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PRIMARY RESEARCH PAPER

Effects of dietary restriction on lifespan, growth, and reproduction of the clam shrimp *Eulimnadia texana*

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Abstract Diet has been linked to lifespan in a broad range of animals. In particular, pronounced caloric restriction has been associated with increased longevity. Herein, the relationship of feeding environment and longevity is examined experimentally using Spinicaudatan crustaceans ("clam shrimp") from the geographically widespread genus *Eulimnadia*. Two projects examine the effects of food differences on longevity, one comparing food environment early vs later in life and one comparing the effects of dietary restriction on longevity, growth, and reproduction. In the former, dietary restriction increased longevity, but only when diets were reduced later in life. A closer examination of the effects of dietary restriction in the second experiment did not find the expected trade-off of increased longevity and reduced reproductive output in the lowest-food treatment, but instead found an overall increase in both longevity and egg production on the low compared to the high food diet. Finally, a detailed analysis of the relationship of size (carapace length) with egg production was presented. This relationship could serve to obtain a rough estimate of reproductive output potential of fossil clam shrimps, of which typically only the carapace size can be assessed.

Keywords Senescence · Aging · Caloric restriction · Clam shrimp life history · Conchostracan fossils

Introduction

Several experiments, from fruitflies to mice to nematodes (Masoro, 1988; Rose, 1991; Kirkwood & Shanley, 2005; Phelan & Rose, 2005), strongly suggest that food limitation prolongs lifespan. Indeed, correlational data on humans have also noted reduced caloric intake reduces the incidence of a broad range of age-related diseases and generally increases lifespan (Balasubramanian et al., 2017). The specific mechanism for this prolongation has not been determined, but it has been suggested that starvation decreases the investment in reproduction, and that this is the cause of the increased lifespan (Phelan & Rose, 2005). In most of the above studies, starvation does indeed lead to both reduced reproductive investment and a longer lifespan. Thus, these data do suggest at least a correlation (if not a causative relationship) between investment in reproduction and lifespan.

Because manipulative experiments studying aging must, by definition, assess total lifespan, most such studies have used short-lived experimental organisms,

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such as insects (Rose, 1991; Clutton-Brock & Langley, 1997) and nematodes (Johnson & Hutchinson, 1993; Gems & Riddle, 2000; McCulloch & Gems, 2003). Spinicaudata crustaceans ("clam shrimp"), which commonly live in temporary pools (Dumont & Negrea, 2002), should be excellent animals for assessing the effects of diet on longevity. Clam shrimp have been primarily studied taxonomically (Schwentner et al., 2009; Timms & Richter, 2009; Rogers et al., 2012; Timms, 2016; Padhye & Lazo-Wasem, 2018; Tippelt & Schwentner, 2018), although the group is quite important in the fossil record (Tasch & Shaffer, 1964; Tasch, 1987; Vannier et al., 2003; Monferran et al., 2013; Li, 2017; Morton et al., 2017; Geyer & Kelber, 2018; Gueriau et al., 2018; Hethke et al., 2018). Clam shrimp have a "bivalved" carapace that folds around the animal to give it the outward appearance of a small clam, thus the name. It is this tough, multi-laminar "shell" that can survive the fossilization process (Orr et al., 2008; Astrop et al., 2015), allowing these crustaceans to be so numerous in the freshwater fossil record (Kobayashi, 1954; Tasch, 1971; Chen & Shen, 1985; Tasch, 1987; Walossek, 1993; Gallego & Breitkreuz, 1994; Shen, 1994; Orr & Briggs, 1999). Clam shrimp are primarily filter feeders and detritivores and can grow quite rapidly in the shallow, freshwater pools they inhabit (Weeks et al., 1997; Dumont & Negrea, 2002; Perez-Bote et al., 2014; Huang & Chou, 2015; Calabrese et al., 2016).

