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# Testing Weissman's Lineage Selection Model for the Maintenance of Sex: The Evolutionary Dynamics of Clam Shrimp Reproduction over Geologic Time

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One of the most perplexing questions within evolutionary biology is: "why are there so many methods of reproduction?" Contemporary theories assume that sexual reproduction should allow long term survival as dispersal and recombination of genetic material provides a population of organisms with the ability to adapt to environmental change. One of the most frustrating aspects of studying the evolution of reproductive systems is that we have not yet been able to utilize information locked within the fossil record to assess breeding system evolution in deep time. While the fossil record provides us with information on an organism's living environment, as well as some aspects of its ecology, the preservation of biological interactions (reproduction, feeding, symbiosis, communication) is exceedingly rare. Using both information from extant taxa uncovered by a plethora of biological and ecological studies and the rich representation of the Spinicaudata (Branchiopoda: Crustacea) throughout the fossil record (from the Devonian to today), we address two hypotheses of reproductive evolutionary theory: (1) that unisexual species should be short lived and less speciose than their outcrossing counterparts and (2) that androdioecy (mixtures of males and hermaphrodites) is an unstable, transitionary system that should not persist over long periods of time. We find no evidence of all-unisexual spinicaudatan taxa (clam shrimp) in the fossil record, but do find evidence of both androdioecious and dioecious clam shrimp. We find that clades with many androdioecious species are less speciose but persist longer than their mostly dioecious counterparts. These data suggest that all-unisexual lineages likely do not persist long whereas mixtures of unisexual and sexual breeding can persist for evolutionarily long periods but tend to produce fewer species than mostly sexual breeding.

**Key words:** Evolution of sex, Sexual dimorphism, Morphometrics, Androdioecy, Chonchostraca.

## BACKGROUND

The functional significance of outcrossing sexual reproduction has intrigued biologists from the very inception of evolutionary biology. Darwin (1859) mused that organisms that refrained from outcrossing sexual reproduction would "diminish vigour and fertility" and that "no organic being self-fertilises itself for an eternity of generations; but that a cross with another individual is occasionally—perhaps at very long intervals—indispensable." Indeed, Weismann (1889) elaborated on this idea by suggesting that "all species with purely parthenogenetic reproduction are sure to die out; not, indeed, because of any failure in meeting the existing

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conditions of life, but because they are incapable of transforming themselves into new species, or, in fact, of adapting themselves to any new conditions." The notion that outcrossing sexual reproduction allowed species to persist in the face of changing environments persisted for more than a century before being challenged as being a "group selection" argument by Williams (1966) in his seminal book. Since then, a plethora of "individual selection" hypotheses have been generated to replace Weismann's hypothesis (Williams 1975; Bell 1982; Kondrashov 1993), most of which have not been successfully borne out (Otto 2009). The lack of definitive support for short-term benefits to outcrossing sexual reproduction has logically led to theoretical re-examination of Weissman's ideas (termed the "lineage selection" model) as a mechanism to maintain sexual reproduction (Nunney 1989; Burt 2000; de Vienne et al. 2013).

Even though the notion that unisexual lineages should be evolutionarily short-lived and less speciose than their outcrossing sexual counterparts (Weismann 1889; Fisher 1930; Muller 1932 1964) is indeed a "group selection" hypothesis, it may nevertheless either partially or wholly explain the predominance of sexual reproduction in the plant and animal kingdoms (Nunney 1989 1999; Burt 2000; de Vienne et al. 2013). Because of the long time frames dictated by the lineage selection model, empirical tests of this hypothesis have only been indirect; the scarcity of major clades of wholly or predominantly unisexual lineages—for example ostracods (Schön et al. 2009), oribatid mites (Norton et al. 1988), spinicaudatan 'clam shrimp'(Weeks et al. 2009) and bdelloid rotifers (Arkhipova and Meselson 2000; Welch et al. 2004; Fontaneto et al. 2007 2012)—has been cited as indicative of the lineage-selection model (Bell 1982).

In order to test such a temporally-dependent hypothesis, we would need a readily fossilized clade that is reproductively labile and from which breeding system type can be assessed. Unfortunately, to date, determination of reproductive mode of fossils has been problematic, and in those taxa that show sexual dimorphism [*e.g.*, ammonites (Longridge et al. 2008; Zatoń 2008), ostracods (Ozawa 2013), and vertebrates (Klein et al. 2012)], reproductive mechanisms are often invariant, disallowing empirical comparison. Because of this, palaeontological tests of the long-term benefits of sexual reproduction in multicellular organisms (Weismann 1889; Fisher 1930; Muller 1932 1964) have been impossible.

There is one clade—branchiopod crustaceans in the suborder Spinicaudata (Fig. 1)—that does fit the above criteria. These clam shrimp exhibit a diversity of reproductive systems: dioecy (males + females), androdioecy (males + hermaphrodites) and selfing hermaphroditism (Sassaman 1995; Brantner et al. 2013; Weeks et al. 2014). Additionally, unisexuality (*i.e.*, selfing hermaphroditism) has independently evolved a minimum of four times from dioecious ancestors (Weeks et al. 2014). These crustaceans readily fossilize, and have a rich fossil record that is well established (Raymond 1946; Novojilov and Kapeljka 1960; Tasch and Shaffer 1964; Zhang et al. 1976; Tasch 1987; Gallego and Martins-Neto 2006; Kozur and Weems 2007; Astrop and Hegna 2015; Hethke et al. 2019). Recent methodological breakthroughs (Astrop et al.

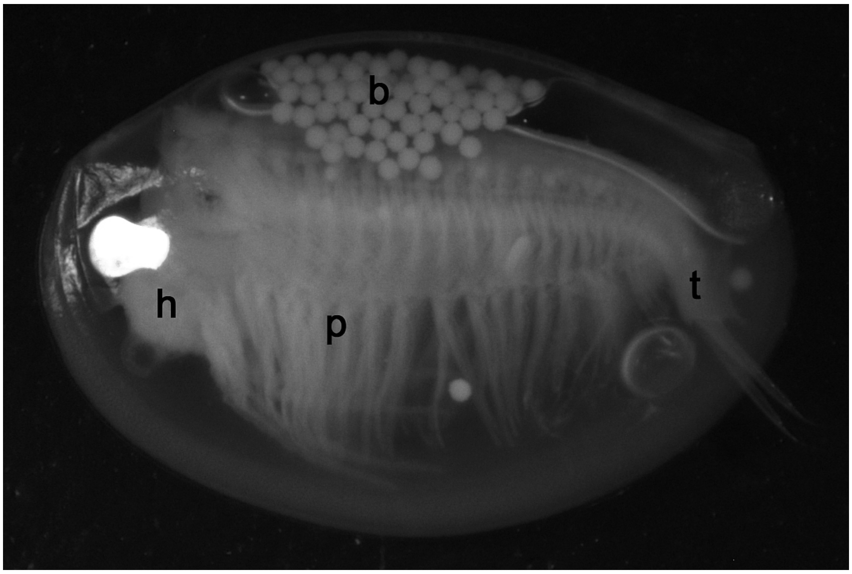


**Fig. 1.** The limnadiid spinicaudatan *Calalimnadia mahei*. b: Brood chamber with eggs, h: Head, p: Phyllopodous thoracic limbs, t: Telson.

2012) have allowed the extraction of sex ratio estimates from fossil clam shrimp to ascertain reproductive systems in fossil populations of Spinicaudata (Monferran et al. 2013; Stigall et al. 2014). The combination of these factors allows us a unique opportunity to test Weissman's original hypothesis: that unisexual species should be short lived and less speciose than their outcrossing counterparts (Nunney 1989; de Vienne et al. 2013).

