THE EVOLUTIONARY DYNAMICS OF SEXUAL SYSTEMS IN DEEP TIME: AN INTEGRATED BIOLOGICAL AND PALEONTOLOGICAL APPROACH

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Dissertation

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ABSTRACT

This doctoral dissertation reports the results of a multi-faceted investigation into the evolutionary dynamics of sexual systems over geologic time using the fossil record of the bivalved branchiopod crustacea in the Order Spinicaudata. The difficulty of assessing the sex of fossils (and by extension, the sexual system employed in extinct organisms) is widespread, and in those taxa that do show sexual dimorphism (e.g., ammonites, some arthropods, and vertebrates), reproductive mechanisms are often invariant, making assessments of reproductive evolution impossible. In this study, new techniques have been developed that will allow the identification of sexes in fossil crustaceans within the taxon Spinicaudata (“clam shrimp”): an enigmatic group of crustaceans with a unique bivalved carapace. Clam shrimp are well-represented in the fossil record, and have a broad array of reproductive types: dioecy (males + females), androdioecy (males + hermaphrodites) parthenogenesis (asexual females), and selfing hermaphroditism. These projects combine the wealth of information about the evolutionary transitions among these various reproductive types gleaned from studies of extant clam shrimp, with the rich representation of clam shrimp throughout the fossil record (from the Devonian to the modern day) to address two canonical hypotheses of reproductive evolutionary theory: (1) that unisexual species should be short lived and less speciose than their outcrossing counterparts and (2) that androdioecy is an unstable, transitional system that should not persist over long periods of time. These studies
provide much needed reviews of existing paleontological and biological research regarding the Spinicaudata, assess their taphonomic fidelity using a number of experimental techniques, develops and tests a morphometric protocol for identifying sexes in fossil populations and subsequently extends these shape comparisons using a large collection of fossil clam shrimp taxa from across the world to characterize sex-specific shape differences among various fossil clam shrimp lineages. We then combine these methods to assess sex ratios in fossil clam shrimp, which are indicative of mating system type in modern taxa, thereby allowing us to assign mating system to fossil species. These combined techniques allow, for the first time, investigation of the two evolutionary questions in the studies noted above and to test theories of breeding system evolution that, to date, have remained elusive to empirical assessment and provide a foundation for future research utilizing the fossil record to perform transformative studies into the evolution of mating systems.
DEDICATION

This dissertation represents the culmination of five years of research which I could not have done without the support of my loving wife, Rebecca and my Family; Sue, Ian, Josh, Rose, Alan, Margaret, Roy, Elizabeth, Andrew, Charlotte, James and Cyril. Thank you all for believing in me and being there when we were so far away.
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Introduction

The reproductive biology of an organism is arguably its most important phenotypic component and the sexual system employed by the species is likely to dominate an integrated organismal bauplan. The biological mechanisms organisms have evolved to reproduce are diverse but poorly understood in extant organisms (Charlesworth 2006), and almost completely unknown in fossil or extinct organisms (Butterfield, 2000; Sassaman, 1995), because they are thought to leave little to no trace in the geologic record.

A sexual system refers to the biological mode of reproduction within a population [e.g., gonochorism (two distinct sexes; also termed "dioecy"), hermaphroditism, parthenogenesis] whereas a mating system refers purely to the physical and behavioral interactions between individuals (e.g., monogamy, polygamy) that ultimately produce offspring.

Among the plethora of sexual systems, two reproductive strategies are dominant among living taxa and have been studied in extensive detail. Clonal reproduction allows for the rapid exploitation of good gene combinations and has been hypothesized to be
characteristic of marginal, high-stress environments where a beneficial genotype would allow for a clonal organism to outcompete others and colonize new habitats, (Barrett, 2002a; Pannell and Barrett, 1998; Schön et al., 2009), but is uncommon in higher metazoans (Bell, 1982). In contrast, the genotypic variability produced by genetic recombination employed in sexually reproducing, dioecious organisms allows for the exploitation of complex but stable environments (Nunney, 1989).

