

Preliminary Study of Temperature Effects on Size and Shape in the Modern Spinicaudatan *Eulimnadia texana* (Crustacea: Branchiopoda)

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Studies of temperature effects on morphology in Spinicaudata have focused on length, with no data on shape. To fill this gap, size and shape variability in response to temperature fluctuations was investigated by rearing the modern spinicaudatan *Eulimnadia texana*. Two days after hydration, juvenile individuals were separated into four different temperature treatments: 20°C, 23°C, 26°C, and 29°C. Hermaphrodite size and shape were analysed by looking at linear combinations of size variables and using Fourier shape analysis; methods that are also used to describe fossil size and shape for better comparison. Size differences were considerable, with reduced growth at low and high temperatures and accelerated growth at the optimum temperature of 26°C, revealing that the reaction of size to increasing temperature is non-linear. The height of the dorsal margin, which is associated with space for egg production in *Eulimnadia texana*, accounts for a high amount of size variability in this species and, presumably, in most of the Limnadiidae. Hermaphrodite shapes reared under temperatures of 20°C and 29°C are statistically distinct while intermediate temperatures yield intermediate shapes. The rate of shape change along temperature is comparatively low between 23°C and 26°C and accelerated at low and high temperatures. With increasing temperature, the highest point of the dorsal margin is shifted towards the anterior of the carapace, while it assumes a median position at 20°C. Our results of strong temperature effects on carapace size and shape imply considerable ecophenotypic variability in Spinicaudata.

Key words: Freshwater ecology, Limnadiidae, Phenotypic plasticity, Spinicaudata, Temperature.

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BACKGROUND

Temporary environments are especially sensitive to temperature fluctuations, which affect growth and life history traits in various branchiopod species (e.g., Belk 1992; Rogers 2015; Huang and Chou 2015 2017). However, there is comparatively little information on the effect of temperature on shape and size variables, other than length, which are key features for palaeontological studies.

Huang and Chou (2015 2017) reported strong temperature effects on life history traits in *Eulimnadia braueriana* Ishikawa 1895, such as earliest hatching time, earliest maturation time, survival, and reproductive mode. Individuals hatched earlier and hermaphrodites matured faster at higher temperatures. In addition, *E. braueriana* grows faster at higher temperatures, and body length will be larger under high temperatures on a particular day. However, there was no significant difference in body length between lower and higher temperatures at earliest maturation time. Hence, Huang and Chou (2015) concluded that there was a significant temperature effect on developmental time rather than body length.

We focused on the spinicaudatan *Eulimnadia texana* (Packard 1871), a model species that has been subject of various studies for the past 25 years (e.g., Marcus and Weeks 1997; Weeks et al. 1997; Astrop et al. 2012; Brown et al. 2014; Weeks 2020; Hethke and Weeks 2020). Belonging to the crustacean class Branchiopoda, *E. texana* is a small clam shrimp (carapace length up to 12 mm, but typically about 5–7 mm; Weeks, 2020) residing within a folded carapace that resembles a clam shell. *Eulimnadia texana* is omnivorous, able to filter feed as well as forage along pond substrates. This species is androdioecious, meaning populations are a mix of males and hermaphrodites (Sassaman and Weeks 1993). Natural *Eulimnadia* populations are typically hermaphrodite-biased (Mattox 1954; Knoll 1995; Weeks and Zucker 1999; Weeks et al. 2008; Rogers et al. 2012), with some populations completely lacking males (Zinn and Dexter 1962; Stern and Stern 1971; Weeks et al. 2008). Hermaphrodites produce desiccation-resistant eggs which they bury within the top several millimetres of the soil (Zucker et al. 2002). These eggs, which are actually encysted embryos (Weeks et al. 2002), hatch 18 to 36 hours after hydration under spring and summer conditions (at