Herein, we use Astrop et al.'s (2012) shape comparison methodology to assess sex ratios in fossil clam shrimp allowing us to assign mating systems to fossil species in a reproductively diverse taxon: the Spindicaudata (Weeks et al. 2008). We then use these analyses to directly address Weissman's (1889) original predictions that unisexual species should be less speciose and shorter-lived than their dioecious counterparts, as well as to assess the prediction that mixtures of males and self-compatible hermaphrodites (androdioecy) should be short-lived (Charlesworth 1984).

## MATERIALS AND METHODS

Sampling for this study was conducted at multiple museums and repositories across the world (Tables 1 and 2). Specimens were processed using a 'portable imaging station' which comprised a Nikon D3000, macro-lens, tripod, lighting, laptop computer and image capture/editing software.

### Morphometric Protocol

The outlines of individual carapaces were digitized using tpsDig v2.10 (Rohlf 2006) and then subjected to standard eigenshape analysis. The protocol and proof of concept utilized in this study is covered in depth in Astrop et al. (2012). A brief description of the methodology follows.

Eigenshape analyses (*sensu* MacLeod 1999) operate via the conversion of the digitized outline of an individual specimen into equidistant, Cartesian (x-y) coordinates. These coordinates are subjected to a generalized Procrustes analysis (GPA *sensu* Bookstein 1996 1997) in order to remove the effect of size, location and rotation and allow the data to projected into a two dimensional space. The Procrustes-aligned coordinates are then transformed into a shape function as angular deviations (phi function: $\varphi$; Zahn and Roskies 1972) from the previous step (coordinate) in order to describe the shape of the curve. This description is derived from a set of empirical, orthogonal shape functions via an eigenfunction analysis of a matrix of correlations between shapes. Eigenshape 'scores' can be then used to project individual specimens into a multi-dimensional morphospace that allows the visualization of individual vectors of shape change and highlight whether particular vectors of deviation from the 'mean shape' are characteristic of a particular group.

Digitized outline data was then processed using modified versions of the Eigenshape v2.6 & Guide to Models v0.7 Mathematica notebooks available via the morphotools site (http://www.morpho-tools.net). The analysis interpolates and standardizes the raw Cartesian data before performing a singular value decomposition to produce eigenvalues, eigenscores and eigenshapes that describe variation of shape within the dataset. Size is removed from the analysis as eigenshape axis one which is manually discarded and the second eigenshape reported by the analysis is treated as the 'true' first eigenshape (ES1) describing shape change. The eigenshapes produced by the analysis describe two-dimensional axes of shape change that can be

**Table 1.** Number of fossil species represented by adequate numbers to be of use in this study from visited institutions

| Collection | # of viable species |
|---|---|
| CONICET | 7 |
| NHM | 5 |
| SMNH | 8 |
| PIN | 2 |
| NIGPAS | 4 |
| AMNH | 3 |

Institution abbreviations: CONICET, National Research Council Scientific and Technical, Corrientes, Argentina. NHM, Natural History Museum, London, UK. SMNH, Smithsonian Museum of Natural History, DC, USA. PIN, Paleontological Institute, Russian Academy of Sciences, Moscow. NIGPAS, Nanjing Institute of Geology and Palaeontology Chinese Academy of Sciences, China. AMNH, Australian Museum of Natural History, Sydney, Australia.

used to construct morphospaces that specimens may be projected onto, allowing trends in shape variation to be observed. The eigenscores can then be used in a simple cluster analysis to evaluate the existence of morphotypes that should correspond to sex.

The current study employed some changes to the protocol outlined in Astrop et al. (2012). Astrop (2014) found that using 10 rather than 500 equidistant points reduced the likelihood that taphonomic and/or human error would enter the analysis while extracting the same level of useful shape information from the carapaces of extant Spinicaudata. Thus, we used this less "noisy" method herein.

### Evolutionary Context

In order to provide a framework for interpreting the evolutionary dynamics of sexual systems over geologic time, hypothesized relationships between extinct taxa were based on Zhang et al. (1976). Trees were constructed manually in Mesquite (V2.75) based on existing literature (*e.g.*, Novojilov 1961; Zhang et al. 1976; Chen and Hudson 1991) to produce files in a nexus format that were manageable by the R language environment and associated phylotools package (Revell 2012).

Unfortunately, most phylogenetic methods and reconstructions do not take into account terminal taxa becoming extinct before the present or the sampling error intrinsic to palaeontological data. Thus, in these analyses, the R package paleotree (Bapst 2012) was utilized, which allowed for time-scaling of branches in the tree and testing for serious issues in assuming the data collected are representative of the actual diversity of the fossil group.

Statistical tests regarding the distribution and duration of sexual systems in fossil groups were performed in R and PAST (Hammer et al. 2001)

## RESULTS

### Monomorphism vs. Dimorphism

In order to establish the presence of different reproductive phenotypes, we must first establish that the methods outlined in Astrop et al. (2012) can effectively discriminate monomorphism (*i.e.*, parthenogenesis

**Table 2.** Metadata of fossil material used in this study. Institution abbreviations same as in table 1

| Species | Familial affiliation | Collection | Specimen # | Age | Useful Eigen-shapes | % variance captured |
|---|---|---|---|---|---|---|
| *Carapacestheria disgregaris* | Eosestheriidae (Shen 1994) | NHM London, Ohio University (OU), SMNH | NHM it2566-81 | Jurassic | 1,2 | 72% |
| *Martinestheria* (*Lioestheria*) *codoensis* | Antronestheriidae (Gallego et al. 2013) | Argentina | Uncurated | Lower Cretaceous | 1,2,3 | 49% |
| *Challaolimnadiopsis mendozaensis* | Eosestheriidae (*Sensu* Zhang et al., 1976)[1] | Argentina | Uncurated | Triassic | 1,2,3 | 72% |
| *Wolfestheria smekali* | Fushunograptidae (Wang) in Hong et al. 1974 | Argentina | Uncurated | Upper Jurassic | 1,2,3 | 36% |
| *Menucoestheria wichmanni* | Eosestheriidae (Zhang et al., 1976) | Argentina | Uncurated | lower Upper Triassic | 2,3 | 20% |
| *Leaia gondwanella* | Leaiidae (Raymond 1946) | SMNH | usnm426155 | Mid-Upper Permian | 1,2 | 34% |
| *Estheria forbesi* (all) | Eosestheriidae (*Sensu* Zhang et al., 1976)[1] | NHM London, Argentina | NHM in44340 - 51883, ARG "New stuff in tissue", TA1-TA7 | Triassic | 1,2 | 59% |
| *Cyzicus* (*Euestheria*) *crustapatulis* | Euestheriidae[1] | SMNH | usnm427800/06 & usnm427807 | Lower Jurassic | 1,2 | 30% |
| *Eosolimnadiopsis santacrucensis* | Eosestheriidae (*Sensu* Zhang et al., 1976)[1] | Argentina | Uncurated | Jurassic | 1,2 | 67% |
| *Lioestheria malacaraensis* | Fushunograptidae (Gallego et al. 2011) | Argentina, SMNH | usnm427989 | Jurassic | 1,2,3,4 | 90% |
| *Euestheria taschi* | Euestheriidae (Monferran et al., 2013) | Argentina | 5718 | middle Late-Jurassic | 1,2,3,4 | 91% |
| *Estheria mangliensis* | Euestheriidae[1] | NHM London | NHM in4961 - 35274 | Upper-Triassic | 1,2 | 68% |
| *Euestheria mangliensis* (?) | Euestheriidae[1] | Argentina | Uncurated | middle Late-Jurassic | 1,2 | 71% |
| *Estheria mangaliensis* | Euestheriidae[1] | SMNH | Uncurated | Upper-Triassic | 1,2 | 54% |
| *Triassoglypta* sp. 3 | Loxomegaglyptidae (Novojilov 1958) | Argentina | Uncurated | Late Triassic | 1,2,3 | 82% |
| *Estheria middendorffi* | Euestheriidae[1] | NHM London | NHM in9262 - un-cataloged | Upper Cretaceous | 1,2 | 62% |
| *Leaia leidyi* | Leaiidae (Raymond 1946) | NHM London | NHM in3088-3114 | Lower Carboniferous | 1,2 | 61% |

