DO BRACHIOPODS SHOW SUBSTRATE-RELATED PHENOTYPIC VARIATION? A CASE STUDY FROM THE BURGESS SHALE

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Abstract: As sessile, benthic filter feeders, brachiopods share an intimate relationship with their chosen substrate. Individuals of *Micromitra burgessensis* in the Burgess Shale Formation are preserved in life position, attached to a range of hard substrates, including skeletal debris, conspecific brachiopods, enigmatic tubes and sponges. Here we investigate the phenotypic variability of *M. burgessensis* associated with differing substrate attachments. We apply geometric morphometrics to test for variation by plotting landmarks on the exterior of ventral and dorsal valves of *M. burgessensis* specimens that are preserved attached to different substrates. Using principal component, canonical variate analyses and ANOVA, we

determine that there is some variation in shape related to substrate. Canonical variate analyses, for ventral valves and dorsal valves, indicates that specimens attached to the same substrate are recognizable in shape from specimens attached to other substrate types. The strength of differentiation however, is not robust and combined with our discriminate analysis of separate populations suggests that substrates potentially only exercise weak control in the morphology of Brachiopoda.

Key words: Substrate, brachiopod, phenotypic variation, geometric morphometrics, Burgess Shale, morphology

BRACHIOPODS exhibit considerable variability in valve morphology (Williams *et al.* 1997). Individual brachiopod valves range in shape from conical, to subcircular, alate, rostrate, ostreiform and some families are even heavily spinose (Williams *et al.* 1997; Holmer & Popov 2000; Brunton *et al.* 2000). Studies have increasingly attributed this variability to the diverse range of life modes and environments that brachiopods inhabit (Thayer & Steele-Petrovic 1975; Alexander 1977, 1984; Thayer 1981; James *et al.* 1992; Wang *et al.* 2012; Topper et al. 2015a). Brachiopods are sessile organisms, the large majority of which attach to hard and soft surfaces via a pedicle (Williams *et al.* 1997). Substrate conditions heavily influence the ability of brachiopods to secure and maintain a stable life position and as a result the association between morphological form and substrate is a central theme in brachiopod studies (Stewart 1981; Curry 1982; Alexander 1977, 1984; Collins 1991; Leighton 2000; Haney *et al.* 2001; Wang *et al.* 2012; Topper *et al.* 2015a). The intimate relationship between brachiopods and their chosen substrate is frequently used as a framework for understanding species distribution (Haney *et al.* 2001; Taylor & Wilson 2003; Solan *et al.* 2004; Bromley & Heinberg 2006) and morphological adaptations between species from different geographical areas (Colmenar *et al.* 2014). However, empirical studies investigating intraspecific phenotypic variation in relation to substrate conditions are rare, despite their relevance in understanding how a species morphologically responds to changes in the ecosystem and surrounding environment.

The few studies that focus on the phenotypic variation of brachiopods in relation to substrate have focused on free-living fossil forms (such as the strophomenids and spiriferids; Shiino & Kuwazuru 2010; Plotnick *et al.* 2013; Shiino & Angiolini 2014). Pedicle bearing brachiopods, both fossil and extant have not seen the same degree of examination, predominantly for two reasons; 1) pedicle bearing brachiopods are generally considered to display only minor variations in shell morphology as they are not in direct contact with the substrate (e.g. Shiino & Angiolini 2014) and 2) brachiopods are also frequently disarticulated and are rarely preserved attached to substrate.

As one of the only Cambrian sites that preserve brachiopods in life position (together with the Chengjiang Lagerstätte; see Zhang & Holmer 2013 for review), the Burgess Shale Formation holds a crucial role in understanding the early ecology and adaptive morphologies of the Brachiopoda. The exceptional preservation of the Burgess Shale has yielded brachiopods preserved attached to a range of substrates, including skeletal debris, conspecific brachiopods, enigmatic tubes, sponges and individuals of *Wiwaxia* (Topper *et al.* 2014, 2015a,b). In a recent investigation, Topper *et al.* (2015a) stressed the importance of substrate choice in brachiopods, suggesting that the distribution of suitable hard substrates was intricately linked with the distribution of brachiopod species in the Burgess Shale community. The ecological relationship between brachiopods and their chosen substrate preserved in the Burgess Shale Formation thus provides an excellent opportunity to test if phenotypic variation exists between forms attached to contrasting substrates.