water temperatures above 18°C), releasing nauplius larvae. Larval and juvenile growth is extraordinarily rapid: the shrimp reach reproductive size in 4–7 days after hatching in the laboratory at 27–30°C (Sassaman and Weeks 1993; Weeks et al. 1997), and in as little as 4–6 days in the field (Vidrine et al. 1987). With an average lifespan of 11–15 days (Weeks et al. 1997; Zucker et al. 2001), *E. texana* is adapted to life in ephemeral freshwater pools, ponds, ditches, dry lakes, and other seasonally astatic freshwater habitats throughout the southern United States, west of the Mississippi River, and into Central and South America (Sassaman 1989; Marcus and Weeks 1997; Pereira and García 2001; Rogers 2020). Early growth of *E. texana* is rapid but decreases dramatically after reproductive maturity. In one study of life-time egg production (Weeks et al. 1997), egg production increased and reached a plateau from day seven to day 17 (average lifespan = 15 ± 0.4 days; maximal lifespan = 22 days), after which average daily fecundity dramatically declined. Hermaphrodites produce thousands of eggs in their lifetime; average-sized individuals generate clutches ranging between 100 and 300 eggs, one to two times a day (Weeks et al. 1997). However, egg production and size follow an exponential relationship, and can reach up to 2,000 eggs per day in the largest hermaphrodites (Weeks 2020).

The current study targets the size and, for the first time, the shape response of hermaphrodite carapaces to four different temperature settings. Our focus on carapaces will allow comparison with fossil species that exhibit a considerable amount of carapace size and shape variability on bedding planes (Hethke et al. 2019). In addition, carapace size will be evaluated using a combination of linear variables. Patterns of single variables allow predictions of temperature effects on life history traits such as egg production; specifically, maximal egg production should correspond with maximal length in the temperature range of 23–26°C, with lower egg production above and below this range.

MATERIALS AND METHODS

Culture

The current study is based on a culture experiment carried out in December 2018 at Freie Universität Berlin, Germany, rearing *Eulimnadia texana* collected from Arizona (USA; “Wallace” pool) in 2003. The experiment required five 40 l tanks, heating rods, sunlight simulating light sources (Nicrew, 28 × 10 × 5 cm, 6 Watt LED) and eight glass cups (500 ml). The tanks remained constantly lit during the experiment. Food was prepared by adding 0.5 g yeast and 0.5 g fine

Spirulina algae powder (AniForte) to 100 ml deionized water and kept in a refrigerator. Each cup received 1 ml of food per day.

The hatching temperature was chosen following Belk (1992), who reported similar numbers of individuals hatching at 15–25°C, but incubation times were longer and hatching was spread over several days at lower temperatures. To ensure that individuals hatched at about the same time, the hatching temperature was set to 25°C. As size has been linked to population density (Hethke and Weeks 2020), we chose an appropriate density of 5 inds/500 ml. Temperatures were maintained by placing 500 ml cups, filled with water from the hatching tank, into baths of deionized water. Water levels and temperatures were monitored daily. If necessary, cups were refilled using water from the hatching tank.

Eggs were hydrated on 3 December 2018 using 12 litres of deionised water, and hatched clam shrimp were separated two days after hydration, on 5 December 2018 (population density: 5 inds/500 ml). Due to high mortality rates at temperatures of 18°C and 30°C during a previous experiment, we adjusted the temperature setting to 20°C, 23°C, 26°C and 29°C. Also, individuals were spread over two cups to reduce the influence of population density on size and shape (= eight cups in total). The experiment was ended after the population size in the 29°C tank dropped to seven individuals on 12 December 2018. The shrimp were preserved in 96% ethanol to avoid shrinking and deformation. Mean temperatures achieved were 19.9°C, 23.0°C, 25.9°C, and 28.9°C. To reduce mortality from handling the clam shrimp, individuals were photographed after ending the experiment, using a Keyence VHX-1000 digital microscope. Sexual maturity and population survival were recorded daily. Earliest maturation time in an individual was defined by the first appearance of an egg clutch.

Analytical methods

All analyses were restricted to a hermaphrodite-only dataset, to eliminate bias from sexual dimorphism (Astrop et al. 2012). Size variables of 27 individuals and outlines of 26 individuals were examined (Tables S1, 2). Only surviving individuals were analysed to reduce further ontogenetic variability.