Clam shrimp in the family Limnadiidae have been more broadly examined for their range of reproductive biology. Limnadiid clam shrimp include dioecious (separate males and females), hermaphroditic, and androdioecious (males + hermaphrodites) species (Sassaman, 1995; Weeks et al., 2006a, 2009; Brantner et al., 2013b). It appears that the ability to self-fertilize has evolved three separate times in this family (Weeks et al., 2014b), and in all cases a mutant "female" has evolved with a small region of the gonad producing sperm with the remainder producing eggs (Zucker et al., 1997; Brantner et al., 2013a, b). Such a "female-biased hermaphrodite" can either spread through the species replacing females and coexisting with males (i.e., androdioecy) or can completely displace both males and females to be all-hermaphroditic (Weeks, 2012). Androdioecious species are much more common within the Limnadiidae, all such species being within the genus *Eulimnadia* Packard, 1874 (Weeks et al., 2008; Reed et al., 2015). At least three limnadiids are known to be all-hermaphroditic: *Eulimnadia agassizii* Packard, 1874 (Zinn & Dexter, 1962; Smith, 1992; Weeks et al., 2005), *Calalimnadia mahei* Rogers et al. 2012 (Weeks et al., 2014b), and *Limnadia lenticularis* (Linnaeus, 1758) (Zaffagnini, 1969; Tinti & Scanabissi, 1996), although a few rare males have been described from the latter species (Eder et al., 2000).

The greatest information about clam shrimp has been reported on species in the genus *Eulimnadia*, primarily due to explorations of their unique androdioecious breeding system. These shrimp live in small temporary pools found in various habitats throughout the world (Mattox, 1939; Mackin, 1940; Belk, 1972; Webb & Bell, 1979; Vidrine et al., 1987; Martin, 1989; Martin & Belk, 1989; Maeda-Martinez, 1991; Pereira & Garcia, 2001; Durga-Prasad & Simhachalam, 2004). They have resting "cysts" that dry out between pond hydrations that will then hatch nauplius larvae after rehydration. These nauplii quickly grow into juveniles in a few days (Olesen & Grygier, 2003). The shrimp either develop as males or hermaphrodites, which is genetically determined in a Z-W (female heterogamety) sex-determining system (Sassaman & Weeks, 1993; Weeks et al., 2010). Hermaphrodites can either self-fertilize or mate with males to produce offspring (Knoll & Zucker, 1995). Offspring produced via selfing have high levels of inbreeding depression (Weeks et al., 1999, 2000, 2001, 2006b; Weeks, 2004). Yet *Eulimnadia* populations are commonly skewed towards hermaphrodites, being 70-80% hermaphroditic (Sassaman, 1989; Weeks & Zucker, 1999; Weeks et al., 1999) suggestive of high rates of selfing in natural populations. *Eulimnadia* grow quickly in early life, maturing in 5–7 days, and then typically produce about one clutch of several hundred eggs per day; lifespan is 3–6 weeks (Weeks et al., 1997).

Herein the reproductive and basic biology of *Eulimnadia texana* Packard, 1871, is further explored. The hypothesis that restricted food extends overall lifespan is tested herein using two experiments that assess the effects of food restriction on lifespan, growth, and egg production in these shrimp. Additionally, the relationship of reproductive output to size (measured as carapace length) was explored on a fine-scale level using measurements on over 7000 individuals. These life-history data should be helpful to researchers on both living and extinct clam shrimp.

Methods

Feeding experiment 1

To assess the effects of diet on clam shrimp longevity, *E. texana* hermaphrodites were reared in individual 500 ml plastic cups and fed three levels of a yeast solution (1 g yeast/100 ml water) added once daily (Weeks et al., 1997). Water levels were checked daily and water was added to replace any water lost due to evaporation. Clam shrimp cysts were hydrated (using deionized tap water) from soil samples taken from New Mexico (WAL location; Weeks et al., 1999) and were initially reared in 27 l aquaria. Once shrimp reached 5 days of age, they were transferred into the 500 ml plastic containers using water from the 27 l hydration aquaria and placed into an environmental chamber set at 25 °C that was fitted with sunlight-simulating fluorescent bulbs. After isolation, the shrimp were fed one of nine feeding regimes (Table 1). The food switch (if there was one) was at 9 days of age, or 4 days into the experiment. Only hermaphroditic shrimp were used for analysis to remove the effect of sex (males commonly have higher mortality than hermaphrodites; Zucker et al., 2001). Shrimp were assayed for survival every day until death.