The most commonly preserved attached brachiopod taxon in the Burgess Shale Formation and the focus of this study is *Micromitra burgessensis* (Resser, 1938; Topper *et al.* 2015a). Here we employ a multilevel geometric morphometrics approach to investigate if phenotypic variation exists within the Burgess Shale *M. burgessensis* population and, if variation does exist, can it be directly associated with differences in substrate conditions. Probing the underlying mechanisms of phenotypic variation provides an invaluable insight into how organisms responded to the rapidly changing niche space in the Cambrian.

MATERIALS AND METHODS

Brachiopod specimens

This study focuses on 50 specimens of *Micromitra burgessensis* preserved in life position (39 ventral valves and 11 dorsal valves) from the Cambrian (Series 3, Stage 5) Burgess Shale Formation, Yoho National Park, Canada. We include an additional 86 specimens of unattached individuals to assess the full morphological variation of the taxon in the Burgess Shale (see Multivariate analysis). The examined specimens (Table S1, Table S2; Topper et al. 2016, Data from: Dryad Digital Repository. http://doi:10.5061/dryad.320h5) housed at the Royal Ontario Museum (acronym: ROM), the National Museum of Natural History, Smithsonian Institution (acronym: USNM) and the Geological Survey of Canada (acronym: GSC). Specimens were collected on Fossil Ridge in British Columbia, from the “thick” Stephen Formation, predominantly from the Walcott Quarry Shale Member and the slightly younger Raymond Quarry Shale Member. Two attached specimens and six unattached specimens included in the analysis were collected from talus picking from the Mount Stephen Trilobite Beds (see Rigby & Collins, 2004 for details on localities). *Micromitra burgessensis* specimens included in the following analyses are grouped according to their attachment on particular substrates; for the ventral valves, the enigmatic tube *Tubulella* sp. (6 specimens), the sponges *Pirania muricata* Walcott, 1920 (28 specimens) and *Vauxia gracilenta* Walcott, 1920 (1 specimen), the chancelloroid *Allonia tintinopsis* Bengtson & Collins, 2015 (3 specimens), conspecific shells (8 specimens), hyoliths (3 specimens) and a trilobite carapace (1 specimen). All specimens included in the analyses are unequivocally considered, based on morphological characters, to be representatives of *M. burgessensis*. Specimens were photographed under normal and cross-polarized light using a Canon EOS6D digital SLR camera.

Landmark configuration

Photographed images of each specimen were used to digitize landmarks. To avoid problems concerning landmark absence, well-preserved brachiopod specimens that were obviously attached to a substrate (see Topper *et al.* 2015a,b) were selected so that the morphological features were easily observable and clearly defined. Landmarks are focused exclusively on the exterior of both valves, as no specimens exhibiting interior features have been observed. To characterize valve shape, 16 landmarks were recorded for each ventral valve and for each dorsal valve 13 landmarks were recorded. As brachiopods are bilaterally symmetrical, all paired landmarks were averaged across the central axis (Klingenberg *et al.* 2002; Zeldtich *et al.* 2004, 2005), reducing the total number of landmarks used to 10 in the ventral valve (Fig. 1H) and 8 in the dorsal valve (Fig. 1I). Taking into account just the symmetric component of shape variation has two benefits: 1) it reduces the dimensionality of variation by half, helping with the requirements for sample size for the subsequent analyses (Klingenberg *et al.* 2002) and 2) it can help eliminate possible artefacts of preservation, such as compaction (as previously documented from shale hosted specimens; Webster & Hughes 1999; Klingenberg *et al.* 2002). Some *M. burgessensis* valves do show fractures, indicating signs of compaction, however overall shell shape does not appear to be distorted or sheared. Ventral and dorsal valves exhibit different morphologies and this is reflected in the landmarks chosen (Fig. 1H,I). The discordant number of selected landmarks meant that separate geometric morphometric analyses were performed for ventral and dorsal valves. For each valve, landmarks were selected that would indicate points of the maximum width (ventral landmark 3; dorsal landmark 3) and maximum length of the valve (ventral landmarks 1, 7; dorsal landmarks 1, 6), the maximum width of the hinge (ventral landmark 5; dorsal landmark 5) and the maximum width (ventral landmark 8; dorsal landmark 7) and maximum length of the larval shell (ventral landmarks 9, 10; dorsal landmarks 6, 8). In order to present a more accurate and complete outline, additional landmarks were chosen on the valve margin that represents the point of maximum curvature of the anterolateral and lateral margin of the shell (ventral landmarks 2, 4; dorsal landmarks 2, 4). For ventral valves, an additional landmark was selected that would indicate the maximum width of the homeodeltidium (ventral landmarks 6). Landmarks were digitized using the TPSDig software package (Rohlf 2010)

