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JOURNAL OF CRUSTACEAN BIOLOGY, 34(5), 611-617, 2014

Journal of Crustacean Biology

POST-LARVAL DEVELOPMENTAL DYNAMICS OF THE SPINICAUDATAN (BRANCHIOPODA: DIPLOSTRACA) CARAPACE

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ABSTRACT

The evolution of reproductive systems is of critical importance to evolution and yet has been difficult to examine using paleontological information. Recent studies on freshwater "clam shrimp" in both extant and fossil populations have demonstrated that sex can be inferred by quantifying morphological differences of the carapaces. However, the extent of morphological continuity between juveniles and mature phenotypes remains unclear, which, if extensive, would confound the determination of sex ratios from fossil specimens. Herein, we report studies of clam shrimp ontogeny using quantitative morphometric techniques to assess carapace shape change through juvenile and early adult development. Intra- and inter-species ontogenetic variance was captured in three species from two families (Limnadiidae, Cyzicidae), three genera (*Paralimnadia*, *Eulimnadia* and *Cyzicus*), and three breeding systems (dioecy, androdioecy and hermaphroditism) using geometric morphometric analyses to identify and characterize discrete phenotypic stages during ontogeny. With this approach, we successfully discriminated juvenile, male and female/hermaphrodite phenotypes. Additionally, we generated models that described the ontogenetic trajectory of carapace shape as species developed into sexually mature phenotypes. These findings verify the ability of these morphometric techniques to distinguish sex ratios using only carapace shape and, thus, validate the use of fossil spinicaudatans for studies of reproductive system evolution in deep time.

KEY WORDS: carapace ontogeny, clam shrimp, morphometrics, paleobiology, shape analysis

DOI: 10.1163/1937240X-00002260

INTRODUCTION

Even though the evolution of breeding systems has been central to evolutionary biology since its inception (Darwin, 1871), the inability to assess breeding systems from most fossils has hampered paleobiological study of this important trait. The fossil record contains relatively few examples of reproductive characteristics. Most of these are instances of dimorphic morphologies in groups like the ammonites (Longridge et al., 2008; Zaton, 2008), dinosaurs (sauropods; Klein and Sander, 2008), mammals (proboscideans, ground sloths, cave bears, bovids and hominids; Lockwood et al., 2007; Reynolds, 2007; Thockeray, 2007; Noubhani et al., 2008; Prothero et al., 2008) and crustaceans (isopods, tanaidceans, decapods and ostracodes; Cohen and Morin, 2003; Hinz-Schallreuter et al., 2007; Narniotko and Martins, 2008; Schallreuter and Hinz-Schallreuter, 2007). These dimorphic taxa are reproductively invariant, except in the ostracodes (Martens et al., 1998), and thus they are generally inadequate for the study of the evolution of breeding systems (Martens et al., 2003; O'Dea et al., 2008). It has become clear that, as the field of evolutionary biology continues to progress, insights gained from evaluations of breeding system evolution across deep geological time will be imperative (Cobbett et al., 2007; Astrop et al., 2012; Legg et al., 2013).

An obscure taxon of crustaceans, branchiopods in the order Spinicaudata ("clam shrimp") appear to have the necessary qualities to allow paleobiological assessment of reproductive evolution over deep time (Gallego et al., 2013; Monferran et al., 2013; Stigall et al., 2014). The most striking feature of Spinicaudata is their bivalved, chitinous carapace, from which they derive their common name, due to its superficial similarity to the shell of bivalved mollusks (Frank, 1988). Even though the carapace is only a weakly biomineralized chitin-calcium phosphate complex, it nonetheless preserves well and is well represented in the fossil record (Frank, 1988; Stigall and Hartman, 2008). Spinicaudata have become a model system for studies of reproductive evolution (Sassaman, 1995; Weeks et al., 1999, 2000, 2006a, b, 2009; Pannell, 2008; Chasnov, 2010). They exhibit a diversity of reproductive systems: dioecy (males + females), androdioecy (males + hermaphrodites) and selfing hermaphroditism (Sassaman, 1995; Weeks et al., 2008; Brantner et al., 2013a, b), with the latter evolving a minimum of four separate times from dioecious ancestors (Weeks et al., 2014). Extant clam shrimp are geographically widespread, occurring on every continent except Antarctica (although there is an excellent Antarctic fossil record for these crustaceans; Tasch, 1987), with a particularly strong freshwater fossil record extending back about 400 million years to the Middle Devonian period (Novojilov, 1961; Tasch, 1969; Woolfe, 1990;