A third, considerably rarer reproductive strategy involves self-compatible hermaphroditism in which individuals benefit from reproductive assurance in low population densities when finding a mate may be problematic. Self-compatible hermaphroditism is also associated with early colonizers or ‘pioneer species’ (Pannell, 2002; Wolf and Takebayashi, 2004). Evolutionary transitions between dioecy and hermaphroditism (Figure 1.1) can result in androdioecy (males and hermaphrodites), gynodioecy (females and hermaphrodites), and in very rare cases, trioecy (males, females and hermaphrodites; not shown). These three transitions are traditionally thought to be highly unstable and thus short lived.
Figure 1.1. Hypothesized dynamics of sexual system transitions from (Weeks et al., 2006)
The case for androdioecy and gynodioecy being transitional and short-lived in nature is strongly supported by frequency of occurrence alone. The rarity of androdioecy and gynodioecy stands in stark contrast to the dominance of hermaphroditism in the plant kingdom (Charlesworth, 1984; Charlesworth, 2006; Pannell, 2002) and dioecy in the animal kingdom (Weeks, 2012). The hypothesized evolutionary dynamics behind a ‘quick’ transition take into account the selective pressures driving the transitions. Transitions from dioecy to hermaphroditism are likely to be the result of selective pressures for reproductive assurance; that is, in populations of dioecious organisms where organismal mobility may be low (Puurtinen and Kaitala, 2002) or individuals occur at low density (Ghiselin, 1969).

A mutation giving rise to a hermaphrodite in a dioecious population will be at an automatic advantage of being able to mate with any individual (and in some cases also to self-fertilize if no mates are available), thus likely outcompeting the mate-limited single sexes, assuming any self-fertilization does not lead to high levels of inbreeding depression (Pannell, 2002). Therefore transitions from dioecy to hermaphroditism involving intermediate sexual systems that include both male and hermaphrodite individuals are predicted to be relatively short lived in a geological time frame (Charlesworth and Charlesworth, 1978).

A similar prediction of a geologically short-lived reproductive type has been levied for unisexual organisms (asexual or completely selfing hermaphroditism), but for an entirely different reason. Mutations resulting in unisexuality will quickly spread
through an otherwise sexual population (due to the advantage of automatic genetic transmission; Williams, 1975), only to lead to longer-term extinction due to the inability of the unisexual to adapt quickly to changing environments resulting from decreased genotypic diversity via the cessation of outcrossing (Nunney, 1989).

Despite seemingly sound biological theory to the contrary, several taxa have been touted as examples of ‘ancient asexuality’ (Judson and Normark, 1996; Normark et al., 2003; Pannell, 2009; Schwander et al., 2011) most notably the bdelloid rotifers (Welch and Meselson, 2000). Several other invertebrate taxa have been proposed to exhibit historical asexuality as they apparently display examples of what (Pannell, 2009) calls a ‘closed marriage’ (Hill et al., 2006; Ross et al., 2012a; Schön et al., 2009; Simon et al., 2002). However, in most cases this does not appear to be strictly true in that males may occur extremely infrequently or some degree of genetic recombination/assimilation may be occurring, albeit in a cryptic way.

The interest in a genuinely ancient asexual lineage seems to be fired by controversy alone at first glance (see Schön et al., 2009). However, the occurrence of unisexual lineages in invertebrates is puzzling and studying ‘ancient’ unisexual lineages may provide us with clues as to the emergence, stability and persistence of sexual systems over geologic time. If these taxa truly do exhibit a long-lived form of unisexuality, or supposedly unstable reproductive mode, how do they accomplish this feat? Why do they do it?
Below I briefly review invertebrate taxa that are hypothesized to exhibit some form of ancient unisexuality or ‘transitional’ sexual system (Figure 1.2) and introduce the subject of this study: the Spinicaudata.

**Bdelloid Rotifers (Bdelloidea: Bdelloida):**

Bdelloid rotifers (Figure 1.2.a) are small aquatic protostomes within the Spiralia. They differ from the other two orders within the phylum Rotifera in that they are exclusively asexual. Much interesting research has been conducted into the persistence of, and diversity within, this group of enigmatic organisms (Arkhipova and Meselson, 2000; Fontaneto et al., 2007; Fontaneto et al., 2012; Welch et al., 2004b).