**Table 2.** (Continued)

| Species | Familial affiliation | Collection | Specimen # | Age | Useful Eigen-shapes | % variance captured |
|---|---|---|---|---|---|---|
| *Cyzicus* (*Euestheria*) *formavariabalis* | Eosestheriidae (*Sensu* Zhang et al., 1976)[1] | SMNH | usnm426198 | Lower Jurassic | 1,2 | 68% |
| *Cyzicus* (*Euestheria*) *crustabundis* | Eosestheriidae (*Sensu* Zhang et al., 1976)[1] | SMNH | usnm427901/4+985 | Lower Jurassic | 1,2 | 59% |
| *Cyzicus* (*Lioestheria*) *antarctis* | Eosestheriidae (*Sensu* Zhang et al., 1976)[1] | SMNH | usnm426177 | Lower Jurassic | 1,2 | 58% |
| *Perilimnadia* sp. | Perilimnadiidae (*Sensu* Zhang et al., 1976)[1] | AMNH | Tray L34-CO1 | Upper Permian | 1,2 | 59% |
| *Hemicyclolaeia mitchelli/ discoidea* | Leaiidae (Raymond 1946) | AMNH | Tray L34-CO3 | Upper Permian | 1,2 | 62% |
| *Cyzicus* (*Lioestheria*) *branchocarus* | Euestheriidae[1] | AMNH | Tray L34-CO5 | Cretaceous | 1,2 | 58% |
| *Estheria simoni* | Euestheriidae[1] | PIN | Uncurated | Upper Carboniferous | 1,2 | 73% |
| *Limnadia volgaica* | Palaeolimnadiidae (*Sensu* Tasch 1956)[1] | PIN | 2141/1 | Upper Permian | 1,2 | 61% |
| *Eosestheria luanpingensis* | Eosestheriidae (Zhang et al., 1976) | NIGPAS | 97438-57 | Early Cretaceous | 1,2 | 76% |
| *Neodiestheria changmaensis* | Diestheriidae (Chen) in Zhang et al. 1976 | NIGPAS | 45564-45566 | Early Cretaceous | 1,2,3 | 71% |
| *Dictyestheria elongata/ ovata* | Halysestheriidae (Zhang et al., 1976) | NIGPAS | Uncurated | Upper Cretaceous | 1,2,3 | 69% |
| *Halysestheria yui* | Halysestheriidae (Zhang et al., 1976) | NIGPAS | Uncurated | Upper Cretaceous | 1,2 | 63% |

| Species | Tot N | N = M1 | %M1 | N = M2 | %M2 | Predicted sexual system[2] |
|---|---|---|---|---|---|---|
| *Carapacestheria disgregaris* | 34 | 17 | 50.0 | 17 | 50.0 | D |
| *Martinestheria* (*Lioestheria*) *codoensis* | 15 | 7 | 46.7 | 8 | 53.3 | D |
| *Challaolimnadiopsis mendozaensis* | 14 | 7 | 50.0 | 7 | 50.0 | D |
| *Wolfestheria smekali* | 33 | 14 | 42.4 | 19 | 57.6 | D |
| *Menucoestheria wichmanni* | 23 | 9 | 39.1 | 14 | 60.9 | D |
| *Leaia gondwanella* | 16 | 6 | 37.5 | 10 | 62.5 | D |
| *Estheria forbesi* (all) | 116 | 63 | 54.3 | 54 | 46.6 | D |
| *Cyzicus* (*Euestheria*) *crustapatulis* | 33 | 10 | 30.3 | 23 | 69.7 | A |
| *Eosolimnadiopsis santacrucensis* | 48 | 17 | 35.4 | 31 | 64.6 | A |
| *Lioestheria malacaraensis* | 55 | 38 | 69.1 | 17 | 30.9 | A |
| *Euestheria taschi* | 20 | 9 | 45.0 | 11 | 55.0 | D |
| *Estheria mangliensis* | 38 | 13 | 34.2 | 25 | 65.8 | A |
| *Euestheria mangliensis* (?) | 38 | 13 | 34.2 | 25 | 65.8 | A |
| *Estheria mangaliensis* | 61 | 33 | 54.1 | 28 | 45.9 | D |
| *Triassoglypta* sp. 3 | 28 | 11 | 39.3 | 17 | 60.7 | D |
| *Estheria middendorffi* | 34 | 11 | 32.4 | 23 | 67.6 | A |
| *Leaia leidyi* | 30 | 10 | 33.3 | 20 | 66.7 | A |
| *Cyzicus* (*Euestheria*) *formavariabalis* | 13 | / | / | / | / | N/A |
| *Cyzicus* (*Euestheria*) *crustabundis* | 17 | / | / | / | / | N/A |
| *Cyzicus* (*Lioestheria*) *antarctis* | 17 | 8 | 47.1 | 9 |  | D |
| *Perilimnadia* sp. | 28 | / | / | / | / | N/A |
| *Hemicyclolaeia mitchelli/discoidea* | 45 | 12 | 26.7 | 33 | 73.3 | A |
| *Cyzicus* (*Lioestheria*) *branchocarus* | 41 | 21 | 51.2 | 20 | 48.8 | D |
| *Estheria simoni* | 17 | 7 | 41.2 | 10 | 58.8 | D |
| *Limnadia volgaica* | 19 | 9 | 47.4 | 10 | 52.6 | D |
| *Eosestheria luanpingensis* | 37 | 17 |  | 20 | 54.1 | D |
| *Neodiestheria changmaensis* | 60 | 28 | 46.7 | 32 | 53.3 | D |
| *Dictyestheria elongata/ovata* | 99 |  |  |  |  | N/A |
| *Halysestheria yui* | 69 | 33 | 47.8 | 36 | 52.2 | D |

[1] suggested change. [2] A = androdioecy; D = dioecy.

or all hermaphrodites) from dimorphism (dioecy or androdioecy). Thus, we began this study by subjecting known sexes of differing combinations to analysis: a sample of 15 males and 15 hermaphrodites of the extant androdioecious *Eulimnadia texana* Packard, 1871 was used as the "dimorphic" population and 15 hermaphrodites as the "monomorphic" population. The first two eigenshape axes contained 82% of the variation for the dimorphic dataset and 57% of the variation observed in the monomorphic dataset. Hierarchical cluster analyses of these respective datasets (Fig. 2) revealed two groups separated by long branch lengths (relative to disparity between either cluster's eigenshape scores) in the dimorphic dataset (Fig. 2B) whereas in the monomorphic dataset (Fig. 2A) branch lengths were considerably lower and did not show the distinct grouping seen in the dimorphic dataset. Thus, the method implemented by Astrop et al. (2012) can successfully distinguish between a monomorphic vs. a dimorphic data set.