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DOI:10.1163/1937240X-00002260

Shen, 1994; Stigall and Hartman, 2008). Despite their ancient divergence, Spinicaudatan gross morphology appears to have changed little since the groups' origin (Novozhilov, 1953; Tasch, 1963, 1969a; Kobayashi, 1973; Jones, 2000; Timms, 2009). Furthermore, the carapace exhibits sexual dimorphism and has been used to infer sex ratios from fossil taxa (Yanbin, 1994; Astrop et al., 2012), which can be further used to infer the breeding system of the fossil species (Stigall et al., 2014). Thus, clam shrimp are a well-studied group of crustaceans that are reproductively labile, have a tractable fossil record, and are sexually dimorphic in a character that readily fossilizes. These combined traits make this an excellent system to explore the long-term evolution of breeding systems.

Despite the bivalved carapace being the most widely recognized morphological component of both extant and fossil spinicaudatans, the ontogeny of this feature has been relatively under studied. Although several excellent studies have documented the ontogeny of spinicaudatans throughout their larval stages (Cannon, 1924; Negrea et al., 1999; Olesen and Grygier, 2003, 2004; Pabst and Richter, 2004), few efforts have focused on post-larval development, with even fewer describing carapace ontogeny (Bishop, 1968; Weeks et al., 1997; Olesen, 1999). Moreover, all efforts to date have relied on qualitative comparisons of discrete larval stages to describe anatomical changes correlated with development (Olesen, 1999). One study attempted to describe the correlation between carapace length and age, but ultimately reasoned that adult carapace size was remarkably plastic (Bishop, 1968). Thus, post-larval carapace ontogeny has remained vague, persisting as an under studied area of branchiopod biology.

To fully elucidate the evolution of spinicaudatan reproductive systems across geological time, it is essential that analytical methods can distinguish within-species ontogenetic variation in carapace shape from variation between sexes or among species (Rohlf, 1993; Adams et al., 2004; Hammer and Harper, 2006; Benítez, 2013). Unfortunately, several popular models that have emerged for analysis of fossil data have not been evaluated for their ability to delineate such variation (Rohlf, 1986; MacLeod, 1999). Here we focus on evaluating ontogenetic variability using eigenshape analysis, a powerful method for outline-based morphometrics (Lohmann, 1983; MacLeod, 1999). In this study, we use a novel method for quantifying sex- and species-specific shape changes during ontogeny. We then characterize the morphological dynamics of the spinicaudatan carapace, quantifying ontogenetic changes across clam shrimp development. Finally, we provide a model that can be used in future ecological, paleontological and biological studies concerning the evolution of breeding systems in this interesting crustacean system.

Materials and Methods

Study Organism

Spinicaudata exhibit a tripartite body plan consisting of a head with a laterally compressed rostrum, a trunk with paired phyllopod appendages per segment, and a telson (Fig. 1). Males have two pairs of specialized anterior thoracapods, termed 'claspers,' which are used to attach to females during copulation. Females are readily distinguished via the presence of eggs carried on their dorsal/lateral side in an area termed the "brood chamber." Primarily restricted to ephemeral water bodies, clam shrimp are able to