Multivariate statistics

All raw landmark data were first transformed using Generalized Procrustes analysis (GPA) (Rohlf & Slice 1990; Zelditch *et al.* 2004; Viscosi & Cardini 2011). This transformation filters out differences in landmark location, scale and rotation effects to ensure that any variation in landmark configuration must be a result of shape. All analyses were performed using the transformed dataset.

To identify whether shape variation exists within and among attachment types and the nature of any variation we used both principal component analysis (PCA) and canonical variate analysis (CVA). We used PCA to visualize the shape variation in the dataset (Claude 2008). To identify if group variation exists, we employed CVA, as it maximizes the shape variation between groups that have been identified *a priori* and seeks to find the combination of variables that differentiate those groups best (e.g. Mardia *et al.* 1979; Campbell & Atchley, 1981; Klingenberg *et al.* 2012). The identified groups in our case are the different substrates for attachment (e.g. specimens attached to *P. muricata* are grouped together and specimens attached to *Tubulella* sp. are grouped together). For PCA, we included unattached specimens of *M. burgessensis*, from the Burgess Shale Formation, to assess if our pool of attached specimen represents the full spectrum of morphological variation in the population. Analysis of variance (Procrustes ANOVA) was used to test for significant differences in overall shape for the different attachment groups.

Our specimens come primarily from two distinct geological members (the Walcott and Raymond quarries) that are not considered to be temporally coeval (Collins *et al.* 1983; Fletcher & Collins, 1998; Garcia-Bellido & Collins, 2007). This means the possibility exists that any variation we might observe, rather than reflecting difference in substrate type, may instead be indicative of changes in the abiotic conditions that occur across the sampling interval. To investigate this possibility, we perform discriminant function analysis (DFA) and ANOVA, using geological member (Walcott or Raymond quarry) as our predictor variable. Multivariate statistics were undertaken using MorphoJ (Klingenberg 2011).

RESULTS

The pattern of shape variation for the ventral and dorsal valves of *M. burgessensis* in the Burgess Shale Formation is visualized in Figure 2. The proportion of variance represented by the two primary axes (PC1 and PC2) is 34% and 24% in ventral valves and 33% and 24% in dorsal valves (full values in Fig. 2C,D). For both the ventral and dorsal valves, warped outlines demonstrate that high scores on PC1 correspond to a reduction in hinge line width (compared with low PC1 scores), with the maximum width of the valve located closer to the anterior of the valve and an overall more subcircular outline (Fig. 2). Higher scores on PC2 represent the positioning of the hinge region relative to the anterior margin of the valve (directed towards the anterior of the valve) and a reduced larval shell size relative to the overall size of the shell (Fig. 2). No discernible groupings based upon attachment types for either ventral or dorsal valves can be discriminated based upon PCA. Unattached specimens fall within the total range of shape variation for attached individuals (Fig. 2), indicating that the attached specimens are representative of the total range of shape variation for the Burgess Shale population of *M. burgessensis*.

Results for CVA of the ventral valve show that attachment groups, with some overlap can be clearly delineated along the two major canonical axes (Fig. 3A). Predictive classification by CVA for attachment types with multiple specimens was able to assign taxa to their correct substrate with 72% accuracy. For the dorsal shell, groupings are distinct with no overlap (Fig. 3B) however; predictive classification is weaker than in the ventral valve and only able to assign specimens to their correct substrate group with 63% accuracy. ANOVA identifies a significant difference between the mean shape of the different attachment groups for the ventral valve (p = 0.0128, F=1.63) and for the dorsal valve (p = 0.0046, F=2.54). CVA results show that pair-wise comparisons of difference in mean shape between attachment groups however, is not always significant (see Table S3, S4). For example, specimens attached to *Pirania* are significantly different in shape from all other attachment groups, with the exception of the specimen attached to *Allonia* (Table S3). Whereas the specimen attached to *Allonia* is not significantly different in shape from any attachment group (Table S3). In the dorsal valve (Table S4), specimens attached to *Pirania* are significantly different in shape from specimens attached to both *Micromitra* and *Allonia* (p = 0.0037 and 0.039), however specimens attached to *Micromitra* are not significantly different in shape from those attached to *Allonia* (p = 0.052).