## Fossil Comparisons

A total of 29 species of fossil Spinicaudata represented by between 30–200 individuals were used in these analyses (Tables 1–3). Individual fossil specimens from collections were deemed viable if there was little to no visible taphonomic interference in the outline of the preserved carapace valve (approximately 30% of observed specimens were of suitable preservation). Small shape variations in individual specimens are described by the eigenshape analysis as 'non-affine' or non-uniform. This non-uniform variation is likely to be relegated to lower eigenshapes as 'noise' whereas more uniform or 'affine' shape change, that is, trends in shape change seen across specimens in the dataset, comprised the majority of variance captured by higher eigenshapes. A total of 1,098 specimens from 29 species (Table 2) were analyzed using the morphometric protocol outlined above.

Observing the branching patterns in the extant (Fig. 2) examples and fossil examples (Fig. 3), similarities and differences are clear. The fossil taxon *Lioestheria malacaraensis* Tasch 1987 (Fig. 3B) definitively displays a strong basal dichotomy in shape variation of a magnitude similar to that seen in the dimorphic dataset of the extant *Eulimnadia texana* (Fig. 2B). Conversely *Palaeolimnadia* sp. (Fig. 3A) exhibits no clear clusters, reminiscent of the monomorphic dataset of *E. texana* (Fig. 2A). A major difference between the patterns seen in *Palaeolimnadia* sp. versus that seen in the monomorphic *E. texana* data is the size of the Euclidean distance between specimens. This distance measure is an effective way of discerning groups because data contained in the vectors are all in the same physical units (a measure of disparity in shape, with size, scaling and rotation removed). The distance between specimens in the *Palaeolimnadia* sp. dataset (Fig. 3A) is of an order of magnitude higher than that seen in the monomorphic *E. texana* dataset (Fig. 2A) and is very similar to distance measures in other dimorphic taxa studied (Astrop et al. 2012). This can be simply interpreted as there being very little difference in shape between individuals in the monomorphic *E. texana* data set and differences in shape between multiple individuals in the *Palaeolimnadia* sp. data set of a magnitude similar to

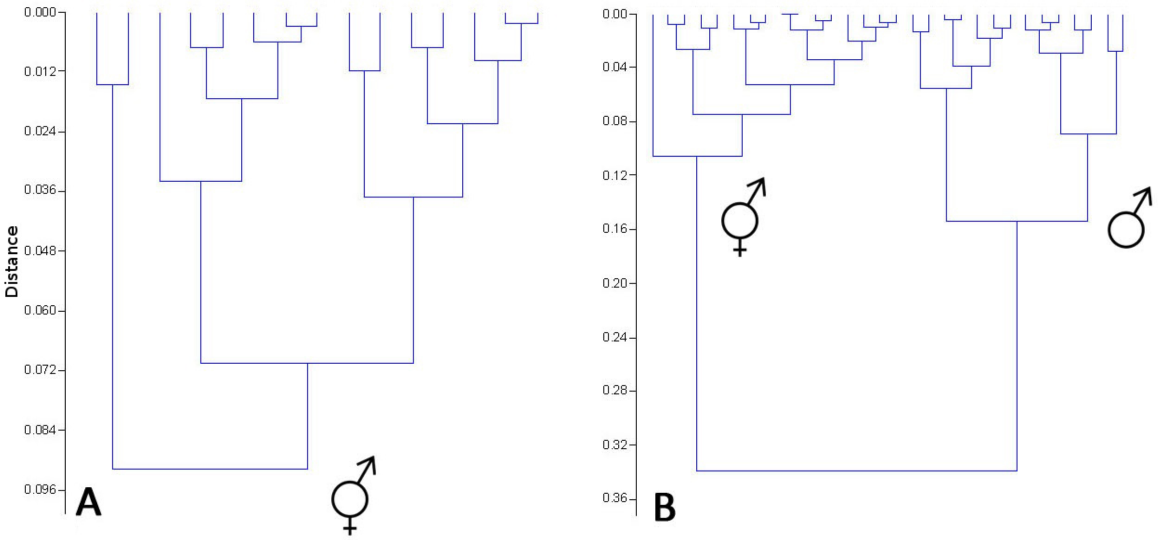


**Fig. 2.** Cluster analyses of a monomorphic (hermaphrodites only) sample of *Eulimnadia texana* (A) and a dimorphic (males + hermaphrodites) sample (B) based on scores of individuals along the first four eigenshape axes. Note the differing distances along the Y-axes in the two graphs.

that seen in the dimorphic data. Therefore, we interpret these patterns in *Palaeolimnadia* sp. (Fig. 3A) as either reflective of the presence of multiple species in a collection labeled as a single species, or as being caused by severe taphonomic interference in these specimens. We found these patterns in four of the 29 (~14%) taxa examined: *Dictyestheria elongata* / *D. ovata* Chang and Chen 1963, *Palaeolimnadia* sp., *Cyzicus* (*Euestheria*)

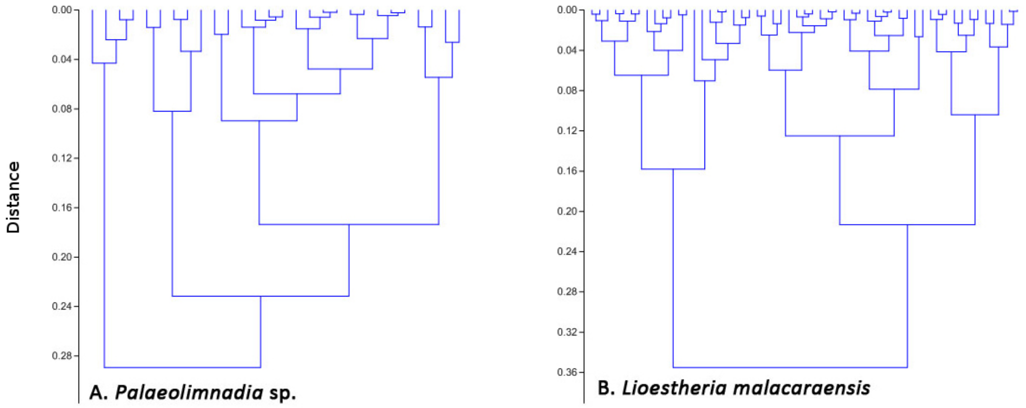


**Fig. 3.** A, Cluster analysis of *Palaeolimnadia* sp. based on informative eigenshapes (ES1 & 2); no discernible major groupings and multiple long branches of similar length interfere with deducing sexual system based on a clear morphotype ratio. B, Cluster analysis of *Lioestheria malacaraensis* based on informative eigenshapes (ES1 & 2) exhibiting a clear basal dichotomy with branch lengths much larger than any subsequent groupings.