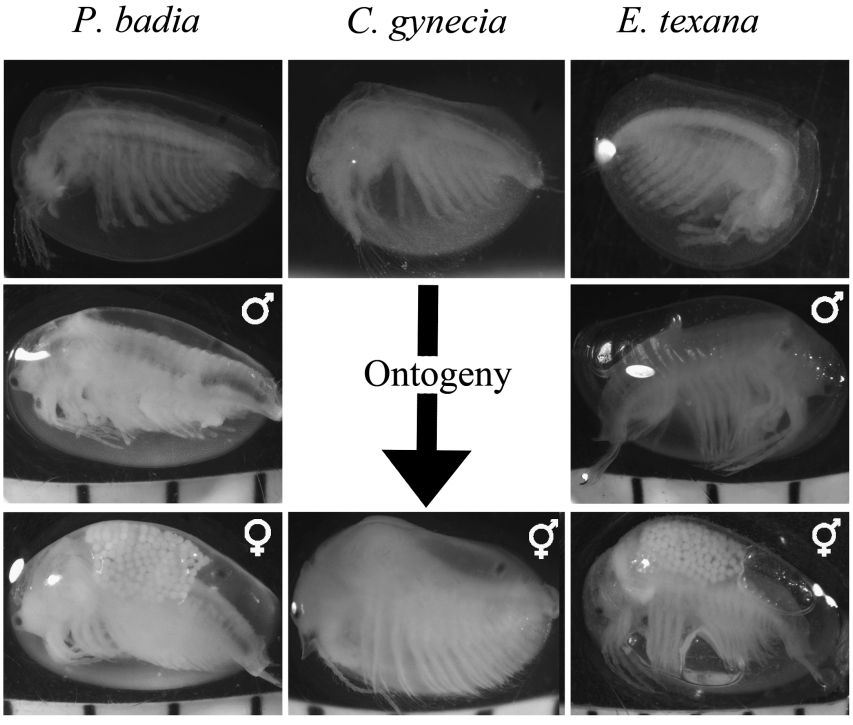

Fig. 1. Images of specimens from each of the three species studied. Where applicable, gender is denoted in the upper right hand corner of each cell. The first row displays the juvenile phenotype of each species. The second row displays the male phenotype (where available) and the third row displays the female or hermaphrodite phenotype.

produce desiccation-resistant cysts that may remain in diapause for years to decades until re-hydration (Dumont and Negrea, 2002; Weeks et al., 2009; Dobrynina, 2011).

Husbandry Protocol

Soil from New Mexico (previously noted as the WAL population; Weeks et al., 1997) was used for rearing *Eulimnadia texana* Packard, 1871. *Paralimnadia badia* (Wolf, 1911) were raised from collections from the Elachbutting region of Western Australia. *Cyzicus gynecia* (Mattox, 1950) was reared from soil obtained from Pymatuning, PA, USA. Soil samples were hydrated in separate, 27-liter aquaria. Each aquarium was filled with approx. 25 l of deionized water maintained at 23 ± 1°C. Populations were raised in a temperature-controlled laboratory held at approx. 35% relative humidity. All aquaria were kept under constant lighting by Vita-Lite synthetic-sunlight fluorescent bulbs (Duro-test, Philadelphia, PA, USA) and oxygenated via air stones (Weeks et al., 1997). Specimens were fed 20 ml of food (2.5 g Spirulina + 2.5 g yeast suspended in 500 ml distilled water) for the first day and 15 ml each subsequent day. Slight deviation from this protocol occurred for populations of *C. gynecia* due to the high population density; they received 20 ml of food every day of the study.

Sampling

Populations were sampled at 8-hour intervals each day. Collections began 72 hours post-hydration (hph, corresponding to the end of the naupliar stage) and continued for 7 days (Olesen, 1999; Olesen and Grygier, 2003, 2004; Pabst and Richter, 2004). *Cyzicus gynecia* only had six full days of sampling. The 72-hour mark corresponds with carapace formation during stage III of larval development (Pabst and Richter, 2004). At each sampling interval, five specimens were isolated from each tank and immediately euthanized in 95% ethanol to prevent bacterial and morphological degradation; specimens were kept in this solution for less than one week between collection and analysis (Bishop, 1968). Thus, for *E. texana* and *P. badia*, 105 shrimp were measured (7 sampling days × 3 samples per day × 5 shrimp per sample), whereas for *C. gynecia*, 90 shrimp were sampled (6 sampling days × 3 samples per day × 5 shrimp per sample).