Size

Nine linear variables (Fig. 1) were measured using the vector graphics software CorelDRAW X7 to evaluate size following Defretin-Lefranc (1965), Tasch (1987), Scholze and Schneider (2015), and Hethke et al. (2018). Each size variable was investigated by visualizing one-

sigma standard deviations in mean and whisker plots. Differences in size between individuals of different temperature treatments were explored by means of non-parametric MANOVA (NPMANOVA) using the software Past4.02 (<https://folk.uio.no/ohammer/past/>; Hammer et al. 2001). General carapace size, perceived as a linear combination of height, length and the height of the dorsal margin (u), was addressed by using principal component analysis (PCA) of the log-transformed measurements of H , L , and u based on a variance-covariance matrix. As all loadings on $PC1$ were positive, we considered $PC1$ to reflect a general size variable used for further analyses.

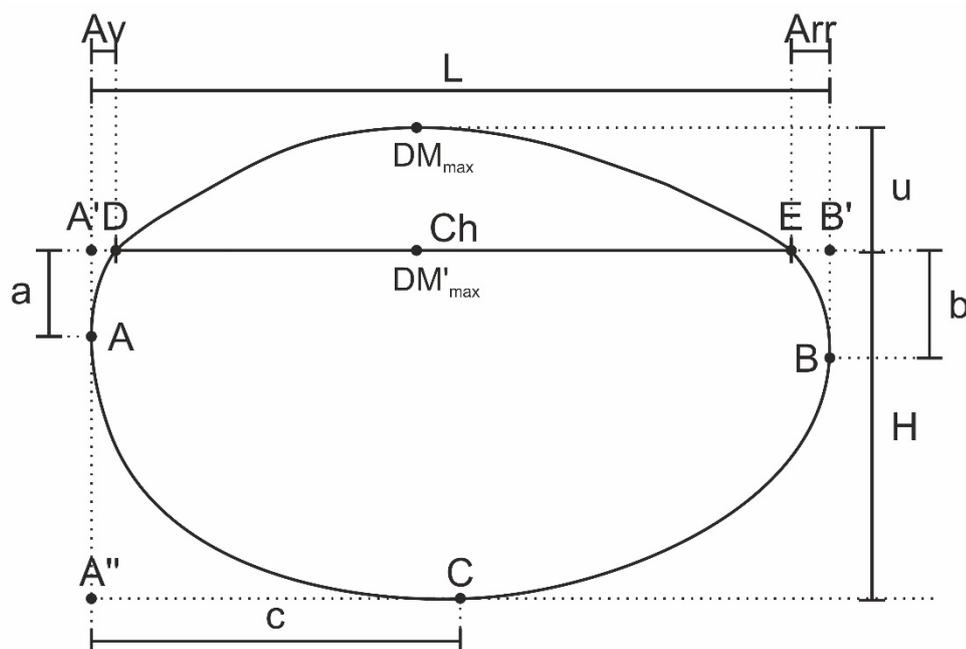


Fig. 1. The nine linear carapace variables obtained, following Defretin-Lefranc (1965) and Tasch (1987). A, most anterior point of the valve; B, most posterior point of the valve; C, most ventral point of the valve; D, anterior extremity of the dorsal margin; E, posterior extremity of the dorsal margin, DM_{max} – highest point of the dorsal margin. a , vertical distance of A to A'; b , vertical distance of B to B'; c , horizontal distance of C to A''; Arr , horizontal distance of E to B'; Av , horizontal distance of D to A'; Ch , length of the dorsal margin; u , vertical distance of DM_{max} to DM'_{max} ; L , valve length; H , valve height. (Abbreviations derive from the French words longueur, hauteur, charnière, avant, arrière.)

Shape

Landmark data of hermaphrodite outlines were captured using the software tpsDIG2 (Rohlf 2010) that offers a tool to trace outlines automatically. As starting position, we chose the homologous anterior extremity of the dorsal margin (landmark D in Fig. 1). For each outline 2500 xy-coordinates were captured. Software packages HANGLE, HMATCH and HCURVE (Crampton and

Haines 1996; Haines and Crampton 2000) were used for Fourier shape analysis and the calculation of mean synthetic outlines. Resultant Fourier coefficients were evaluated using PCA based on a variance-covariance matrix.

