman

1963, *Cambarincola ingens* Hoffman 1963, *Cambarincola fallax* Hoffman 1963, *Cambarincola branchiophila* Holt 1954, and *Pterodrilus alvicornus* (Moore 1895) were associated with *Cambarus sciotoensis* Rhoades 1944 at mainstem (i.e., Strahler order 3 – 7) sites (Figure 1). In the James River watershed, the branchiobdellidans *Xironogiton instabilis* and *Cambarincola holostomus* Hoffman 1963 were associated with the crayfish *Cambarus longulus* Girard 1852, and *Ankyrodrilus koronaeus* Holt 1965 was correlated with *Cambarus acuminatus* Faxon 1884 in the Roanoke River watershed.

Branchiobdellidan occurrences showed individualistic responses to Strahler stream order (Figure 2). Two species were most frequently observed in headwaters (*C. philadelphicus* and *B. illuminatus*), and two species were most frequent in lower reaches (*P. alvicornus* and *C. fallax*). Four species showed hump-shaped responses to stream order (i.e., quadratic term included in best model; *X. instabilis*, *C. holostomus*, *C. ingens*, *C. branchiophila*). Only two species showed no relationship with stream order (i.e., intercept-only model was chosen by AIC model selection, *C. heterognathus* and *A. koronaeus*). The highest branchiobdellidan species richness occurred at Strahler order 3 – 4, and declined upstream and downstream (Figure 2).

Seasonal variation: There were strong correlations between total branchiobdellidan abundance and crayfish size, stream identity, and season; all highly significant (Table 2). There was also a significant interaction between crayfish size and season; worm abundance varied greatly with season on large crayfish, but varied less on smaller crayfish, and worm abundance was higher in Sinking Creek than Big Stoney (Figure 3; left panel). Branchiobdellidan

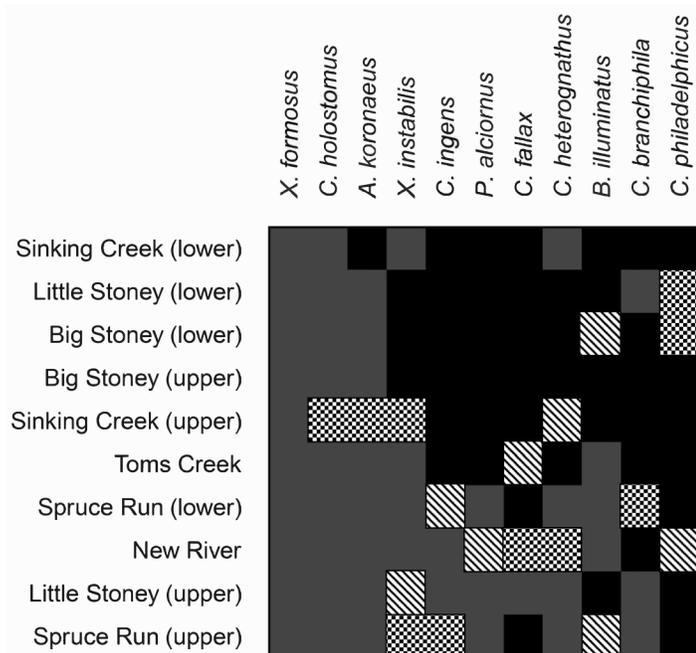


Figure 4. Graphical comparison of species records observed in the present study and those from Hobbs et al. (1967). Rows represent sampling areas composed of aggregated sampling sites, columns represent branchiobdellidan species. Gray cells = absent in both studies, black = present in both studies, checkered = present in Hobbs et al. (1967) and absent in current study, crosshatched = absent in Hobbs et al. (1967) and present in current study.

species composition also varied with crayfish size, stream identity, and season (Table 3). The first principal coordinate of worm species composition (PCoA1) captured 36% of variation in the full dataset and was strongly positively correlated with *P. alciornus* ($r = 0.75$) and strongly negatively correlated with *C. fallax* ($r = -0.94$). Thus, the relative abundances of these two taxa varied antagonistically along PCoA1. Overall, Big Stoney had higher relative abundances of *P. alciornus*, whereas Sinking Creek had higher relative abundances of *C. fallax* (Figure 3; right panel). Shifting PCoA1 values throughout the year indicated increasing relative abundance of *P. alciornus* and decreasing relative abundance of *C. fallax* through the winter and spring months, and opposite trends during summer months (Figure 3; right panel). Crayfish size effects indicate decreasing relative abundance of *C. fallax*, and increasing relative abundance of *P. alciornus* as crayfish grow larger.