Eigenshape Analysis

Specimens were transferred to a Petri dish and imaged using an Olympus B061 microscope (Olympus America, Center Valley, PA, USA). Images were recorded with a ProgRes C5 camera (Jenoptik Optical Systems, Easthampton, MA, USA) using ProgRes software. To ensure that carapace shape was accurately captured, all samples were placed on a small volume of water to prevent carapace rolling. Once afloat, the carapace was consistently stable, allowing accurate image capture.

For digital conversion and subsequent analysis, we assigned 500 equidistant Cartesian coordinates along the outline of each carapace with the aid of TPSDig2 software (Rohlf, 2000). Once converted, TPSUtil (Rohlf, 2000) was used to add individual.tps files into an appended.tps. Morphometric analyses were performed using standard eigenshape analysis (MacLeod, 1999). These were executed using modified versions of Jonathan Kreiger's series of Mathematica notebooks, Standard Eigenshape Analysis 2.6 and Guide to Models v0.5 (available online at morpho-tools.net).

Models of shape change during early shrimp development were generated as part of a separate eigenshape analysis. These models were generated by performing eigenshape analysis, with mean eigenshape generation, on samples at approximately the same stage in carapace development. Thus, these models describe mean carapace ontogeny from its emergence (approx. 72 hph) through sexual maturity.

"Morphotype" classifications were denoted either a "male," "juvenile," or "female/hermaphrodite" (where appropriate). Classifications were assigned based upon visual assessment: males have observable "claspers," females/hermaphrodites have eggs, and juveniles lack both (Weeks et al., 2009). For morphotype demarcation, a covariance (rather than correlation analysis) was appropriate as the interest of this study was the variance of phenotype through development. Discriminant matrices were utilized to derive discriminant values from raw eigenscore data.

Statistical Analysis

We implemented a hierarchical cluster analysis utilizing Ward's method to cluster eigenscore data from the first four eigenshapes. Our distance matrix was based on Euclidean distance. To generate support for groups, we conducted 10,000 bootstrap resamplings of the data for each species.

A one-way ANOVA was employed to determine statistical significance of each morphotype. Each morphotype was represented by 15 randomly subsampled specimens of that morphotype. The analysis of variance was conducted with morphotype as the categorical predictor and eigenscores from the second eigenshape as the dependent variable. The second eigenshape was chosen due to its relatively large description of morphological variance. Whereas the first eigenshape likely accounted for variation in carapace size, the second described pure morphological variation, as discerned from our closed eigenshape analysis. Statistical analyses were performed using R 2.14.1.

Results

Figure 2 models gender-specified ontogenetic variance in discrete 24-hour intervals. In all images, each successive outward radiation approximates mean carapace shape 24 hours further into development. The relative surface areas of each layer were normalized among species to more clearly present shape differences, i.e., carapace size differences among species were removed for this comparison. Several studies have noted that anatomical scale is negatively correlated with population density; this has been illustrated in a laboratory setting with *Branchipus schaefferi* Fisher, 1834 (Anostraca) and further observed in wild populations of *Paralimnadia stanleyana* (King, 1855) (Bishop, 1968; Hössler et al., 1995).

Definite clusters appeared when we plotted the eigenscores ($n = 30$) from the second eigenshape against those of the third (Fig. 3). These clusters reflected each of the morphotypes we observed: three for *P. badia* and *E. texana* and two for *C. gynecia* (Fig. 3). Among all morphotypes for each species studied, we found a significant difference between morphotypes, when analyzing eigenscores from the second eigenshape (*C. gynecia*: $F_{1,28} = 222.8$, $P < 0.0001$; *E. texana*: $F_{2,27} = 99.25$, $P < 0.0001$; *P. badia*: $F_{2,26} = 123.88$, $P < 0.0001$).