Feeding experiment 2

To explore further the effects of diet on growth, egg production, and longevity, *E. texana* hermaphrodites were isolated in individual 500 ml plastic cups and fed either low (0.5) or high (1.0 ml) food levels throughout their lives. Clam shrimp were hydrated as noted above and then transferred into 500 ml plastic containers at 5 days of age in the same manner explained above. In this experiment, the cups were placed on shelving in a wet laboratory under sunlight-simulating fluorescent bulbs. The temperature in the laboratory ranged from 23 to 25 °C. After isolation, the shrimp were fed one of the above two levels of food. The shrimp were assayed daily until death and water was added to replace any water lost due to evaporation. Shrimp maturing as males (identified by the development of "claspers" on the first two trunk limbs; Dumont & Negrea, 2002) were removed from the study. Each day, the shrimp were removed from their cups, their carapaces were measured (maximal length), and the eggs in their cups were removed and counted. Each shrimp was returned to its cup and then fed. The entire measurement and egg removal process took ~ 5 min per cup. At death, the carapaces were measured and the final eggs were removed and counted from the rearing cups.

Table 1 Experimental design for feeding experiment 1

Treatment	Food level (ml)		*N*
	Days 1–4	Days 5 +	
Low–low	0.5	0.5	15
Low–med	0.5	1.0	15
Low–high	0.5	1.5	14
Med–low	1.0	0.5	15
Med–med	1.0	1.0	16
Med–high	1.0	1.5	14
High–low	1.5	0.5	15
High–med	1.5	1.0	16
High–high	1.5	1.5	14

Days day of experiment, *N* number of replicates

Carapace length and egg production

The relationship of size (measured as carapace length) and egg production was assessed using previously published data on hermaphrodites from three projects (Weeks, 2004; Weeks & Bernhardt, 2004; Weeks et al., 2014a), totaling 1,000,495 eggs counted from a total of 7463 clam shrimp. Two of these studies had shrimp reared in the field in artificial pools (n = 1763; Weeks & Bernhardt, 2004; Weeks et al., 2014a). The former location was a pool near Portal, Arizona (designated "WAL" in previous publications) while the latter were several pools in the USDA/ARS Jornada Experimental Range and NSF-LTER Jornada site in New Mexico. A third had shrimp collected from naturally filled pools (n = 260; Weeks et al., 2014a), also from the Jornada sites in New Mexico. A fourth had shrimp reared under laboratory conditions (n = 5440; Weeks, 2004). These shrimp were from four populations: three from the Jornada sites in New Mexico (previously termed "JD1", "JT4", and "SWP5") and one from the population from Portal, Arizona (WAL). The size/egg production relationship was assessed across all of these rearing conditions to present the broadest array of sizes and rearing conditions possible. In all experiments, egg production was assessed using pictures (collected using an

Olympus microscope equipped with a COHU High Performance CCD Camera) of both lateral sides of the animal and then directly counting the eggs through the clear carapace (for detailed information on the egg counting technique, see Weeks et al., 1997). The size of the shrimp was then assessed from these images using the Scion Image software package to measure the maximal carapace length.

Statistical analyses

The effect of food treatments on lifespan (feeding experiment 1) was assessed using a two-way ANOVA, with three "before" food treatments crossed with three "after" food treatments (Table 1). Age at death was natural-log transformed to normalize the residuals.

The effects of high vs low food were assessed on size, egg production, and longevity (feeding experiment 2) using three one-way ANOVAs, one for each of the three dependent variables.

All statistical analyses were conducted in JMP Pro 13.2.1 (SAS Institute, Inc.).

The relationship of size (measured as carapace length) to egg production was assessed using the nonlinear regression function in SigmaPlot 12 (Systat Software Inc., 2011).

Results

Feeding experiment 1

Longevity (age at death) was influenced by food treatment, but only the food treatments experienced later in life (Table 2). The highest food diet (1.5 ml food/day) later in life had significantly lower longevity relative to either medium (1.0 ml food/day) or low (0.5 ml food/day) food later in life (Fig. 1). In all three early-food treatments, the medium food later in life ranked the highest in lifespan (Fig. 1), but these rankings were not significantly different (Table 2).