Unisexuality in bdelloid rotifers is characterized by the complete absence of males, recombination and meiosis (Welch et al., 2004a). Reproduction occurs via individuals that produce only eggs containing viable embryos. The bdelloids are often touted as the ‘genuine article’ when it comes to ancient asexuality. Some studies (Fontaneto et al., 2007) claim that the order is as much as 80-100 million years old, meaning the bdellooids are flagrantly flying in the face of established biological cannon that the reduced genotypic diversity inherent in all forms of unisexuality should doom lineages to extinction in a relatively quick evolutionary timeframe.

However, all is not as it seems when the fidelity of this ‘closed marriage’ is scrutinized more thoroughly (a theme common to most instances of supposedly strict, closed unisexuality). Bdelloid rotifers, like many other unisexual invertebrates (e.g., branchiopods, tardigrades, nematodes) can enter a desiccation resistant state called
anhydrobiosis, a form of cryptobiosis likely evolved as an adaptive mechanism for surviving in the absence of water, in which they can remain for an undetermined amount of time. Upon recovery from diapause, it has been reported that “bdelloid rotifers accumulate foreign genes and render them transcriptionally competent” (Boschetti et al., 2011) during a process that repairs DNA damaged during anhydrobiosis. Foreign genes, in this case, can be sourced from anywhere in the individual's immediate environment, even from food. This novel adaptation to harsh environments may have had unforeseen advantages, acting very much like recombination in sexual reproduction, allowing the bdelloids to incorporate and utilize diverse new genetic material (Gladyshev et al., 2008). In light of this discovery, it seems likely that their persistence and diversity as a ‘unisexual’ order may be, in part, the result of a novel and cryptic method of acquiring exogenous genetic information in the absence of sex. Thus, the bdelloid system is likely not the ‘closed marriage’ it was previously thought to be.
Figure 1.2. Some examples of unisexual invertebrates; a) Bdelloid rotifer (Tree of Life Web Project) b) Darwinulid ostracod (Schon et al 2009) c) Timema phasmid (Maderspacher 2011) d) the Nematode ceanorhabdites elegans (Schon et al 2009) e) Aphid (Schon et al 2009) and f) Scale insects (Schon et al 2009).
**Darwinulid Ostracods:**

The ostracod crustacean family Darwinulidae (Figure 1.2. b) contains three genera (*Alicenula*, *Darwinula* and *Penthesilenula*) all of which have been reported as asexual (Schon *et al*, 2009). Three non-functional (no sperm or sperm producing organ) males have been reported in the genus *Darwinula* from a total of thousands of collected individuals, which has been suggested as “positive negative evidence for asexuality” (Birky, 2010): the existence of functional males posited by skeptics of the unisexual Darwinulidae would have to occur in such a low frequency to have evaded detection, suggesting that outcrossing (if it exists) must occur at such a low level that it would have no effect on the sexual system.

The darwinulids have a good fossil record extending back to the Devonian some 360mya, again, with no evidence of males in any of the fossils studied. It is thought that the group was actually more diverse during the Palaeozoic, but has suffered a severe loss of lineages during the Permian-Triassic extinction event. The seven recorded asexual darwinulid species have been the subject of intense debate since they were initially proposed as ‘ancient asexual scandals’ (Judson and Normark 1996) with many responses (Butlin *et al.*, 1998; Schön *et al.*, 2009; Smith *et al.*, 2006) arguing for and against the applicability of either the ‘ancient’ or ‘asexual’ moniker.