**Table 3.** Fossil clam shrimp measured

| Name | Reference |
|---|---|
| *Carapacestheria disgregaris* | Tasch 1987 |
| *Challaolimnadiopsis mendozaensis* | Shen et al. 2001 |
| *Cyzicus* (*Euestheria*) *crustabundis* | Tasch 1987 |
| *Cyzicus* (*Euestheria*) *crustapatulis* | Tasch 1987 |
| *Cyzicus* (*Euestheria*) *formavariabalis* | Tasch 1987 |
| *Cyzicus* (*Lioestheria*) *antarctis* | Tasch 1987 |
| *Cyzicus* (*Lioestheria*) *branchocarus* | Talent 1965 |
| *Dictyestheria elongata/ovato* | Chang and Chen 1964 |
| *Euestheria luanpingensis* | Zhang et al. 1990 |
| *Eosolimnadiopsis santacrucensis* | Gallego 1994 |
| *Estheria forbesi* all | Jones 1862 |
| *Estheria mangaliensis* L3 | Jones 1862 |
| *Estheria mangliensis* L1 | Jones 1862 |
| *Estheria middendorffi* | Jones 1862 |
| *Estheria simoni* | Pruvost 1911 |
| *Euestheria mangliensis* L2 | Jones 1862 |
| *Euestheria taschi* | Vallati 1986 |
| *Halysestheria yui* | Chang 1957 |
| *Hemicylcolaeia discoidea/mitchelli* | Mitchell 1925; Etheridge 1892 |
| *Leaia gondwanella* | Tasch 1987 |
| *Leaia leidyi* | Lea 1855 |
| *Limnadia volgaica* | Novojilov 1970 |
| *Lioestheria malacaraensis* | Tasch 1987 |
| *Martinsestheria codoensis* | Cardoso 1962 |
| *Menucoestheria wichmanni* | Gallego 2010 |
| *Neodiestheria changmaensis* | Shen and Chen 1982 |
| *Paleolimnadia* sp. | Tasch and Oesterlen 1977 |
| *Triassoglypta* sp. 3 | Gallego 2005 |
| *Wolfestheria smekali* | Mongerran et al. 2013 |

*formavariabalis* Tasch 1987 and *Cyzicus* (*Euestheria*) *crustabundis* Tasch 1987. Thus, for these taxa, no sexual system could be inferred.

For the remaining taxa, morphotypes were assigned when cluster analysis of the informative eigenshape scores either produced two distinct groups (such as in Figs. 2B and 3B) or a single grouping (such as in Fig. 2A). We found that none of the remaining 25 taxa showed a pattern indicative of a single sex (*i.e.*, as in Fig. 2A). Instead, all 25 taxa had two distinct groupings separated by large Euclidian distances, as seen in figures 2B and 3B. Thus, these analyses resulted in two sexes being present among the samples of each of these 25 taxa.

Actual sexes may be assumed for the two distinct morphotypes in two ways. First, although naturally occurring extant populations often display fluctuating frequencies of sexes (often seasonally), the average ratio over the season remains indicative of the sexual system employed by the species (50:50—dioecious; 30:70 male: "female"—androdioecious). This has been recorded in extant, wild populations of dioecious species such as *Cyzicus tetracerus* (Krynicki 1830) (Popović and Gottstein-Matočec 2006), *Leptestheria nobilis* Sars, 1900 (Karande and Inamdar 1959) and the androdioecious species *E. texana* (Strenth 1977). When considering ecological observations, alongside the fact that fossil-bearing strata usually represent multiple generations living and dying over time, obtaining morphotype (or 'sex') ratios for a fossil taxon from such a deposit should reflect the ratio representative of the sexual system of that taxon. Second, rare instances of soft part preservation have allowed the matching of claspers (male copulatory appendages) to specific carapace shapes; for instance, soft part preservation in the Jurassic euestheriid *Euestheria luanpingensis* (Zhang et al. 1990), where claspers are associated with more elongated sub-quadrate carapace shapes and eggs are preserved within sub-spherical carapaces.

Fossil taxa were interpreted as displaying a sex ratio indicative of androdioecy if one morphotype comprised less than 35% of the sample. This percentage was used because it is close to the 30/70 ratio (male/female respectively) that is observed in most extant androdioecious species (Weeks et al. 2008) but leaves some room for sampling error. Examples of morphotypes in fossil species can be seen in figure 4.

Seven of the 25 fossil taxa included in this analysis exhibited distinct morphotypes with a skewed frequency where the less common morphotype made up 35% of the sample or less. These taxa occurred in three of the nine families studied (Fig. 5): the Leaiidae, Fushunograptidae and Euestheriidae. Two of three taxa in the Leadiidae, one of two taxa in the Fushunograptidae and four of eight taxa in the Euestheriidae exhibited androdioecious sex ratios. Interestingly, where androdioecy was suggested in a fossil family, it seemed to occur in at least half of the species sampled in that family (Fig. 5).

Polytomies in the tree presented in figure 5 originate from uncertain intra-familial relationships inferred by Zhang et al. (1976) and revised by Astrop and Hegna (2015). However, by time-calibrating the tree using the software package Paleotree (Bapst 2012), it was possible to bound first occurrences in the fossil record to branches and resolve polytomies according to (in this case) the range of geologic stages through which the genera occur (Fig. 6). This revised analysis adds information that would otherwise be lost and that is often ignored in modern phylogenetic studies that incorporate extinct taxa. Time-scaling the tree shows that androdioecious lineages have occurred multiple times since the Devonian. The durations of the branches (Fig. 6) are reflective of the first and last known occurrences of species within that genus in the fossil record.

It is clear that the fossil clam shrimp have two distinct clades both originating ~300 mya (Fig. 6). In this case, clade A has 15 species and only a single case of a skewed sex ratio (*Lioestheria malacaraensis*). The breeding system of three of the species in this clade were not determined (*Dictyestheria elongata / D. ovata*, *Cyzicus* (*Eustheria*) *formavariabalis* and *Cyzicus* (*Eustheria*) *crustabundis*), but the remaining 11 species were determined to be dioecious. Thus, the range of dioecy possible for this clade is ~73–93% dioecious. Clade A is determined to be a primarily dioecious (PD) clade. Clade B has only seven species, of which four are androdioecious. Thus, this clade is 57% androdioecious. Clade B is considered the primarily androdioecious (PA) clade. We can compare the PD to the PA clade in two ways: number of species per clade and average species duration in the fossil record. For the former, we used a binomial test to assess the likelihood of equal numbers of species being distributed in the two clades because the clades appear to be approximately equally old (300–320 my). The likelihood that the two clades are actually equally speciose is 0.041, and thus the PD clade has significantly more species than the PA clade. Using the species duration estimates from figure 6, we found that the PA average duration (57.4 ± 8.7 MY) was significantly longer ($F_{1,20} = 11.45$; $P < 0.0030$) than the PD average duration (21.9 ± 5.9 MY) indicating that the PD clade has more, shorter-lived species than the PA clade (Fig. 6).

## DISCUSSION

Although the theory that sexual reproduction has been maintained because it allows organisms to adapt to evolutionarily rapid changes in the environment (Weismann 1889) has been largely discounted because it was determined to be "group-selectionist" (Williams 1966; Maynard Smith 1971 1978), an effective empirical test of this theory in metazoans has been impossible until now. Herein, we have made the first such test, using fossil species from the reproductively labile clam shrimp as our study organism. From these comparisons, we have made two important discoveries: (1) all-female/hermaphrodite species were not observed in the fossil data studied (2) there were several fossil lineages with sex ratios indicative of androdioecy, and those species were non-randomly divided into two clades that differed in average species duration and species number.