Historical versus present composition: A comparison of current records with those of Hobbs et al. (1967) revealed little change in branchiobdellidan species composition across the 10 sampling areas during the intervening ~50 years. Overall, the species matrix from the presented study was 84% similar to that of Hobbs et al. (1967; grey and black cells in Figure 4). Correlation between the two matrices was significant (Mantels $r = 0.64$, $P = 0.002$). We confirmed 45/55 (82%) of species records from Hobbs et al. (1967), and recovered an additional eight species records not reported by Hobbs et al. (1967).

DISCUSSION

The Mountain Lake Region is composed of three major watersheds; the New River which joins the Mississippi River via the Ohio River, and ultimately the Gulf of Mexico, and the James and Roanoke which are Atlantic drainages. Hobbs et al. (1967) noted that the larger tributaries of the New River watershed were dominated by *Cambarus sciotensis*, whereas those of the James and Roanoke were dominated by *Cambarus longulus* in riffle habitats and *C. acuminatus* in pools and deeper runs. In all watersheds within the region, and in fact much of the Appalachian Mountains, headwater streams are dominated by *Cambarus bartonii*. From our analysis, NMDS ordination revealed patterns in branchiobdellidan species composition that mirrored variation in host species composition. Headwater streams throughout the region were typified by *C. philadelphicus* and *B. illuminatus*, and to a lesser degree *X. instabilis* and *C. holostomus*. However, the composition of branchiobdellidans of the three watersheds diverged in mainstem tributaries. The mainstem tributaries of the Atlantic drainages (James and Roanoke) were typified by *A. koronaeus* and *X. formosus*, whilst the mainstem tributaries of the New River were typified by *C. branchiphila*, *C. fallax*, *P. alciornus*, *C. heterognathus*, and *C. ingens*. One possible explanation for the disparity among drainages is simply that separate biogeographic histories led to colonization and/or speciation events which gave rise to the unique faunas of these drainages, and the mountain tops that separate the Gulf and Atlantic drainages has prevented subsequent mixing. This explanation is unlikely as all but two branchiobdellidan species (*C. ingens* and *X. formosus*) were observed in both Gulf and Atlantic drainages, but at variable frequencies. Likewise, strict host specificity fails as an explanation because rampant promiscuity has been observed in the host associations of the Mountain Lake branchiobdellidans (Mc Manus 1960; Hobbs et al. 1967). It appears that these emergent patterns of symbiont diversity are the result of ongoing ecological processes, not the outcome of strict host specificity or complete dispersal barriers.

One hypothesis to explain changing branchiobdellidan species composition from headwaters to mainstem tributaries is that species vary in their individual tolerances to underlying habitat variables, giving rise to patterns of species turnover along habitat gradients. This “Gleasonian Hypothesis” is an enduring central theme of community ecology and emphasizes the importance of environment over species interactions (Gleason 1926). Our analysis revealed patterns of species occurrences along a gradient of Strahler stream order that appear to perfectly conform to the Gleasonian view. The occurrences of each species showed an individualistic relationship with stream order (Figure 2). The emergent effect of these individualistic responses was that branchiobdellidan richness peaked at intermediate stream orders where both headwater and mainstem species could be found. Species-specific variation in thermal tolerances and optima could explain some species turnover along stream corridors, as headwaters tend to be cooler than mainstems and branchiobdellidans vary in their temperature tolerances (Berry and Holt 1959). Although this explanation could explain the limited distributions of species restricted to headwaters, it cannot explain variation among

downstream sites with presumably similar temperature profiles. In contrast to Gleason's view, species interactions almost certainly influence geographic variation in branchiobdellidan species composition. Recent experimental work has demonstrated that interactions among branchiobdellidans cause successional patterns in species composition on individual hosts (Skelton et al. 2016a; Thomas et al. 2016), and thus the effects of such interactions on the composition of branchiobdellidan assemblages at broader scales seems probable. Similarly, differences in host affinities and variation in host species composition are likely to influence local symbiont species composition.