RESULTS

Eulimnadia texana earliest maturation time was strongly dependent on temperature (29°C: 5 days after hydration, 23°C and 26°C: 6 days after hydration, 20°C: 8 days after hydration). Hence, our results reflect clam shrimp that are of the same age rather than the same ontogenetic stage.

Size

PC1 and *PC2* of the log-transformed variables *H*, *L*, and *u* explain 88.9% and 10.7% of the variance in the dataset, respectively (Fig. 2A, Table S1). According to NPMANOVA (Table 1), the size of clam shrimp kept at 20°C is distinctly smaller than that of individuals raised under near-optimum temperatures of 23°C and 26°C, illustrated by figure 2B. Growth rates increase with temperature until an optimum temperature has been reached and then decrease again. Hence, the response of size to temperature is non-linear.

Table 1. Non-parametric MANOVA of log-transformed linear variables *H*, *L*, and *u* of *Eulimnadia texana*. Summary and pairwise comparisons. Significant pairwise comparisons ($p < 0.05$) are shaded

NPMANOVA of <i>PC1</i> scores (linear combination of log-transformed <i>H</i> , <i>L</i> , <i>u</i>)			
Permutation <i>N</i> :	9999		
Total sum of squares:	0.179		
Within-group sum of squares:	0.123		
<i>F</i> :	3.471		
<i>p</i> (same):	0.035		
Pairwise comparisons			
	23°C	26°C	29°C
20°C	0.0229	0.0102	0.087
23°C		0.6572	0.7232
26°C			0.4637

The investigation of single linear variables (Fig. 3) indicates that the size pattern in Fig. 2B is driven by variable *u* (20°C distinct; $p_{\text{(same)}} = 0.0046$). Mean and whisker plots of variables *L*, *H*, and *Ch* show a similar pattern as *u*, with optima at 26°C but just one significant pairwise

comparison of 20°C and 26°C for the dorsal margin. Qualitative data of a previous experiment indicates that length is significantly reduced in specimens kept at 18°C. The pattern is inverted for variables *Av*, *Arr*, and *a* (29°C distinct), representing a shift in shape in reaction to high temperatures that lead to high *Av* and *Arr* relative to the dorsal margin (*Ch*).

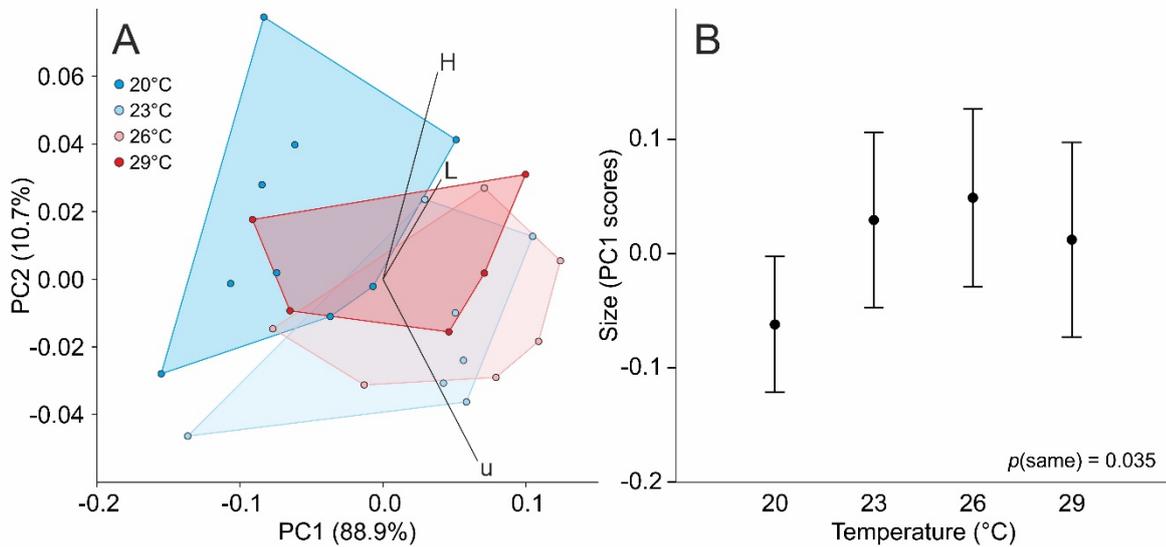


Fig. 2. Temperature effect on size in hermaphrodites of *Eulimnadia texana*. A, PCA of log-transformed variables *H*, *L*, and *u* (compare with Table 1). B, Means and standard deviations (one sigma) of *PC1* scores, which represent a measure of general size.