To further investigate the disparity between morphotypes inferred from each of the eigenshape plots, we performed hierarchical cluster analyses to discriminate each morphotype within each of the three species (Suzuki and Shimodaira, 2006; Fig. 4). There were clearly three major groupings in *P. badia* and two major groups in *C. gynecia*. Within *E. texana*, there was minor variation within the juveniles, but they were clearly distinct from the males and hermaphrodites.

Discussion

The long-term evolution of reproductive systems has been hampered by the difficulty of assigning sex to fossil specimens in taxa with labile breeding systems (Martens et al., 2003; O'Dea et al., 2008). Astrop et al. (2012) proposed a method for extracting sex ratio information from Spinicaudata. This method uses the sexually dimorphic shape of the carapace to distinguish males from females/hermaphrodites, thus allowing the assignment of sexes to fossils and the subsequent inference of breeding system on the basis of sex ratios in the fossil samples, e.g., in the Jurassic *Hardapestheria* (Stigall et al., 2014). A potential problem with this methodology would be if the juvenile Spinicaudata were to have the same shape as one of the two sexes, then those juveniles would be assigned to that sex when in fact their sex was not yet knowable.

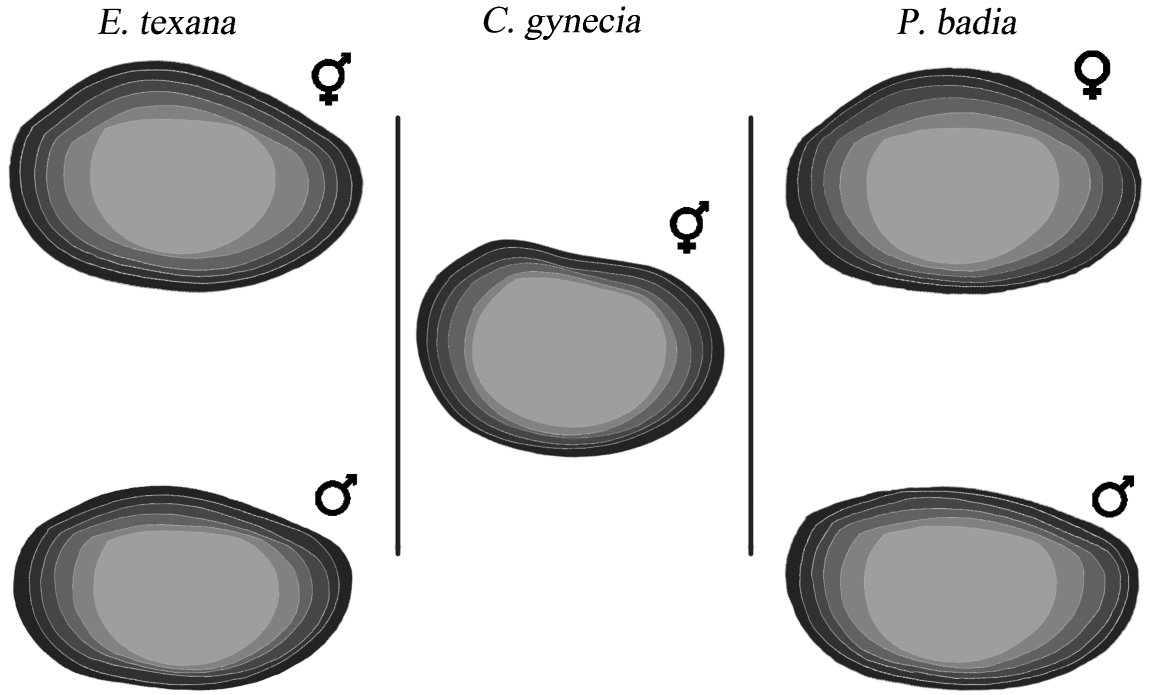

Fig. 2. Characterizations of mean ontogenetic carapace shape of the three species studied. The central figure of all nested images represents mean juvenile shape. Each radiation represents an illustration of mean carapace shape approx. 24 hours further into development. Because *C. gynecia* only had 6 full days of sampling, only 6 images are shown for each species. Species identity is noted above each shape "column." The radiations are not meant to represent relative size over time, but rather illustrate shape changes over 24-hour time blocks.