Could variation in host affinities among symbionts explain geographic variation in symbiont composition? Overall variation in branchiobdellidan species composition was better explained by changes in crayfish species composition than stream order alone. While the results of PERMANOVA indicated highly significant effects of stream order on the composition of branchiobdellidans, subsequent variation partitioning analysis indicated that a greater fraction of variation was explained exclusively by crayfish composition (21%), than what was explained exclusively by stream order (3%), though a considerable fraction (8%) was shared between crayfish composition and stream order. These results suggested that branchiobdellidans, though not strictly host-specific, may have affinities for certain hosts. Experimental evidence for a few branchiobdellidan species has shown that some potential host species are difficult to colonize (Farrell et al. 2014a), or not preferred (Brown and Creed 2004; Tanaka et al. 2016). This correlation suggests a stronger relationship between symbiont species composition and host species composition than symbiont species composition and physical habitat. However, stream order is merely a proxy for environmental conditions, not an actual environmental factor. Furthermore, crayfish species composition was closely related to environmental conditions. Because of this confounding correlation, surveys alone are not likely to disentangle the main and interactive effects of habitat and host species composition on branchiobdellidan diversity. We suspect future efforts utilizing field and laboratory experiments will elucidate the mechanisms that drive the correlations between host species composition, physical habitat, and branchiobdellidan species composition. Moreover, molecular studies will likely uncover additional layers of geographic diversity in the branchiobdellidans of the southern Appalachians. We and Hobbs et al. (1967) distinguish species by morphology. A recent molecular treatment demonstrated cryptic diversity among morphologically similar branchiobdellidan taxa, particularly taxa identified by morphology as *C. philadelphicus* (Williams et al. 2012). It is very possible that branchiobdellidans that we and Hobbs et al. (1967) identified as *C. philadelphicus* as the dominant branchiobdellidan of headwater tributaries throughout the region may in fact be multiple cryptic species.

In addition to high species turnover observed among and within streams, branchiobdellidan abundance and species composition followed strong seasonal cycles. Model fits estimated a greater than four-fold increase in total branchiobdellidans on large crayfish (40 mm CL) from Sinking Creek during the spring months, and a similar pattern of lesser amplitude in Big Stony.

Table 1. Results of permutations multivariate analysis of variance (PERMANOVA) for the effects of stream identity and Strahler order on branchiobdellidan species composition in the Mountain Lake Region, Virginia USA. 1,000 Permutations were constrained to within watersheds.

Parameters	df	Mean SS	F	R ²	P
Strahler order	1	4.61	25.80	0.14	0.001
stream	14	0.74	4.16	0.32	0.001
order × stream	11	0.30	1.69	0.10	0.004
residuals	78	0.18		0.43	
total	104				

Table 2. Negative binomial generalized linear model showing main and interactive effects of crayfish size, stream, and seasonality on total abundance of branchiobdellidans in two streams in southwestern Virginia. Seasonality was modeled as the sine and cosine of Julian day centered and converted to radians. Null deviance = 861.11 (df = 421), residual deviance = 477.08 (df = 413), theta = 1.589 (SE = 0.113).

Coefficients	Estimate	SE	z	P
intercept	1.359	0.315	4.307	< 0.001
crayfish size (CL)	0.055	0.008	6.562	< 0.001
stream (Sinking)	0.739	0.125	5.897	< 0.001
sin(radians day)	-1.053	0.410	-2.571	0.010
cos(radians day)	1.202	0.310	3.876	< 0.001
sin(rad) × CL	0.051	0.011	4.480	< 0.001
cos(rad) × CL	-0.042	0.008	-5.005	< 0.001
sin(rad) × stream	-0.624	0.177	-3.530	< 0.001
cos(rad) × stream	-0.377	0.137	-2.757	0.006

Table 3. Generalized linear model showing main and interactive effects of crayfish size, stream, and seasonality on total abundance of branchiobdellidans in two streams in southwestern Virginia. Seasonality was modeled as the sine and cosine of centered Julian day and converted to radians. Composition was modeled as the first principal coordinate of a Bray-Curtis distance matrix based on relativized branchiobdellidan abundances. Null deviance = 30.272 (df = 408), residual deviance = 17.622 (df = 402).

Coefficients	Estimate	SE	z	P
intercept	-0.141	0.063	-2.217	0.027
crayfish size (CL)	0.010	0.002	5.801	< 0.001
stream (Sinking)	-0.377	0.026	-14.565	< 0.001
sin(radians day)	0.310	0.091	3.401	< 0.001
cos(radians day)	0.006	0.029	0.207	0.836
sin(rad) × CL	-0.005	0.002	-2.114	0.035
cos(rad) × stream	-0.174	0.034	-5.122	< 0.001

Based on observations made of *Branchiobdella kozarovi* Subchev 1978, De Witt et al. (2012) identified temperature as the principle driving force behind seasonal changes in branchiobdellidan abundance. Although in partial congruence with their conclusions, our findings do not corroborate temperature as the only important