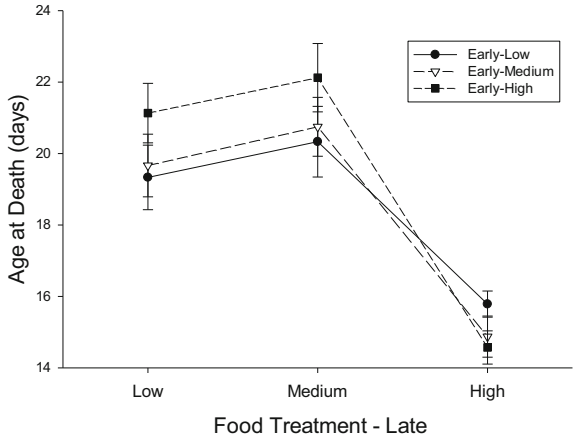

Fig. 1 Longevity (age at death) for clam shrimp fed 9 types of feeding treatments (feeding experiment 1): low, medium and high food from age 5 to age 9 crossed with the same three food levels fed from age 10 until death. Error bars portray one standard error of the mean

The food treatment in early life ranked from lower to higher longevity with more food in the low and medium late-diet treatments, but this order reversed in the high late-diet treatment. However, there was no significant effect of early diet on lifespan and no significant interaction of early and later diets on total lifespan (Table 2; Fig. 1).

Feeding experiment 2

A second feeding experiment was conducted to assess whether diet affected life-history variables consistently or there may have been a trade-off of longevity with growth or egg production. This experiment used the two lower food treatments (half = 0.5 ml/day; full = 1.0 ml/day) to maximize the likelihood of detecting any life-history trade-offs, if they existed. Growth (carapace length) and lifetime egg production were measured along with longevity (age at death).

Once again, the higher food treatment had marginally significantly lower longevity (Table 3). It was clear that longevity did not trade off with increased

Table 2 ANOVA of age at death for feeding experiment 1

Source	df	Sum of squares	*F*-ratio	*P* value
Food treatment-before	2	0.039	0.797	0.4528
Food treatment-after	2	2.773	56.563	< 0.0001
Before × after	4	0.141	1.435	0.2263
Error	127	5.013		

egg production nor increased growth rate: lower food also resulted in increased egg production and there was no difference in growth between full and half food treatments (Table 3; Fig. 2).

Interestingly, although the trend of lower food resulting in higher performance was the same between feeding experiments 1 and 2, the performance differed in the two experiments for the actual level of food: the best performance in feeding experiment 1 was at 1.0 ml/day whereas the best performance for feeding experiment 2 was at 0.5 ml/day (Fig. 1 vs. 2).

Carapace length and egg production

The relationship of size and egg production was clearly nonlinear (Fig. 3). The nonlinear regression produced a high *R*-squared (0.7664) and an exceptionally significant fit of eggs to carapace length ($F_{(1,7461)} = 24.481$; $P < 0.0001$). The relationship between these two measures was eggs = $0.16 \times \text{length}^{3.78}$ (Fig. 3).

Discussion

The current projects were designed to address two complimentary questions: how does diet affect longevity and what is the fine-scale relationship of size (estimated via maximum carapace length) and reproduction. The former is an extension of a previous paper on the basic life history of *Eulimnadia texana* (Weeks et al., 1997), adding the dimension of a variable food environment. The latter provides a detailed relationship between clam shrimp size and egg production in *E. texana*. Each of these will be discussed separately below.

Table 3 Size, egg, and longevity data for two food treatments in feeding experiment 2: high (1.0 ml/day) and low (0.5 ml/day)

Source	df	Sum of squares	*F*-ratio	*P* value
Size at death				
Food	1	0.482	0.6249	0.4328
Error	53	40.900		
Lifetime eggs				
Food	1	1,621,345	4.0884	0.0482
Error	53	21,018,328		
Age at death				
Food	1	17.42	3.7309	0.0588
Error	53	247.42		