Herein, we have specifically assessed this potential problem. We have quantified iterative stages during ontogeny and compared our data across all three species representing the three known sexual systems within Spinicaudata. Previous research concerning branchiopod ontogeny has concentrated almost exclusively on larval development; where post-larval development has been mentioned, it is primarily a call for further study (Bishop, 1968; Olesen, 1999).

Bishop (1968) summarized the potential of carapace morphometrics for paleontological purposes but did not suggest how to resolve identification issues. Oleson (1999) added resolution to the field when he assessed the larval and post-larval development of a spinicaudatan, using SEM imaging to designate six larval and three post-larval 'stages' of development. Here, we have simplified these post-larval designations and corroborated the existence of intra-species morphotypes through a quantitative analysis.

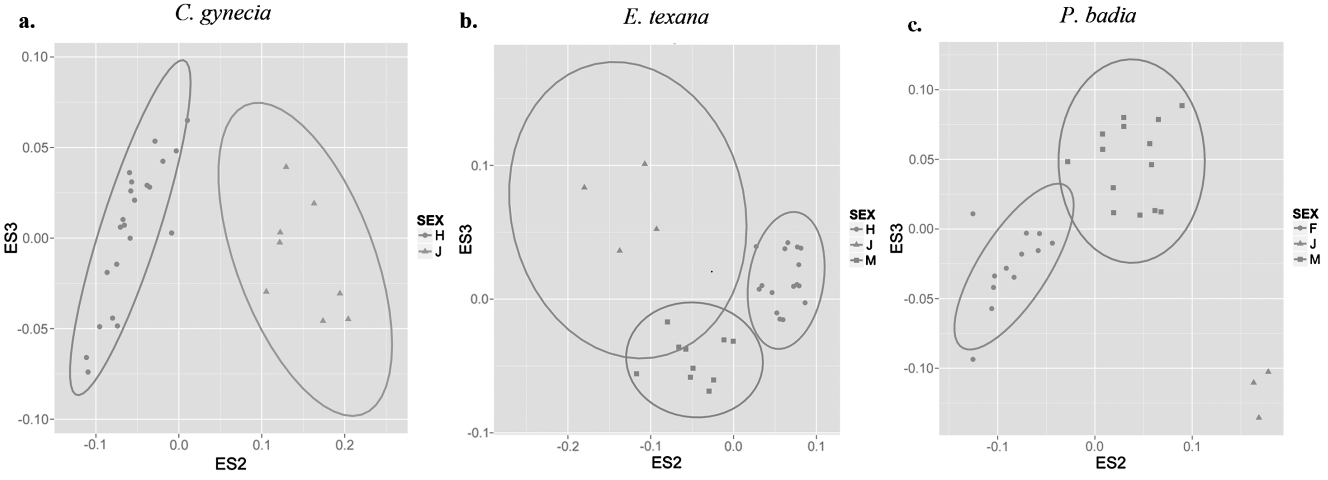

Fig. 3. Loading plots of eigenscores along eigenshapes 2 and 3. Samples and ellipses are shaded by morphotype. Ellipses represent the 95% confidence region for the indicated morphotype, where applicable. Panels a, b and c represent *Cyzicus gynecia*, *Eulimnadia texana* and *Paralimnadia badia*, respectively. Abbreviations: J = juveniles; H = hermaphrodites; F = females; M = males.