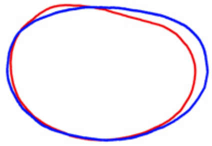
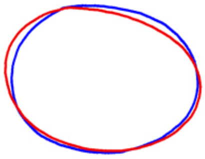
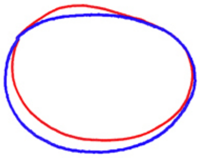
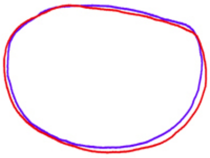
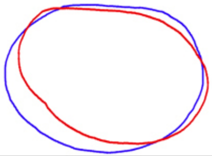
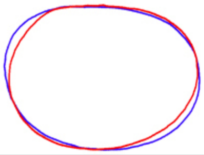
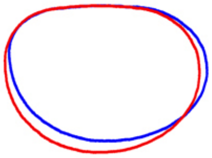
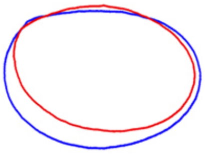
| | | |
|---|---|---|
| ***Martinestheria codoensis***<br>**(Antronestheriidae)**<br>**Lower Cretaceous**<br>**Argentina** | ***N=15***<br>***M1=46.5%***<br>***M2=53.5%***<br>***Dioecy*** |  |
| ***Wolfestheria smekali***<br>**(Fushunograptidae)**<br>**Upper Jurassic**<br>**Argentina** | ***N=33***<br>***M1=43%***<br>***M2=57%***<br>***Dioecy*** |  |
| ***Carapacestheria disgragaris***<br>**(Eoestheriidae)**<br>**Jurassic**<br>**Antarctica** | ***N=34***<br>***M1=50%***<br>***M2=50%***<br>***Dioecy*** |  |
| ***Leaia gondwanella***<br>**(Leaiidae)**<br>**Mid-Upper Permian**<br>**Antarctica** | ***N=16***<br>***M1=37.5%***<br>***M2=62.5%***<br>***Dioecy*** |  |
| ***Challaolimnadiopsis mendozaensis***<br>**('Pemphilimnadiopsidae' Eosestheriidae)**<br>**Triassic**<br>**Argentina** | ***N=14***<br>***M1=50%***<br>***M2=50%***<br>***Dioecy*** |  |
| ***Cyzicus (Euestheria) crustapatulus***<br>**(Euestheriidae)**<br>**Lower Jurassic**<br>**Argentina** | ***N=33***<br>***M1=30%***<br>***M2=70%***<br>***Androdioecy*** |  |
| ***Estheria forbesi***<br>**(Eosestheriidae)**<br>**Triasic**<br>**Argentina** | ***N=116***<br>***M1=54%***<br>***M2=46%***<br>***Dioecy*** |  |
| ***Menucoestheria wichmanni***<br>**(Eosestheriidae)**<br>**Upper Triassic**<br>**Argentina** | ***N=23***<br>***M1=40%***<br>***M2=60%***<br>***Dioecy*** |  |

**Fig. 4.** A sample of the fossil taxa studied, their diagnosed sexual systems and overlaid mean-shapes of the detected morphotypes (M1 = Morphotype 1; M2 = Morphotype 2).

## Lack of All-Unisexual Fossil Clam Shrimp

Out of the 29 fossil clam shrimp species examined, we found no instances of genuine monomorphism and therefore no cases of inferred unisexuality. Although we were unable to determine the sexual system of four fossil species, none of these four species had patterns indicative of a single-sexed species (Fig. 2A). Instead, these four species had long-branch patterns indicative of either mixed species assemblages or taphonomic interference causing carapace outline distortion (see Fig. 3A for an example). Thus, out of the 25 species of which we could determine breeding system type, we found no evidence of all-unisexual species, suggesting that if purely hermaphroditic taxa occurred in the Spinicaudatan clade in the geologic past, they are not represented in the fossil record so far examined (covering the past 370 million years).

This dearth of all-unisexual species in the fossil record is mirrored in the extant clam shrimp species so far studied. Among all of the extant Spinicaudata, unisexuality (*i.e.*, selfing hermaphroditism) has evolved four independent times (Weeks et al. 2014), with a fifth independent derivation proposed but not verified (Roessler 1995). Of the four transitions away from dioecy, three have been to unisexuality (*i.e.*, species comprised solely of self-fertilizing hermaphrodites) and one has been to androdioecy (*i.e.*, ~30% males + ~70% self-fertilizing hermaphrodites). One of the three unisexual taxa is the monospecific *Calalimnadia mahei* Rabet & Rogers, in Rogers et al. (2012). The genus *Limnadia* comprises three unisexual species (Bellec et al. 2018). The third (*Cyzicus gynecius* (Mattox 1950)) is in a genus of 26 species (Rogers 2020). The fourth derivation is in the genus *Eulimnadia*, which appears to be predominantly androdioecious (Weeks et al. 2009) and contains about 45 species (Rogers 2020). Overall, the Spinicaudata are primarily dioecious (Sassaman 1995), containing approximately 200 total species (Rogers 2020).

The combined evidence of a complete absence of carapace monomorphism in the fossil taxa in our study, with the few examples of all-unisexual species among extant clam shrimp species, supports the hypothesis that all-unisexual species (in the case of the Spinicaudata, species that are exclusively self-fertilizing hermaphrodites) should be prone to extinction and less speciose than their dioecious counterparts (Weismann 1889; Fisher 1930; Muller 1932 1964).

Because we found no unisexual species in any of the 25 fossil species surveyed, we can infer that less than 4% (fewer than one out of 25) of fossil clam

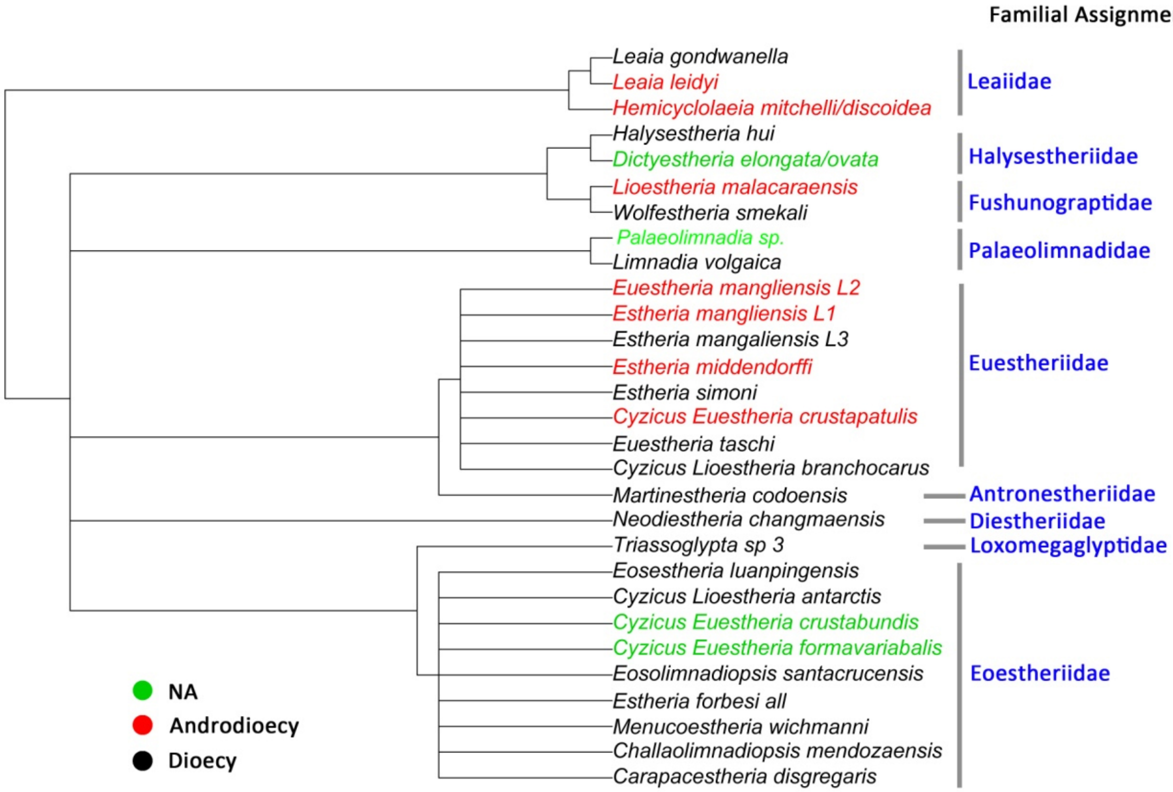


**Fig. 5.** Distribution of predicted sexual systems in fossil taxa analyzed in this study. NA = taxa in which sexual system prediction was impossible (see MATERIALS AND METHODS).

shrimp are all-unisexual. Only four of ~200 extant clam shrimp species (~2%) are all-unisexual, and those few all-unisexual species tend to be in monomorphic clades or at the tips of their respective phylogenetic trees (Weeks et al. 2014). These data suggest that all unisexual lineages are shorter-lived and less speciose than their outcrossing counterparts, as originally suggested by Weismann (1889).