Measuring internal transcribed spacer region diversity in darwinulids and their related lineages provided empirical support for earlier suggestions that these ancient asexuals avoid the classic problem of deleterious mutation accumulation via the
cessation of evolution. Studies comparing within- and between-lineage molecular divergence revealed that darwinulid ostracods appear to have slowed their evolutionary rate to 10% of that of related lineages (Schön et al., 1998; Schön and Martens, 2003). Coupled with an efficient DNA repair system, small brood size and relatively long life span, the ancient asexual title appears secure for darwinulid ostracods, albeit at the cost of extreme reduction in lineage diversity (*Alicenula* is monospecific, *Darwinula* is known from two globally distributed species and the *Penthesilenula* are the most specious with four species). Although this group is definitely an excellent candidate for studying the persistence of asexuality over evolutionary time, the lack of species and genetic diversity, along with the apparent ‘locking’ of the entire lineage into asexuality for nearly 200 ma, renders them of little use in elucidating evolutionary dynamics of differing sexual systems or their responses to different sources of selection over time. However, in a larger inter-organismal study of sexual system evolution, they would undoubtedly be of pivotal importance.

**Oribatid Mites:**

Oribatid ‘beetle mites’ (Arachnida, Oribatida) (Figure 1.3) are small, soil-dwelling mites consisting of an estimated 50-100 thousand species worldwide (Norton et al., 1988). Oribatids are represented in the fossil record from strata dating back to the Devonian (Norton et al., 1988) and are relatively well preserved as they have a thicker cuticle than related groups. This extensive record has lent credence to the hypothesis that their current global distribution is the result of continental drift.
Within the order Oribitada there are several lineages proposed to exhibit unisexuality in the form of parthenogenesis, including the suborders Trhypochthoniidae (51 species), Malaconothridae (137 species), Camisiidae (78 species), Brachychthoniidae (158 species), Lohmanniidae (179 species), Nanhermanniidae (58 species) and the genus Nothrus (67 species) (Heethoff et al., 2007).

This high frequency of unisexuality stands in stark contrast to the low lineage splitting seen in Darwinulid ostracods. Indeed, it has been estimated that up to 10% of oribatid lineages are unisexual, considerably higher than the 1% suggested for other animal taxa (Maraun et al., 2003). Also of import, and again, in opposition to the pattern seen in darwinulids, is the high number of suggested independent instances of unisexuality within the oribatid mites (six suborders).

Oribatids are another unsuspected candidate for the ‘ancient asexual’ crown and have more recently become contenders after the age of some unisexual lineages was estimated to be over 80ma (Heethoff et al., 2007). The biological mode of reproduction in this group is hypothesized to be automictic thelytoky (polar bodies fuse producing offspring that are genetically identical to parent), although precise cytological mechanisms remain understudied (Schön et al., 2009). Meiosis appears to be occurring but it is likely that diploidy is achieved via re-absorption of a polar body (one of the three daughter cells produced during meiosis acts as ‘sperm’ fertilizing the parent egg cell (Stenberg and Saura, 2013)). Therefore some recombination is occurring and subsequently many would argue against this as a case of ‘strict’ parthenogenesis.
(Neiman and Schwander, 2011; Schaefer et al., 2006). Homogenization of the nuclear genome, similar to that noted in darwinulid ostracods has also been noted and is likely the result of similar suggested mechanisms.

Coupled with the conserved morphology noted in the fossil examples of oribatids, this high frequency of unisexuality in multiple lineages suggests oribatid mites would be an excellent study organism for the investigation of sexual systems over an evolutionary timescale. However, much more biological research into the cytology and genetics of their reproductive system must be undertaken before sense can be made of their evolutionary persistence.

Timema Stick Insects:

The genus *Timema* (Figure 1.2.c) is a basal phasmatid stick insect indigenous to the west coast of the United States. Five of the 21 species described in this genus are reported to be unisexual and likely parthenogenic (Sandoval et al., 1998). These stick insects have been the focus of more recent research regarding geographic parthenogenesis, an extension of Bell’s (Bell, 1982) hypotheses that parthenogenic populations should occur at the peripheral, northern-most extant of a species range due to the distinct colonization advantage inferred via the ability to produce offspring without the need to find a mate (Figure 1.4). These studies have not only found that this is, in fact, the case (Law and Crespi, 2002), but that of the five independent derivations of parthenogenic lineages from sexual progenitors, three may be candidates for ancient
asexuality; thus the prefix ‘scandalous’ has been applied to these insects (Schwander et al., 2011).