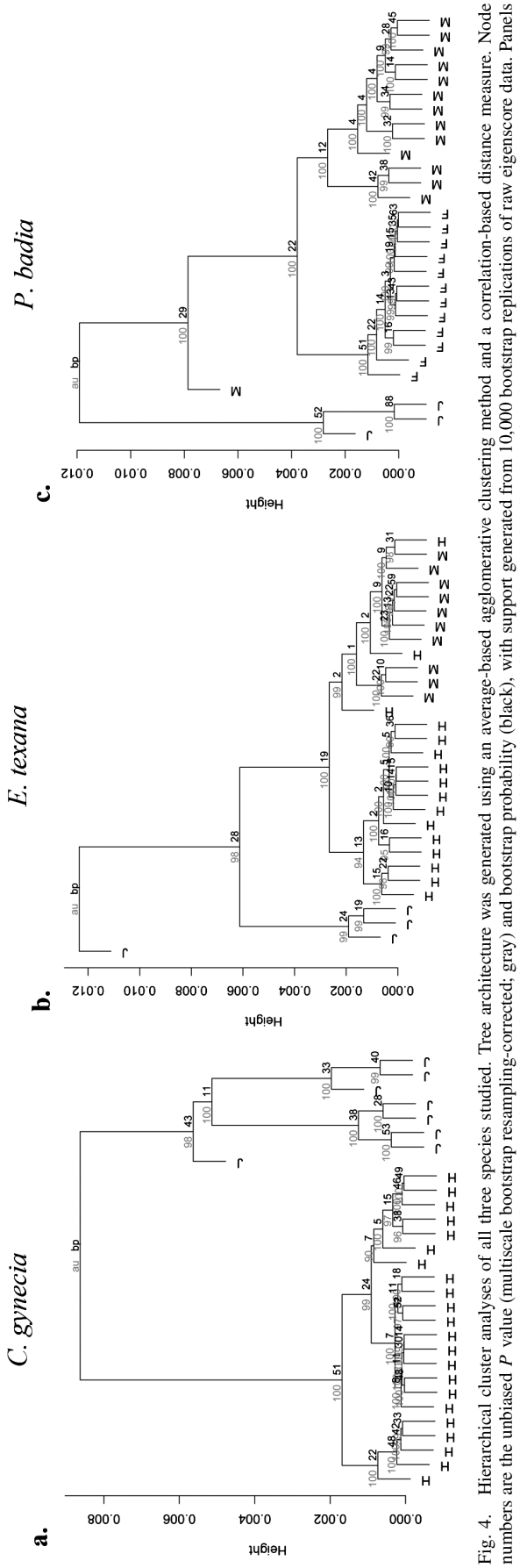

Fig. 4. Hierarchical cluster analyses of all three species studied. Tree architecture was generated using an average-based agglomerative clustering method and a correlation-based distance measure. Node numbers are the unbiased *P* value (multiscale bootstrap resampling-corrected; gray) and bootstrap probability (black), with support generated from 10,000 bootstrap replications of raw eigenscore data. Panels a, b and c represent *Cyzicus gynecia*, *Eulimnadia texana* and *Paralimnadia badia*, respectively. Abbreviations: J = juveniles; H = hermaphrodites; F = females; M = males.

Juvenile Carapace Morphometrics

To assess the existence and degree of morphological distinctiveness of the nascent carapace, we analyzed juveniles of three species representing three distinct genera, two families and three breeding systems. We found that the juvenile stage has a discrete and readily quantifiable shape relative to the adult stage in all three species. Furthermore, we did not detect significant morphological differences between the three species during the initial 24 hours of carapace development, suggesting similar carapace shapes among juveniles of these three species ($F_{2,32} = 2.95$; $P = 0.0666$). Therefore, we propose that juvenile carapace morphology is relatively consistent across species and that the disparity observed between adult phenotypes of different species is the result of allometric trajectories in the realized morphospace. Noting the phylogenetic distance between these families, we feel that this conclusion is indicative of a general trend and could be extrapolated across the Spinicaudata (Schwentner et al., 2009). This conservative juvenile shape may be a remnant of a canalized developmental pathway shared by all spinicaudatans.

Despite initial carapace homology, we observed an increasingly significant amount of morphological variation within the initial 24 hours of post-naupliar development (96 hours post-hydration), in nearly all cases. This phase encompassed the first two carapace molts, which are in line with previous observations (Weeks et al., 1997). Within this timeframe, the trajectory along which the carapace developed in the realized morphospace was at its steepest. This early shape modification was consistent across all three species and corresponded with the high rates of exoskeleton molting that occur during the earlier portions of their life cycle (Weeks et al., 1997). Subsequent molts involved a general increase in scale, though conferred minimal shape change (Hössler et al., 1995). This trend was captured by our models (Fig. 2), as well as the subtle variation in brood chamber positioning between the two Limnadiid species (*E. texana* and *P. badia*).