driver of seasonal changes. We saw increasing numbers throughout the spring, however, peak abundance was reached in May and June in Big Stoney and Sinking Creek respectively, and declined during the warmest months of July and August. A similar pattern was observed in a small Texas pond by Young (1966), in which total branchiobdellidan abundance peaked in June and subsequently declined in July and August. Another author reported highest abundances in fall and winter in a southern Canadian stream (Bishop 1968). Thus, for the branchiobdellidans studied here, by Young (1966) and Bishop (1968), the linear relationship between branchiobdellidan abundance and temperature reported by De Witt et al. (2012) is an insufficient explanation for seasonal dynamics, and a more complete explanation remains to be discovered. We suspect multiple factors interact to create these seemingly ubiquitous seasonal changes. Previous studies have demonstrated negative effects of molting on branchiobdellidan populations (Bishop 1968; Koepp 1975). Perhaps branchiobdellidan reproduction is stimulated by increased resource availability and increased metabolic rates because of warming water temperatures during spring, but losses accrued by increased rates of host molting, particularly in larger hosts, may counteract increased rates of branchiobdellidan reproduction in late summer. Interactions among branchiobdellidans may also influence seasonal dynamics as larger species become more abundant later in the year and consume smaller and more numerous species, as was observed by Thomas et al. (2016). Seasonal changes in branchiobdellidan abundance could have important repercussions on host biology because high densities of branchiobdellidans can cause damage to host tissues and have negative effects on host growth (Brown et al. 2012; Rosewarne et al. 2012; Rosewarne et al. 2014).

In contrast to large crayfish, small crayfish showed little seasonal variation in total worm abundance. Frequent host molting, size-specific host choice, and host grooming behaviors are known to limit branchiobdellidan abundance on small crayfish, and these effects probably supplanted any seasonal changes brought on by increased metabolic rate or resource availability. Host size-specific effects on branchiobdellidan populations and assemblages have been discussed at length in previous work and we direct the interested reader to them (Bishop 1968; Skelton et al. 2013; Skelton et al. 2014; Skelton et al. 2015; Skelton et al. 2016a; Thomas et al. 2016).

Seasonal changes in species composition manifested as the aggregate effect of species-specific differences in the magnitude of seasonal dynamics and antagonistic dynamics between the two most abundant branchiobdellidan species. The abundance of all branchiobdellidans increased during the spring, however, the abundance of *Pterodrilus alcicornus* increased at a greater rate, causing a concurrent increase in the relative abundance of that species (Figure 3 – right panel). Subsequently, this species experienced a greater rate of loss during the summer and autumn, leading to decreased relative abundance, and increased relative abundance of *C. fallax*. One possible explanation is that *P. alcicornus* has a shorter generation time, allowing their populations to increase at a greater rate than the other branchiobdellidans during the spring. Though data on the generation time of this species are not available, *P. alcicornus* was the smallest species present at

our study sites and size is often correlated with generation time (Blueweiss et al. 1978). While the reason for their subsequently higher rates of attrition are completely unknown, this result illustrates how species-specific responses to seasonal drivers may create predictable cyclic changes in the species composition of a symbiont assemblage.

There has been a recent trend in the field of parasitology to identify patterns of community structure and repeatability in parasite communities to better understand the underlying mechanisms that determine patterns of species co-occurrences within and among hosts. Work primarily focused on fish parasites suggests that parasite communities are extremely variable across space and time (Poulin and Valtonen 2002; Timi and Poulin 2003; González and Poulin 2005; Poulin 2007). In our study, the tremendous seasonal fluctuations observed in Sinking and Big Stoney suggest that branchiobdellidan assemblages might experience frequent local extinction and recolonization events that would give rise to a dynamic patchwork of species distributions and populations that blink in and out of existence over relatively small timescales. Thus, the results of our short-term contemporary study might suggest that high temporal variability would make branchiobdellidan assemblages non-repeatable and randomly variable at longer timescales, as has been observed in communities of fish parasites (Poulin and Valtonen 2002; Timi and Poulin 2003; González and Poulin 2005). Contrary to this view, a comparison of historical and contemporary data revealed surprising constancy of branchiobdellidan occurrences throughout the contiguous range of *Cambarus sciotensis* in the New River drainage. Most branchiobdellidan species were found in most of the same places in historical and contemporary records. For example, *Ankyrodrilus koronaeus* was found by Hobbs et al. (1967) mostly in the tributaries of the Atlantic drainages, except for two isolated populations within the New River Watershed; one at four sites in the mid-stretches of Sinking Creek and one in Wolf Creek. During our survey, we confirmed the presence, and in fact, abundance of these populations within the same restricted ranges, despite the continuous distribution of the dominant host (*C. sciotensis*) throughout the New River Watershed. There are no obvious physical or biological barriers to prevent the spread of these populations, yet they remained confined to short stretches of these streams for over half a century. And, despite vast seasonal changes in abundance, they persisted. From these observations, we conclude that branchiobdellidan assemblages appear to be more stable across space and time than has been commonly observed for communities of aquatic parasites, and thus branchiobdellidan assemblages may be driven by different or more deterministic processes than aquatic parasite assemblages. The cause for the discrepancy between aquatic parasite communities and the branchiobdellidans is unknown, but once discovered, could expand our understanding of the ecological processes that structure diverse and ubiquitous symbiont communities that include not only parasites, but commensals and mutualists too.

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