_Timema_ appears to reproduce via functionally mitotic parthenogenesis; that is, eggs are formed fully diploid without the need for fertilization. Of the unisexual taxa discussed in this overview, _Timema_ is the only group that may be considered ‘strict’ parthenogens; there is no crossover in genetic material between generations and, as such, are subject to, and indeed display evidence of, the ‘Meselson effect’ (Welch and Meselson, 2000).

The Meselson effect describes the accumulation of non-homologous mutations, leading to allelic divergences that are larger in long-term asexual lineages than in sexual lineages, essentially resulting in ‘independent’ genomes in individual lineages of a historically asexual species. The result in asexual lineages is an allelic phylogenetic tree that is not congruous with species/population phylogeny. This effect leads to an effective test of true ‘ancient asexuality’, of which, three of the five parthenogenetic _Timema_ stick insect species apparently pass, with ages ranging from 500,000 to 2,000,000 years (Schwander and Crespi, 2009; Schwander et al., 2011).

The lack of homogenizing effects that are associated with recombination, DNA repair and cryptic sex that have been suggested as evidence of absence of sex in other unisexual species enables us to identify asexuality and its age in this group. Although these ages are debatably ‘ancient’ (a relative term given either a biological or palaeontological context), some species of _Timema_, as previously mentioned, are
indeed asexual in the strict biological sense. There is no recombination event between mother-daughter lineages, therefore creating a ‘closed marriage’ of isolated lineages that allelically diverge from separate populations over time via the Meselson effect.

Although undoubtedly asexual, *Timema* is not considerably old in an evolutionary sense and lacks a fossil record. Therefore this interesting arthropod is of little use in looking for the persistence of unisexuality or transitional sexual systems over geologic time.
Figure 1.3. The Devonian oribatid *Devonacarus sellnick* (left) from Nortan *et al.*, 1988 and the recent oribatid *Hypochthonius* sp.

Figure 1.4. Left: phylogenetic relationships within *Timema* with distribution of parthenogenesis. Right: geographic distribution of *Timema* on the Eastern coast of the US (note more northerly distribution of unisexual populations). Taken from (Law and Crespi, 2002).
Caenorhabditis Nematode Worms:

*Caenorhabditis elegans* and *C. briggsae* are widely renowned by biologists as model organisms, *C. elegans* having the privilege of being one of the first few animals to have their entire genome sequenced (Gerstein et al., 2010). While the majority of Caenorhabditis nematodes (Figure 1.2.d) are gonochoristic, these two species are androdioecious, often occurring as purely hermaphroditic populations (Hill et al., 2006). The hermaphrodites are capable of self-fertilization but are protandrous, producing only one initial ‘batch’ of sperm that is subsequently used to fertilize their own eggs. The gonadal material then switches to oogenesis shortly after maturation. These individuals lack any kind of copulatory structure so cannot outcross as males. Males are produced either by existing males mating with the female-biased hermaphrodites (hermaphrodites allocating the majority of their resources to producing eggs), producing 50% male offspring, or through spontaneous generation of males by self-fertilizing hermaphrodites via non-disjunction of sex chromosomes (Chasnov 2010). Therefore, androdioecious populations of *C. elegans* consist of haploid males and diploid females.

Although it has been determined that male sperm frequently out-compete the sperm produced by the hermaphrodites, males are rare in natural populations, often comprising less than 1% of a population (Chasnov, 2011; Chasnov, 2010). Such a scarcity of males suggests that males cannot be selectively maintained.

Recent studies investigating the evolution of hermaphroditism in these species suggests self-fertilization has independently arisen in *C. elegans* and *C. briggsae* as a
result of inter-species hybridization events (Kiontke et al., 2004; Hill et al., 2006; Woodruff et al., 2010). Some estimations place the emergence of these clades at 80mya. However, studies employing molecular clock estimates of divergence times for nematodes have relied heavily on speculative prior assumptions such as the validity of an ecdysozoan clade, an arthropod-nematode divergence circa 1,000,000,000 years ago, and a universal clock. Therefore it is deemed unwise to place too much credibility in such an estimate. This is made even more apparent by the absence of a fossil record for this group (Kiontke et al., 2004), which also limits the applicability of *Caenorhabditis* to evolutionary questions on a geologic timescale.