Performing DFA and ANOVA using geological member as our predictor variable, we find no difference between the Walcott and Raymond Quarry specimens for either ventral or dorsal valve outline. There is considerable overlap for the two groupings in the DFA plots for both valves (Fig. 4), predictive classification is inaccurate (ventral outline = 61%; dorsal outline = 77%) and, based upon ANOVA, there is no significant difference between the two groups ($p_{ventral\ outline}$ = 0.31, F=1.21; $p_{dorsal\ outline}$ = 0.14, F=1.59).

DISCUSSION

Our results demonstrate that the shape variation in the Burgess Shale population of *M. burgessensis* is at least partly controlled by differences in substrate type. In our study, the strength of differentiation is not robust; the PCA does not show discrete groupings and whilst CVA does identify discrete predictable groupings based upon substrate type and ANOVA shows significance, individual groupings when compared to each other are not always statistically significant (Figs 2-3, Table S3, S4). Because the correlation we identify is weak, we cannot assert that substrate is a major control on *M. burgessensis* morphology, but the fact that we find any correlation, suggests it does have some influence. There are two possible explanations for the variation we identify. First, that the differences we observe represent ecophenotypic plasticity. Differences in the nature of the substrate potentially act as a forcing mechanism influencing or inhibiting shell growth in specific directions or planes. Or alternatively; each substrate type could be harbouring discrete populations of *M. burgessensis* that are morphologically distinct. This would mean that our signal is phylogenetic, and that *M. burgessensis* in the Burgess Shale Formation consists of a number of cryptic species. Because our overall signal is weak, it would be presumptuous to claim that the open morphospace between some of our CVA groupings indicate cryptic speciation. We therefore take a conservative approach and propose that the Burgess Shale population of *M. burgessensis* is conspecific and the variation we observe likely represents ecophenotypic plasticity.

Our results are somewhat harmonious to studies of living and sub-fossil brachiopod assemblages that noted the conservative morphological nature of brachiopods (e.g. Kowalewski *et al.* 1997; Krause 2004; Tomašových *et al.* 2008). Comparable to our results herein, the shape variation observed in *Glottidia* (Kowalewski *et al.* 1997) and *Terebratalia* (Krause 2004; Tomašových *et al.* 2008) was not considered to have crossed the threshold of shape change that would invite assignment to another new or existing species and the subtle shape variation detected in these studies was instead attributed to factors such as geographic separation (Kowalewski *et al.* 1997), depth (Krause 2004) and ontogenetic changes associated with reorientation due to current strength (Tomašových *et al.* 2008). Kowalewski *et al.* (1997) and Krause (2004) highlighted the remarkable consistency of shape variability in time-averaged brachiopod assemblages, a result similar to that obtained herein, where there is little shape change evident between *M. burgessensis* populations from the Walcott Quarry Member and the younger Raymond Quarry Member (Fig. 4).

Phenotypic variation is the ability of an organism to react to an environmental input with a change of form (Pfenning *et al.* 2010) and is considered ubiquitous in nature (Schlichting & Pigliucci 1998; Fordyce 2006; Pfenning *et al.* 2010). Most fossil taxa are identified to the species level on the basis of preserved phenotype, which in the case for brachiopods is principally the morphology of the shell. The morphometric characters employed here are routinely reported in systematic descriptions of brachiopod taxa (Williams *et al.* 1997; Holmer & Popov 2000) and are collectively considered diagnostic to *M. burgessensis* (Resser 1938; Topper *et al.* 2015a,b). The presence of open morphospace between variants in a fossil species typically results in the recognition of distinct morphospecies and the establishment of discrete taxa (Aldridge 1981; Hohenegger & Tatzreiter 1992; Reyment & Kennedy 1998; Douglas *et al.* 2001; Rufino *et al.* 2006; Leyva-Valencia *et al.* 2012; Neubauer *et al.* 2013). Intraspecific variability is often not taken into account or quantitatively analyzed resulting in an artificial inflation of diversity (De Baets *et al.* 2013, 2015).