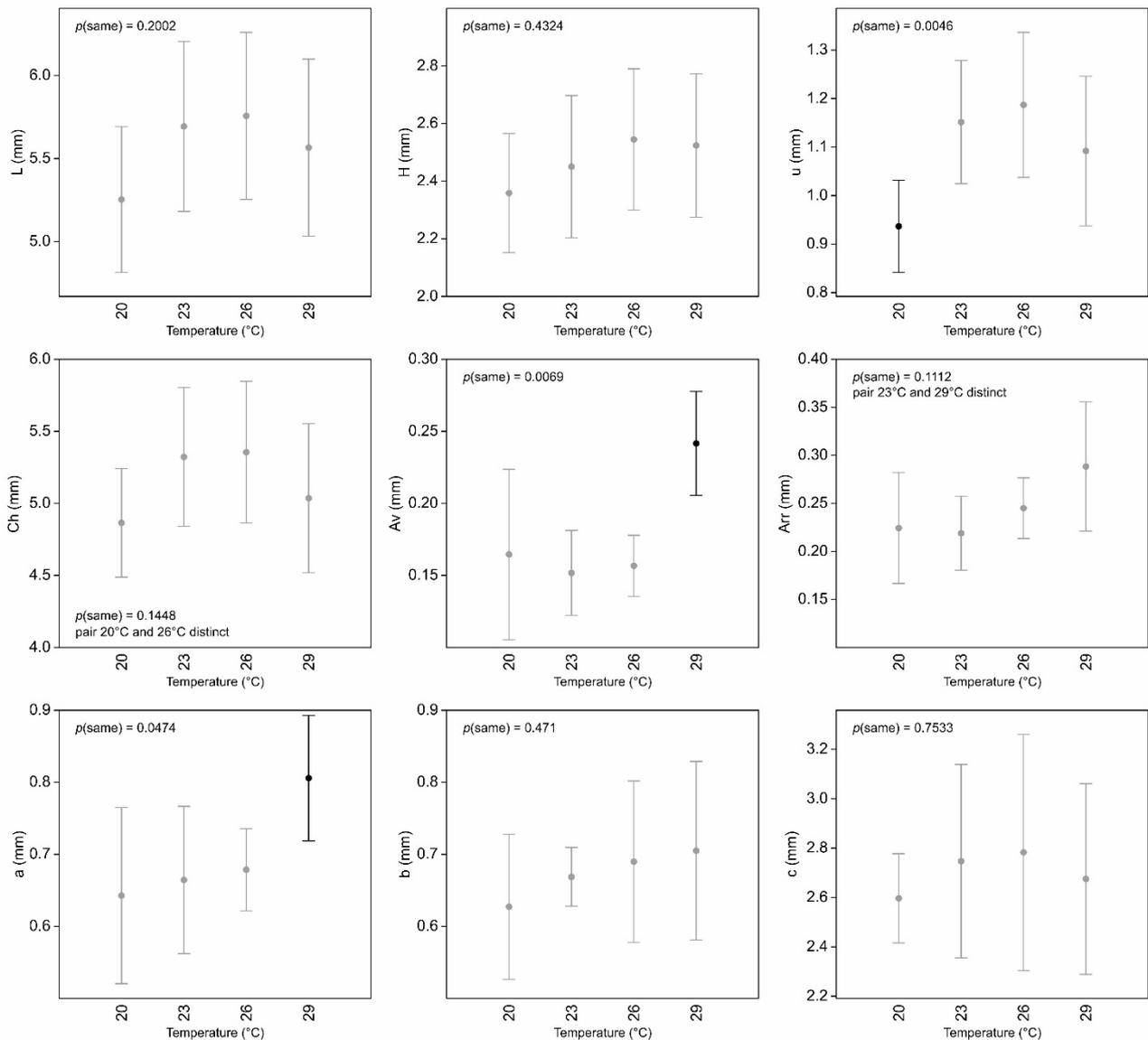


Fig. 3. Means and standard deviations (one sigma) of nine linear variables per temperature. Non-parametric test results for significant difference between the four temperature groups given. Distinct temperatures in black, non-distinct temperatures in grey. Significant comparisons ($p < 0.05$) are mentioned.