**Aphids:**

The ubiquitous hemipteran Superfamily Aphidoidea (Figure 1.2.e) are a cosmopolitan group well known as agricultural pests (Wilson et al., 2003) are also commonly used as text-book example of asexuality, which is somewhat erroneous as the majority of aphid species are actually cyclically parthenogenic, seasonally engaging in sexual reproduction with males, and therefore not strictly unisexual. Several suggested instances of ancient asexuality have been investigated in the group (Delmotte et al., 2003; Normark et al., 2003) and all have failed to find evidence of asexuality being ancient despite the occurrence of an impressive amount of heterozygous karyotypes in relatively diverse asexual lineages.

Explanations for the occurrence of parthenogenic lineages involve hybridization events (Simon et al., 2002; Delmotte et al., 2003) between contiguous, cyclically
parthenogenic species as well as heterochronic developmental discontinuity in that maturity is hormonally controlled and the lack of environmental cues may cause the cessation of male production, a process seen in wild populations. This essentially means that these species can be sexual given the right environmental circumstances, which may be absent for long periods of time.

Despite the Aphidoidea being an important and interesting group for studying the ecological interaction of sexual systems, no convincing evidence supports the persistence of unisexuality over deep time. However, the widespread occurrence of cyclic parthenogenesis in the group is intensely interesting and likely ancient in origin (Normark, 1999). Unfortunately the homogenizing effects of seasonal outcrossing would render impossible any attempts at deciphering the origin of this sexual system by allelic divergence, and the complex sexual systems, inter-species hybridization and the formation of intra-specific ‘races’ on particular host plants still confounds efforts to resolve phylogenetic relationships within the group.

**Scale Insects:**

Scale insects (Coccoidea) of the order Hemiptera (Figure 1.2.f) contain approximately 8,000 species, have a patchy fossil record extending only as far back as the Cretaceous (Gullan and Cook, 2007) and like aphids, are renowned as agricultural pests. They are highly derived insects adapted for parasitism of plants. The female is neotenetic while the male undergoes drastic metamorphosis and is often gutless and ephemeral (Gullan and Kosztarab, 1997). The sessile morphology and lifestyle of the
female severely limits mate search efficiency, as the females are not mobile and cannot actively search for males. This unique biology has a probable ancestral influence in the evolution of unisexual lineages (Gullan and Kosztarab, 1997).

The Coccoidea display a variety of sexual systems, including dioecy, parthenogenesis and self-fertilizing hermaphroditism. It is thought that the effective elimination of males in some species (which has evolved multiple times in the group) results from widespread haplodiploidy (males develop from unfertilized, haploid eggs, as in most hymenoptera) associated with male-phenotype-killing endosymbionts (Ross et al., 2012b).

Recent research has suggested that large population size in some scale insects may promote the evolution of asexuality (Ross et al., 2012a). However, considering the two-fold advantage of unisexuality occurring from the loss of males and ability to reproduce constantly and quickly, the arrow of causality seems to be pointing in the opposite direction. The suggestion that large population size may preclude the emergence of unisexual mutants remains avant garde in light of the accumulated scientific knowledge from other taxa regarding unisexuality which interpret it as an adaptation that precedes range expansion, by improving colonization ability and increasing the effective population size.
Table 1.1. Sexual systems and hypobiotic mode of Invertebrate taxa discussed in this chapter