As the substrate for attachment consists of a range of biological organisms, the substrate therefore has its own character traits that presumably inhibit or promote shell growth in particular directions. One of the primary foci of many living brachiopods is to attach to a hard substrate and retain the ability to grow and feed efficiently (Thayer & Steele-Petrovic 1975; Alexander 1977; James *et al.* 1992). The shape variation observed in *M. burgessensis* in the Burgess Shale Formation is most likely a result of individuals adapting to the character traits of the substrate in a way that would provide the most stable environment for unimpeded growth and feeding. For example, skeletal elements such as *Tubulella* sp. (Fig. 1A) and hyoliths (Fig. 1E) provide a straight and uniform surface and this is reflected in a relatively straight hinge line of specimens attached to *Tubulella* sp. and hyoliths as the hinge line continues to abut the substrate as the valve grows (as evident in the warped outlines in Fig. 3). This is noticeably different to the prominent homeodeltidium and concave hinge line displayed by the specimen attached to a curved and bent trilobite carapace (Fig. 3A). Regardless of the contrasting architectural aspects of the attachment site, attachment to isolated sclerites (hyoliths and trilobite carapace) would result in largely unimpeded valve growth, as no obstructions are obviously present (Fig. 1C,E). The same could be argued for specimens attached to *Tubulella* sp, however the protracted length of the slender tubes does intermittently result in a number of individuals attaching to the same *Tubulella* sp. specimen, potentially inhibiting growth (Topper *et al.* 2015a).

The chancelloriid *A. tintinopsis* and the demosponges *P. muricata* and *V. gracilenta* provide a relatively straight and invariable surface for attachment (Fig. 1D, F, G) and the ventral and dorsal valves of specimens attached to those substrates have a relatively wide and straight hinge line, (Fig. 3A,B). The point of maximum width of the valve in specimens attached to *P. muricata* is more towards the anterior margin compared with specimens attached to *A. tintinopsis* and *V. gracilenta* that are, at least in the ventral valve, sub-rectangular in outline (Fig. 3A). *Pirania muricata* is a branching demosponge and in addition to branches, *P. muricata* also possesses numerous long spicules that emerge from the external wall (Rigby 1986). Protruding spicules may potentially places constraints on the valve growth of attached individuals, forcing individuals to grow longitudinally rather than laterally (compared with *V. gracilenta*). Attachments to *A. tintinopsis* and *V. gracilenta* however are only represented by a single data point and the single specimen attached to *A. tintinopsis* shares a similar morphospace to the grouping of specimens attached to *P. muricata* (Fig. 3A). Additional attached specimens of either substrate would help clarify these shape changes.

Ventral valve specimens attached to *M. burgessensis* (Fig. 1B, 3A) exhibit a well-defined and prominent homeodeltidium. A feature that could be linked to providing added stability on the rounded anterior margin of *M. burgessensis*. Ventral and dorsal valves of specimens attached to conspecific brachiopods also exhibit a near subquadrate outline with raw linear measurements approaching a 1:1 ratio in terms of maximum width/length of the valve (Fig. 3, Table S1). These characters are not shared by other attachment groupings and it is possible that an increase in valve width may have placed strain on the attachment, a mechanism that forced an increase in longitudinal growth. The majority of attachments on conspecific specimens occurred whilst the brachiopod host substrate was alive (Fig. 1B; Topper *et al.* 2015a) and the shape variation we observe may also be a response of the epibiont's susceptibility to the recurring movement of the brachiopod host opening and closing its valves.