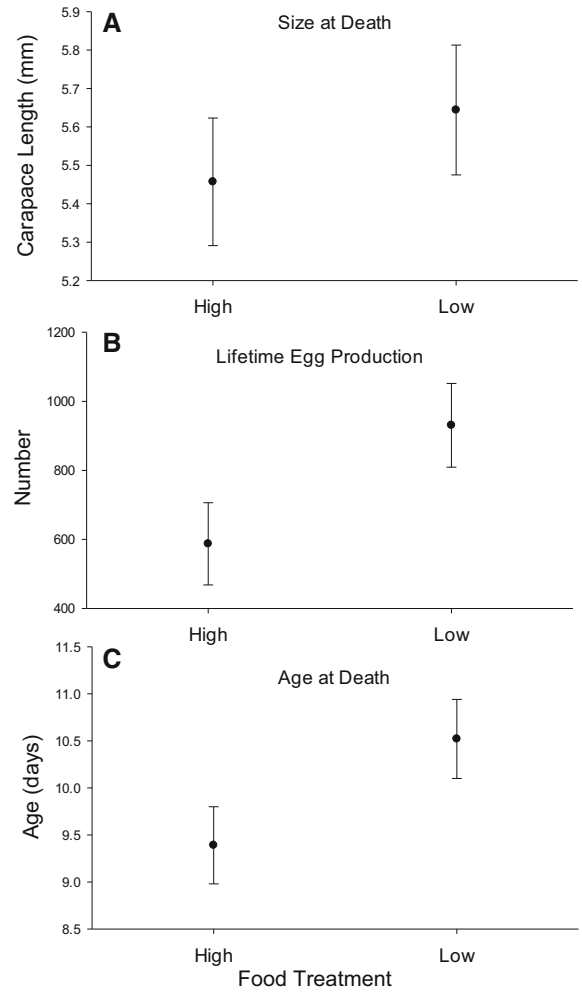

Fig. 2 Growth (**A**), egg production (**B**), and longevity (**C**) for clam shrimp fed a low and high food diet (feeding experiment 2). Error bars portray one standard error of the mean

Dietary restriction and longevity

In the feeding experiment 1, a lower level of food increased longevity for the shrimp in the lower fed treatments. This effect was only significant for the later feeding treatments (i.e., after 9 days of age). A possible explanation for the differences in early vs

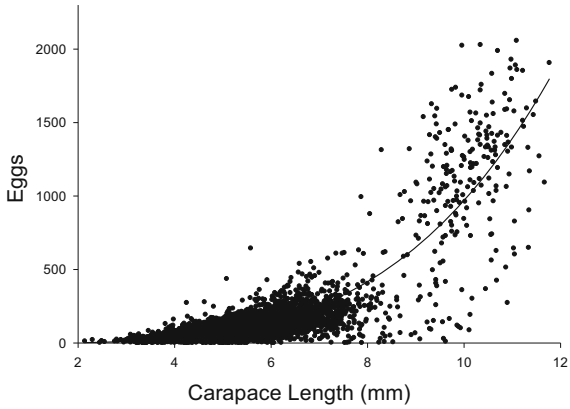

Fig. 3 The relationship of egg production and size (carapace length) in *Eulimnadia texana* Data from Weeks (2004), Weeks & Bernhardt (2004) and Weeks et al. (2014a)

later effects of food restriction is that the fixed food additions may have been sufficiently high for all three food levels when the shrimp were younger (and thus smaller). Such an increasing effect of environment on size with increasing age is common in plants experiencing competition in dense vs. sparse plantings, wherein the effects of crowding are not observable until the plants are older and larger (Harper, 1977). A better experimental design to note effects of food restriction on early growth may have been to increase food level daily throughout the course of the experiment rather than keeping feeding environments constant.

Food restriction is known to increase longevity in a number of animals: mammals (Rose, 1991; Phelan & Rose, 2005) fish, crustaceans, arachnids, molluscs and insects (Comfort, 1979; Weindruch & Walford, 1988; Rose, 1991; Kirkwood & Shanley, 2005). Mice fed half to two-thirds less than ad libitum diets live up to 65% longer, on average, than normal-diet mice (Weindruch et al., 1986). However, in most of these instances, longevity increases while other life history measures (e.g., growth and/or reproduction) usually decline with dietary restriction (Rose, 1991; Kirkwood & Shanley, 2005; Phelan & Rose, 2005). Thus, the first project could not assess whether longevity traded off with growth and/or egg production as diet was restricted.

To assess whether longevity traded off with growth or egg production under dietary restriction, feeding experiment 2 was conducted under the two lower food treatments used in the first experiment. Because of the lack of effect of restricted diets on younger shrimp in feeding experiment 1, diet remained constant throughout the experiment. Once again dietary restriction increased lifespan, but it did not have an effect on growth. Egg production also increased on the lower diet. Thus in these clam shrimp, it appears that the higher food level produced poorer outcomes than the lower food level. Additionally, in feeding experiment 2, *E. texana* longevity did not trade off with growth and/or reproductive output.