Shape

Variables *PC1* and *PC2* explain 49.8% and 10.5% of the variance in the Fourier dataset, respectively (Fig. 4, Table S2). 95% concentration ellipses of temperatures 23°C and 26°C show a high degree of overlap (Fig. 4A), represented by almost identical mean shapes (Fig. 4B), while temperatures 20°C and 29°C are fully separated. NPMANOVA of the Fourier coefficients reveals that shape differences are significant across different temperature settings ($p_{\text{same}} = 0.0001$). In

particular, individuals reared at 20°C are morphologically distinct from shapes of all other temperatures (Table 2).

In general, temperature is increasing towards positive scores of *PCI* in figure 4A. The superimposition of mean outlines in Figure 5 shows that the highest point of the dorsal margin is shifting towards the anterior at higher temperatures. Temperature and shape (represented by group-median *PCI*-values) follow a non-linear relationship, with a decelerated rate of change between 23°C and 26°C.

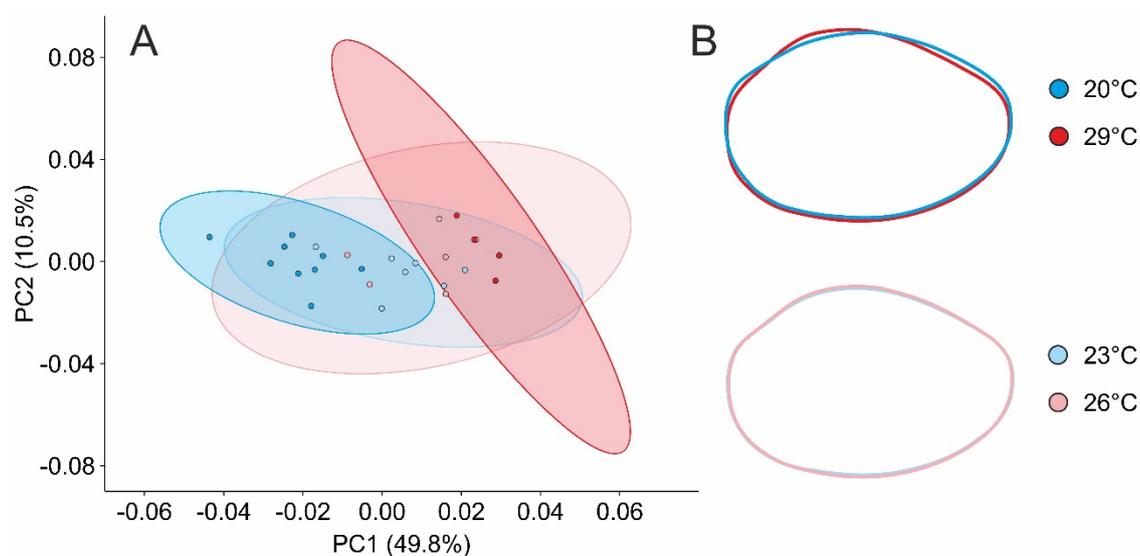


Fig. 4. A, PCA of the Fourier dataset. B, Superimposed mean outlines. While shapes of extreme temperatures (20°C, 29°C) are distinct, intermediate shapes (23°C, 26°C) are almost congruent.