In Weismann's original formulation of this hypothesis, unisexual lineages would be evolutionarily short-lived because of their inability to evolve quickly enough to keep up with a changing environment (Weismann 1889; Muller 1932). A more modern twist on Weismann's original idea suggests that unisexual lineages are short lived because of the accumulation of deleterious alleles (*i.e.*, 'Muller's ratchet'; Muller 1964) and the subsequent 'mutational meltdown' that occurs when population sizes decline due to the effects of this mutation accumulation (Lynch et al. 1995). This is the first corroborative evidence of Weismann's original idea in a multicellular animal lineage, and suggests that the longer-term "group selection" forces of differential speciation and extinction (Nunney 1989) may select for outcrossing sexual reproduction within the Spinicaudata. Clearly, because of the long time frames of this comparison, we have no direct evidence of "group selection," per se. Nonetheless, the patterns shown among the fossils and mirrored in the extant species are indicative of low speciation and short duration for all-unisexual species of Spinicaudata.

## Parity of Androdioecious and Dioecious Clam Shrimp Species

Seven of the 25 fossil species in which breeding system could be determined where found with sex ratios indicative of androdioecy, with the remaining 18 species having sex ratios indicative of dioecy (Table 2). The average number of fossil specimens examined for the eight androdioecious species was ~40 (Table 2), which would yield an expected ~2% chance of finding a sex ratio of 35% males if the true sex ratio was 50% males. Because we sampled 25 species, we would expect less than one of these 25 species to be mistakenly categorized as androdioecious when it was actually dioecious, given our sex ratio cut-off and the average sample sizes of those species found to be androdioecious. Given that we instead found

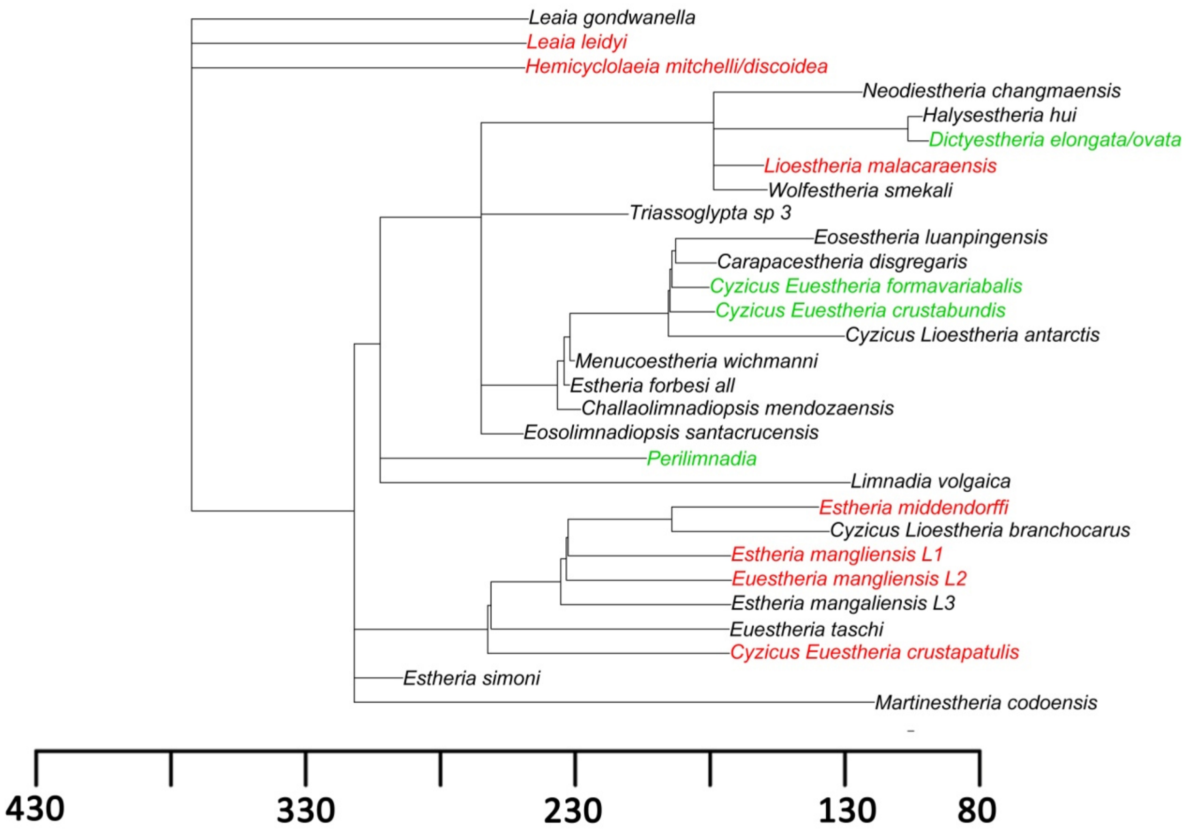


**Fig. 6.** A time-calibrated phylogeny with polytomies largely resolved by stratigraphic occurrence and branch lengths approximate to generic range. X axis = millions of years.

seven species to be androdioecious suggests that the reproductive lability in extant clam shrimp (Weeks et al. 2008) is reflective of a general tendency towards reproductive lability in clam shrimp over geologic time (Fig. 6). A similar level of lability (also in the form of androdioecious and dioecious species) has been described in the closely related branchiopod crustacean group Notostraca (Mathers et al. 2013), suggesting that the Branchiopoda have a genetic system that tends towards the repeated evolution of sperm production in females."

The causes and mechanisms of this suggested reproductive lability remain relatively unknown. However, Weeks et al. (2006) proposed that "females" with small amounts of sperm production can be produced via low levels of crossing over between the sex chromosomes in these crustaceans; these "intersexes" could then be further modified over evolutionary time to be increasingly fit hermaphrodites. Such repeated evolution of sperm-producing "females" can lead to the spread of hermaphrodites, which may eventually outcompete either (a) females to form androdioecy or (b) both males and females to produce unisexual (*i.e.*, selfing hermaphrodite-only) species. The selective pressure for such a hermaphroditic spread is likely an adaptation to harsh, fluctuating environments where reproductive assurance is beneficial (Pannell 1997 2002). However, the current data suggest that any lineage that subsequently loses males essentially passes a 'Rubicon' after which it is doomed to extinction. This 'point of no return' is likely associated with the mode in which hermaphroditism occurs in the Spinicaudata: if the frequency of hermaphroditic individuals becomes high enough to establish large numbers of monogenic populations (as monogenic hermaphrodites have the greatest reproductive assurance, always producing self-fertilizing hermaphrodites that cannot produce or cross with males), these populations may eventually out-reproduce and succeed any amphigenic populations (consisting of female-biased hermaphrodites that are able to produce both hermaphrodite and male offspring) maintaining males. This would quickly eliminate outcrossing from a lineage, ultimately leading to extinction.