The lack of a trade-off of longevity with growth and/or reproduction was unexpected. Basic life-history theory predicts that increased longevity should be associated with lower allocations to growth and especially reproduction early in life, which is reported in many animals (Rose, 1991; Phelan & Rose, 2005; Lemaitre et al., 2015). Thus, it is possible that the higher food regimes used in these two studies produced negative general effects on these clam shrimp, as has been suggested in some rodent feeding studies assessing longevity (Masoro, 1988).

A complication for comparing these two experiments is that feeding experiment 2 may have been generally stressful to the shrimp, for some reason. The highest average survival in feeding experiment 2 (10.5 days) was much lower than the lowest average survival in experiment 1 (14.6 days). Thus, some other environmental variable that was not controlled (e.g., being reared in an environmental chamber in feeding experiment 1 vs a larger wet laboratory in feeding experiment 2 or a constant vs a slightly variable, respectively, temperature level) or the disturbance of being removed periodically for size measurement in Experiment 2 may have caused differential mortality rates between the two experiments. The ages at death in both feeding experiments 1 and 2 are within the range of average lifespan for laboratory-reared *E. texana* (Weeks et al., 1997), so neither project was outside the normal longevity range for this species.

Future work on these shrimp should assess a broader range of dietary options to assess whether lower food does indeed cause a trade-off of increased longevity with lower reproductive output at lower dietary levels than used in these studies. The lower food diets (0.5 and 1.0 ml/day) were chosen in feeding experiment 2 specifically because we expected that the lowest of our measured diets would be most likely to produce a trade-off. Clearly, this was not the case in

our experiment, so even lower levels of food addition may be needed to show the predicted trade-off, assuming one exists in these crustaceans.

Size and reproductive output

The best fit relationship between egg production and size (measured by carapace length) was that egg production increased in a power function of size from shrimp measured in the three combined previous studies (Fig. 3). A previous report on the relationship of egg production to size in *E. texana* (Weeks et al., 1997) noted a simple linear relationship between size and reproductive output. Similarly, Huang & Chou (2015) noted linear relationships of clutch size to body size in *Eulimnadia braueriana* Ishikawa 1895. Huang & Chou (2017) also reported linear relationships between clutch size and overall size in *Eulimnadia braueriana* and in the fairy shrimp *Branchinella kugenumaensis* (Ishikawa 1895). A linear relationship between egg output and size was also reported for the clam shrimp *Cyzicus grubei* (Simon, 1886) (Perez-Bote et al., 2014). However, all of these studies compared $\sim$ 600 or fewer branchiopods and none of the authors explored the possibility of egg/size relationships other than linear. The large sample size herein (over 7400 total shrimp) allowed a more subtle relationship between size and egg production to be discernable.

The observation that larger clam shrimp disproportionally increase egg production is a common finding in crustaceans (Green et al., 2009; Currie & Schneider, 2011). Various populations of the American lobster (*Homarus americanus*, H. Milne Edwards, 1837) range in their exponent from $b = 2.77$ to 3.27 (Currie & Schneider, 2011). Similarly, a log-linear relationship produced the best fit of egg production to size in the rock lobster [*Jasus edwardsii* (Hutton, 1875)] (Green et al., 2009). In this current comparison, there is a broad range of reproductive hermaphrodites, ranging from $\sim$ 2.5 to 11 mm. Such a broad range signifies how flexible growth rates are in *E. texana* and underscore the notion that these clam shrimp minimize time to reproduction, even at the cost of lower overall productivity, to allow reproduction before their pools dry. Because growth rate significantly declines after the start of egg production (Weeks et al., 1997), this drive towards earliest possible reproduction can be costly compared to delaying reproduction. Such a delay would otherwise allow hermaphrodites to capitalize on the greater per-individual reproductive output that larger size affords these crustaceans.

Clam shrimp are extremely abundant in fossil formations of freshwater lakes and ponds (Tasch, 1987; Chen & Hudson, 1991; Shen, 1994; Hethke et al., 2018). Commonly, only the clam shrimp carapace is fossilized, likely due to its durability arising from its multi-layered composition (Astrop et al., 2015). Therefore, paleontologists hoping to glean ecological processes from these ancient freshwater systems must use information derived from fossilized clam shrimp carapaces (e.g., Hethke et al., 2019). The detailed relationship of carapace size to reproductive output described herein could serve as a rough estimate of reproductive output potential of fossilized clam shrimps, of which typically only the carapace size can be assessed.

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