Table 2. Non-parametric MANOVA of Fourier coefficients of *Eulimnadia texana* according to temperature. Summary and pairwise comparisons. Significant pairwise comparisons ($p < 0.05$) are shaded

NPMANOVA of Fourier coefficients			
Permutation <i>N</i> :	9999		
Total sum of squares:	0.020		
Within-group sum of squares:	0.012		
<i>F</i> :	5.251		
<i>p</i> (same):	0.0001		
Pairwise comparisons			
	23°C	26°C	29°C
20°C	0.0011	0.0002	0.0018
23°C		0.5161	0.0117
26°C			0.2077

DISCUSSION

Temperature effect of size and shape in *Eulimnadia*

An evaluation of temperature effects on clam shrimp growth requires notes on life history traits. The present study investigates a single cohort of individuals (same age); nevertheless they reflect slightly different ontogenetic stages. Earliest maturation times vary according to temperature in *Eulimnadia braueriana* (~18.3 days at 15°C; ~ 5.3 days at 30°C; Huang and Chou 2015 2017). As data suggested no significant difference in body length at maturity under different temperature treatments in *E. braueriana*, Huang and Chou (2015) concluded that temperature affects developmental time rather than body length, corroborated by our observations here. Time to maturation in *E. texana* increased with decreasing temperature: Individuals at 29°C matured three days before those at 20°C. Therefore, some of the size and shape variability in response to temperature must reflect the presence of different ontogenetic stages. But, if the temperature effect was purely ontogenetic, then the largest carapace size (at 26°C) should correlate with the most extreme shape (at 29°C). Instead, size and shape (Figs. 2, 4) seem to follow two different trajectories, suggesting an interplay of pure temperature effects and ontogenetic variability.

Temperature effects on shape in *E. texana* include distinct shapes at extreme temperatures and intermediate shapes at optimum temperatures (Fig. 4, Table 2). Hence, the response of shape to temperature is non-linear, described by a decrease in the rate of change between 23°C and 26°C (Fig. 5). In turn, the size response of *E. texana* (same age) to temperature follows an optimum pattern (Fig. 2B), corroborated by estimated body lengths at earliest maturation time of *E. braueriana* (same ontogenetic stage): 2.3 mm, 2.7 mm, 3.2 mm, and 2.9 mm at 15°C, 20°C, 25°C, and 30°C, respectively (Huang and Chou 2017). Although these data are not fully comparable, they suggest a non-linear size response to temperature.

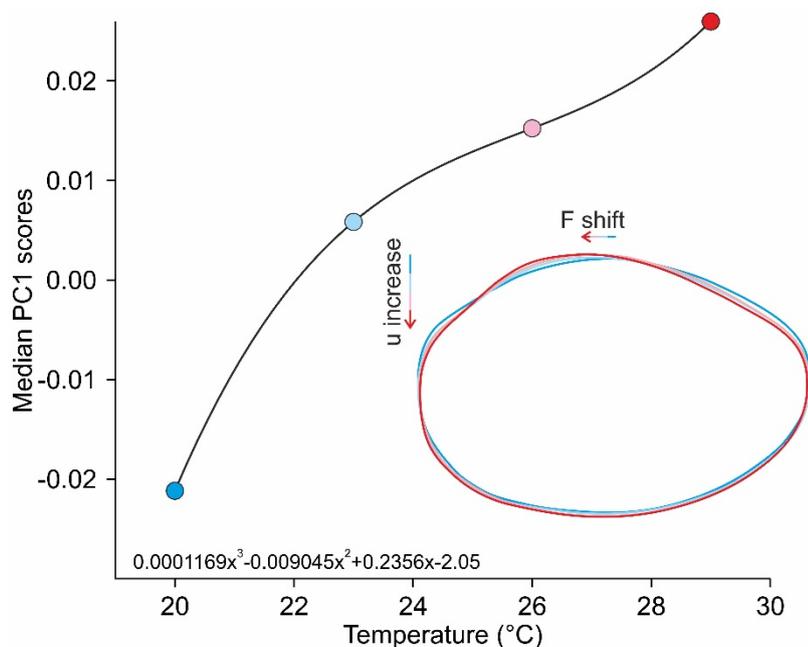


Fig. 5. The relationship between temperature and shape (represented by the group-median *PC1*-values of the Fourier dataset) is non-linear, with a decrease in the rate of change between 23°C and 26°C. Superimposed mean outlines reveal an increase in *u* and a shift of the highest point of the dorsal margin towards the anterior of the carapace at higher temperatures.