The macroevolutionary patterns of lineage duration and speciation rates in the primarily dioecious (PD) and primarily androdioecious (PA) clades did not conform to expectations. Extant androdioecious clam shrimp species commonly form all-unisexual populations (Weeks et al. 2009) and in some cases whole species are unisexual (Weeks et al. 2005, Bellec et al. 2018). Thus, extending Weismann's (1889) original argument, we expected that PA clades would produce androdioecious species that were both shorter lived and less speciose than PD clades. We did indeed find that the PD clade was significantly more speciose than the PA clade, but the PA clade has species that survived significantly longer than the average PD species (Fig. 6). The extant, primarily androdioecious clade *Eulimnadia* is also quite long-lived (Weeks et al. 2006), suggesting that androdioecy, per se, does not doom a lineage to extinction. These combined data also fail to support the idea that androdioecy is an unstable, transitionary sexual system (Charlesworth 1984), given that androdioecious fossil taxa as old as 370 million years apparently persisted within families for at least 70 million years (Fig. 6).

The pattern seen between PA and PD clades could have been produced in a number of ways. It is possible that the PD clade tends to spin off more species, but many of those species are shorter lived than in the PA clades. This might suggest that dioecy allows a more "exploratory" evolutionary trajectory than androdioecy, but that many of those "experiments" fail. An alternate explanation is that the androdioecious breeding system is very stable in these environments (temporary freshwater pools) and that any evolutionary "exploration" may spin off exceptionally short-lived unisexual lineages (*i.e.*, all selfing hermaphrodites) that quickly go extinct. Androdioecy has been suggested as a mechanism to assure reproduction in habitats with high population turnover (Pannell 1997 2002). It is possible that the PA crustacean lineage has historically populated such habitats and thus that androdioecy has been an optimal strategy for them for these longer time frames. Likewise, the PD clade may occupy niches that are relatively more stable.

We are well aware that the comparative fossil data among these two clades of clam shrimp are not strong enough to make broad generalizations, nor to discern which (if any) of the above explanations may have caused the patterns we observed. However, these data allow us to begin to speculate as to causation for the patterns observed and to address questions that have heretofore not been addressable in metazoans. We will need more reproductive data from fossil clades in reproductively labile groups before we can better understand the patterns of persistence and speciation differences between dioecious and unisexual lineages.

## Macroevolutionary Patterns within the Branchiopoda

The exact pattern of the emergence and maintenance of androdioecy and dioecy in the Spinicaudata and Branchiopoda is only beginning to be explored (Hoeh et al. 2006; Weeks et al. 2009 2014; Mathers et al. 2013). However, our exploration of the spinicaudatan fossil record begins to shed light on the duration, emergence and disappearance of lineages that exhibit different breeding systems within the Branchiopoda. Figure 7 represents two possible patterns of breeding system dynamics in the Branchiopoda based on the data collected in this study. The first represents a scenario where each occurrence of androdioecy in living and fossil taxa is independent and all arise from a dioecious ancestor. The second scenario entails one single occurrence of androdioecy in an ancestral phyllopod and four subsequent losses in spinicaudatan clades. Despite the second hypothesis being the most parsimonious, involving only five state changes (occurrence/disappearance of sperm production in females), the first scenario is not only more biologically intuitive (given the sex determination deduced for Spinicaudata in neontological studies) but also is supported by recent molecular analyses and attempts at ancestral state reconstruction (Mathers et al. 2013; Weeks et al. 2014).

The fossil taxa in this study carry a clear palaeontological signal that suggests the hypothesized reproductive lability inferred from studies of living Spinicaudata is ancient, occurring multiple times over the past 370 million years. It appears that this unique crustacean clade has been able to use this lability successfully throughout the geologic past to claim both the benefits of unisexual and sexual reproduction while avoiding the long term negative effects of engaging in prolonged periods of selfing. These crustaceans have clearly been highly successful throughout time (often bestowed the dubious moniker of "living fossils"), and it is likely that their reproductive lability has contributed to this long term success.

## CONCLUSIONS

The patterns seen in the Spinicaudata offer the first empirical observations of multiple reproductive systems occurring in the fossil record of a single clade and provides a framework for future integrated biological and palaeontological studies to elucidate the evolutionary dynamics of biological phenomena over geologic time. By integrating palaeontological and biological approaches, we have recovered definitive evidence for a hypothesized microevolutionary phenomenon occurring at a macroevolutionary level. The absence of fossil monomorphic populations in the Spinicaudata adds weight to the idea that closed, unisexual lineages are doomed to extinction through reduced genetic variability and accumulation/exposure of deleterious mutations. This study also finds evidence contradicting the idea that androdioecy is an unstable, transitionary breeding system, occurring in fossil taxa as old as 370 million years old and persisting within families that are at least 70 million years old. The value of these results would be greatly enhanced with additional molecular evidence and fossil-calibrated divergence time estimates to increase the accuracy of predicted lineage durations. It is our hope that the integrated, multi-pronged approach to investigating the evolution of breeding systems in living and fossil Spinicaudata be utilized for similar investigations of biological interactions in other fossil taxa.

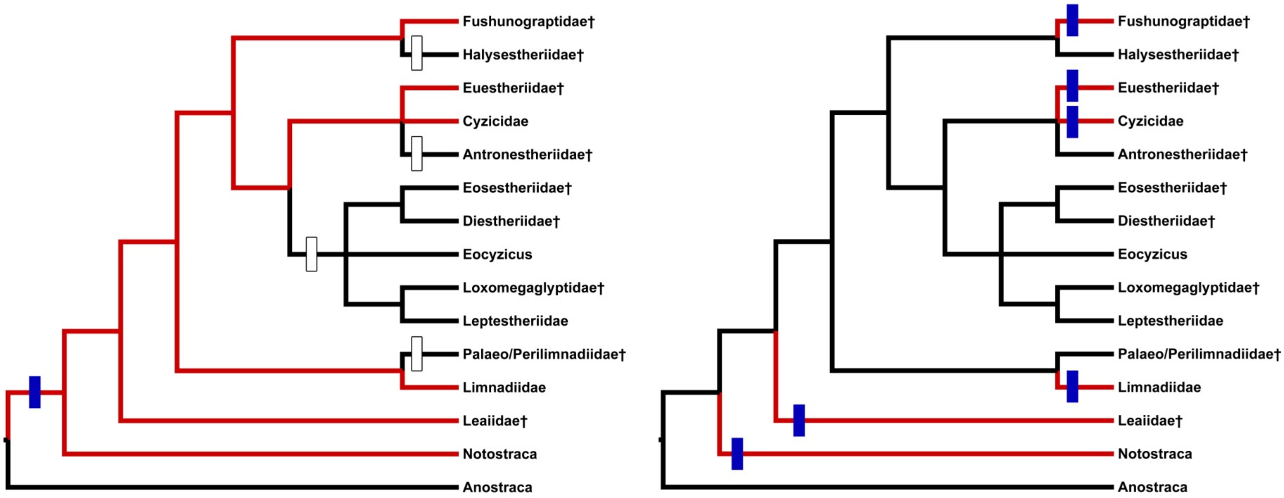


**Fig. 7.** Two possible scenarios of the evolutionary dynamics of hermaphroditic lability in Spinicaudata at the family level. Red branches: lineages with some degree of hermaphroditism present in some taxa. Black branches: lineages devoid of hermaphroditic 'females' in all taxa. Blue boxes: sperm production in females occurring. White boxes: Sperm production lost in hermaphrodites recreating females. † = Extinct group.

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**Availability of data and materials:** This manuscript does not contain any personal data beyond that belonging to the authors.

**Competing interests:** TIA, LPB and SCE declare that they have no conflicts of interests.

**Consent for publication:** No consent needed.

**Ethics approval consent to participate:** Not applicable.

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