CONCLUSION

Our results demonstrate that the phenotypic variation in the Burgess Shale Formation population of *M. burgessensis* is to some degree affected by differences in substrate type. The character traits of different biological substrates presumably acting as a mechanism that influences shell growth in particular directions. When organisms are faced with new or changing environments, a central challenge is the coordination and origin of different phenotypic traits that would increase the chance of survival. For *M. burgessensis* in the Burgess Shale this meant adapting to the character traits of the substrate in such a way that would provide and maintain stability for uninhibited growth and feeding. Individuals attached to relatively straight and uniform substrates exhibiting a straighter hinge and less prominent homeodeltidium compared to specimens attaching to variably curved and bent substrates. Brachiopod specimens attached to sponges potentially influenced by the presence of projecting large sponge spicules and specimens attached to conspecifics adapting to attachment on a rounded anterior margin. The strength of our signal between attachment groupings does not provide sufficient support for recognition of discrete morphospecies within *M. burgessensis* and suggests that although the morphology of *M. burgessensis* does react to some degree to substrate type, the signal is weak. Concepts such as phenotypic plasticity are of great interest in evolutionary studies and despite the invaluable evolutionary evidence that fossil taxa can offer, studies are few. The present study has shown that the morphology of *M. burgessensis* does react to some degree to substrate type, however the weakness of the signal indicates that influence of substrates on the morphology of the Brachiopoda is relatively minor.

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DATA ARCHIVING STATEMENT

Data for this study are available in the Dryad Digital Repository: https://doi:10.5061/dryad.320h5)

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Figure captions

Fig. 1 *Micromitra burgessensis* (Resser, 1938) from the 'middle' Cambrian (Series 3, Stage 5) Cambrian 'thick' Stephen Formation and landmark configuration. A, ROM63170, RQ +8.2 m, *M. burgessensis* attached to *Tubulella* sp. B, ROM63350, RQ + 11.6 m, *M. burgessensis* attached to the anterior margin of another *M. burgessensis*. C, ROM56952, BW-150 cm, *M. burgessensis* attached to tergite fragment of an unidentified trilobite. D, ROM6215, RQ + 8.8 m, *M. burgessensis* attached to *Allonnia tintinopsis* Bengtson & Collins, 2015. E, ROMxxxx, *M. burgessensis* attached to an unidentified hyolith. F, ROM63339, RQ + 8.4 m, *M. burgessensis* attached to *Vauxia gracilenta* Walcott, 1920 G, ROM63187, BW-170 cm, *M. burgessensis* attached to *Pirania muricata* Walcott, 1920. H, Landmark configuration of ventral valve. I, Landmark configuration of dorsal valve. RQ refers to Raymond Quarry and BW refers to below the base of the phyllopod bed in the Walcott Quarry, succeeding numbers are an indication of stratigraphical level, see Caron and Jackson (2008) for details. All scale bars 5 mm. (Figure 166 mm wide)

Fig. 2 Principal Components Analysis (PCA) of shape variation in *M. burgessensis*. A, First two principal components (PCs) of ventral valve shape. B First two PCs of dorsal valve outline. C, PCA of ventral valve showing full range of PCs and respective percentage of variance. D, PCA of dorsal valve showing full range of PCs and respective percentage of variance. Warped outlines visualize shape variation and colours of data points correspond to attachment types as indicated in the provided legend. (Figure 166 mm wide)

Fig. 3 Canonical variate analysis (CVA) of valve shape variation in *M. burgessensis*. A, CVA of ventral valve outline showing attachment groupings. B, CVA of dorsal valve shape showing attachment groupings. Attachment group mean shapes are visualized using warped outlines. Colours of attachment mean shapes correspond to data point colours in the CVA and those used in Figure 2. (Figure 166 mm wide)

Fig. 4 Discriminate Function Analysis (DFA) of *Micromitra burgessensis* populations from the Walcott (blue) and Raymond (red) quarries. Frequencies of discriminant scores predicted by a jackknife cross validation are shown using histogram bars. Population mean shapes are visualized using warped outline drawings, scale 1. (Figure 166 mm wide)

Table S1 Details of *Micromitra burgessensis* ventral valve specimens included in the analysis, including information regarding attachments, locality and individual measurements. WQ denotes the Walcott Quarry Shale Member, RQ denotes, the slightly younger Raymond Quarry Shale Member and Ta refers to material collected as talus.

Table S2 Details of *Micromitra burgessensis* dorsal valve specimens included in the analysis, including information regarding attachments, locality and individual measurements. WQ denotes the Walcott Quarry Shale Member and RQ denotes the slightly younger Raymond Quarry Shale Member.

Table S3 CVA results of valve shape variation in the ventral valves of *M. burgessensis*. In the results of the CVA, significant *p* values are indicated by light blue boxes.

Table S4 CVA results of valve shape variation in the dorsal valves of *M. burgessensis*. In the results of the CVA, significant *p* values are indicated by light blue boxes.