It is well established that in *E. texana*, carapace length is strongly correlated with egg production (Weeks et al. 1997; Weeks 2020). In fact, egg production exponentially increases with increasing body size, measured as carapace length (Weeks 2020). Thus, the current data suggest that egg production should be non-linearly affected by temperature: higher egg production should accompany the higher growth rates found in the temperature ranges of 23–26°C (Fig. 2B). Egg production is predicted to be lower above and below this temperature range.

Conversely, the current study contrasts previous results of increasing body length with increasing temperature in *E. braueriana* on a particular day (Huang and Chou 2015; day 4 mean body lengths are 0.53 mm, 0.67 mm, 1.68 mm, 2.84 mm at 15°C, 20°C, 25°C, and 30°C). However, these numbers represent body lengths prior to maturation, while the size pattern at earliest maturation time reflects that of our study.

Comparison to other branchiopod crustaceans

Morphological variability in response to temperature has been reported from other branchiopod crustaceans as well. Rogers (2015) found that the anostracan genus *Branchinecta* matured and reproduced rapidly at higher temperatures, but with a much smaller body and egg clutch size, still producing eggs that are not reduced in size. However, at lower temperatures

Branchinecta matured slowly, at a larger body size, but produced a much larger egg clutch. This suggests that *B. lindahli* sacrifices body size to produce at least a few proper eggs in support of the egg bank during unfavourably warm conditions. In contrast, *Branchinella kugenumaensis* (Ishikawa 1895) is reported to alternate growth with reproduction, spending energy on either one or the other, in an indeterminate growth strategy (Huang et al. 2010). Centeno et al. (1993) found that the nutritional value of the food ingested by parent anostracans does not seem to affect egg viability or offspring sensitivity. Thus, it is inferred that the adult stage function is to produce as many viable eggs as possible in whatever time is available (Rogers 2015).

It will be interesting to look at variable u to understand size variability in response to temperature: increased u at optimum temperatures (Fig. 3) allows space for larger egg clutch sizes. In agreement, Huang and Chou (2015) also reported largest clutch sizes at 25°C for *E. braueriana*. Also, Weeks (2020) identified that egg production increased with size, following an exponential relationship.

Furthermore, growth and shape variation are affected in wild populations by other factors as well. Rogers et al. (2012) provided preliminary data on developmental differences in *E. texana* cultures exposed to predation pressures side by side with cultures without such influence. Rogers et al. (2012) also demonstrated that *E. texana* carapace variation could be derived from the nutritive quality of the food provided in culture. Animals kept to a limited diet matured at a slower rate, had fewer growth lines, smaller clutch size and smaller body length, corroborated by related population density experiments of Hethke and Weeks (2020). Conversely, cultures fed a diet with greater nutritive value had larger body length, a faster maturation rate, more growth lines, growth lines more clearly defined, and larger clutch size.

All these extrinsic factors together exacerbate the great morphological malleability in clam shrimp; indeed, this malleability is why there are no reliable morphological characters to separate adults of *Eulimnadia* and most *Paralimnadia* (Rogers 2020). Clearly, the optimum size hypothesis and the non-linear response of shape to temperature need further testing, including various ontogenetic stages. In combination, such morphometric data might provide an excellent tool to identify stress and optimum environments.

CONCLUSIONS

Temperature strongly affects morphology and developmental time in *Eulimnadia texana*. The size response of *E. texana* (same age) to temperature follows an optimum pattern, implying largest sizes at optimum temperatures and smaller sizes at low and high temperatures. The size

pattern can partly be explained by high egg production at optimum temperatures. In contrast, the shape response to temperature includes distinct shapes at extreme temperatures and intermediate shapes at optimum temperatures. The rate of shape change is comparatively low between 23°C and 26°C and accelerated at low and high temperatures.

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Ethics approval consent to participate: Not applicable.

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Supplementary materials

Table S1. *Eulimnadia texana* size measurements. Hermaphrodite-only dataset. (download)

Table S2. Fourier coefficients of 26 outlines of *Eulimnadia texana* hermaphrodites. (download)