Carapace Morphometrics of Sexually Mature Phenotypes

Across all three species studied, adult carapace divergence was pronounced and readily quantifiable from the juvenile morphotype as early as 24 hours after carapace formation (96 hours after initial hatching), though this was most distinct in *E. texana* and *P. badia*. *Cyzicus gynecia* developed a mature phenotype later (∼120 hours post-hatching) than both other species. Future studies will expand these observations to additional species and employ the model described herein to assess phenotypic divergence as a function of development.

When two adult sexes were examined (*E. texana* and *P. badia*), the carapace shape of the sexes differed both from each the other and from the juveniles (Figs. 3 and 4). The distinctions between the sexes, i.e., the arched dorsal surface of the females/hermaphrodites and the relatively flattened dorsal surface of the males, were evident in *E. texana* and *P. badia* by 96 hours and then became only slightly more prominent in the following 48 hours, as noted above. Additionally, juvenile carapace shape was distinct from the shapes of both sexes. The data consistently

suggested a higher level of similarity between males and females/hermaphrodites than either had with the juveniles, placing the juvenile branch as the out-group. Thus, juveniles should not be confused with adults for estimates of sex ratios in fossil samples and can be distinguished both on the basis of carapace growth lines and now also on the basis of shape.

In addition to ontogenetic demarcation, our models may also allow for the identification of multiple-species populations of Spinicaudata. As evident in Fig. 2, females of *Paralimnadia badia* position their brood chambers in a medial position along the dorsal periphery of the carapace. Conversely, hermaphrodites in populations of *Eulimnadia texana* position their brood chambers toward the anterior terminus of the dorsal periphery of the carapace. Striking trends such as these may facilitate species-specific identification in populations where multiple species coexist.

CONCLUSIONS

The method detailed herein allows for a quantitative assessment of sexual types as well as differentiation of juveniles from adults. Such analyses, based solely on the Spinicaudatan carapace, may be used to infer breeding systems (Weeks et al., 2009) from fossilized Branchiopoda without the potential misidentification of juveniles as a mature phenotype. As shown in this study, ontogenetic and sexually dimorphic shape changes are ubiquitous and uniform within living Spinicaudata. Herein we have measured two of the three spinicaudatan families (Cyzicidae and Limnadiidae), the latter of which is the most diverse of the three with respect to carapace morphology (Dumont and Negrea, 2002). Cyzicidae and the third family, Leptestheridae, have rather uniform carapace morphology (Dumont and Negrea, 2002). Thus, the results of the current work should apply broadly to most Spinicaudata, but further analyses of the Leptestheridae and more representatives within Limnadiidae (especially the genus *Limnadopsis*) would certainly be warranted to determine the generality of our results.

Destructive taphonomic processes, such as desiccation or decay, are likely to interfere with the fossilization of the carapace, but in distinctly non-affine (non-uniform) ways that add noise to the morphological signals of ontogeny and dimorphism. These interfering phenomena should be considered when performing similar analyses on fossil material. Nonetheless, such noise can be adequately countered with large sample sizes of reasonably well-preserved material. Thus, studies of fossil Spinicaudatans of appropriate sample sizes (Stigall et al., 2014) can allow the comparison of the long-term persistence of various breeding systems in these interesting freshwater crustaceans.

ACKNOWLEDGEMENTS

We thank Alissa Calabrese, Lara Roketenetz, Alex Monnin and Julia Fine for logistical support with population rearing, Jonathan Krieger for his contributions to morphometric analyses, and Edward Ramirez, Alexandria Hannay and Walker Furpahs for thoughtful discussions on the subject. This project was funded in part by the Tiered Mentoring program at the University of Akron.

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RECEIVED: 18 March 2014.
ACCEPTED: 28 June 2014.