<table>
<thead>
<tr>
<th>Taxa</th>
<th># of living species</th>
<th>Sexual Systems</th>
<th>Hypobiotic Mode</th>
<th>Fossil record?</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coccoidea</td>
<td>8000</td>
<td>Gonochorism, self-fertilizing hermaphroditism,Parthenogenesis</td>
<td>Diapause Cysts</td>
<td>moderate</td>
<td>Cretaceous</td>
</tr>
<tr>
<td>Caenorhabditida</td>
<td>20+</td>
<td>Dioecy, Androdioecy, Self-fertilizing hermaphroditism</td>
<td>Adult cryptobiosis</td>
<td>very poor</td>
<td>Cambrian</td>
</tr>
<tr>
<td>Bdelloidea</td>
<td>370</td>
<td>Parthenogenesis</td>
<td>Adult cryptobiosis</td>
<td>moderate</td>
<td>Permian</td>
</tr>
<tr>
<td>Darwinulidae</td>
<td>30</td>
<td>Parthenogenesis</td>
<td>Diapause eggs</td>
<td>excellent</td>
<td>Carboniferous</td>
</tr>
<tr>
<td>Oribatida</td>
<td>100000</td>
<td>Dioecy, Parthenogenesis</td>
<td>Adult Quiescence</td>
<td>moderate</td>
<td>Devonian</td>
</tr>
<tr>
<td>Timema</td>
<td>7</td>
<td>Dioecy, Parthenogenesis</td>
<td>None</td>
<td>none</td>
<td>Recent</td>
</tr>
<tr>
<td>Spinicaudata</td>
<td>60+</td>
<td>Dioecy, Androdioecy, Self-fertilizing hermaphroditism</td>
<td>Diapause eggs</td>
<td>excellent</td>
<td>Early Devonian</td>
</tr>
</tbody>
</table>
Hypobiosis

An evolutionary trend among taxa that exhibit non-gonochoric sexual systems is that all exhibit some form of hypobiosis (Table 1.1) and as such they are able to cease biological processes at a particular life stage allowing them to occupy temporally ephemeral or transitory environments that are not exploitable by sexual lineages (‘Tangled Bank’ hypothesis (Bell 1982)). Many aquatic invertebrates do this by producing desiccation resistant eggs (diapause) where a viable embryo is tightly encased in a proteinaceous shell that will not hatch until favorable conditions arise. Other invertebrates, such as tardigrades, nematode worms and bdelloid rotifers, can enter hypobiosis as adults. It is likely that both unisexuality and hypobiosis are beneficial to pioneering organisms commonly experiencing potentially hostile habitats, as unisexuality relieves selective pressure to find a mate and allows new populations to be founded with a minimal number of individuals. On the other hand, hypobiosis provides an evolutionary ‘loophole’ allowing unisexual species to avoid the problems associated with reduced genetic diversity and adaption to changing environmental conditions, in that if the conditions are unfavorable, adults or eggs can enter hypobiosis and avoid such conditions, re-establishing the population when environmental conditions are more favorable. This essentially releases unisexual organisms from a large quantity of selective pressure that sexual species with temporally continuous lineages must adapt to. Future studies into the evolution of sexual systems would benefit from considering the repertoire of adaptations that may be employed by individual lineages and the
dynamic interplay between such adaptations, sexual systems, their environments and their evolutionary history.
The Order Spinicaudata

Reproduction and mating systems are some of the most important areas of inquiry within evolutionary biology. Yet because of the bias for hard part preservation, the fossil record contains relatively few examples of reproductive characteristics. The invertebrate groups discussed in the previous sections, while displaying a plethora of interesting sexual systems, do not meet all of the requirements needed in order to highlight them as ‘model organisms’ for studying the evolution of sexual systems over deep, geologic time. In order for a group to be useful in this respect, they must have an extensive, diverse and global fossil record. Extant representatives should show different sexual systems distributed across taxa in a way that indicates a long history of sexual system diversity (as opposed to phylogenetically constrained instances of a sexual system, as seen in the Darwinulid ostracod and Timema stick insects, that may offer little information regarding evolutionary dynamics of sexual systems over time) and must display morphological features that are indicative of the sexual system employed in order for useful data to be extracted from the fossil record. Finding a fossil system that allows for a comprehensive examination of mating systems over time would be invaluable in understanding one of the most important evolutionary selective forces shaping the history of life.

Large branchiopod crustaceans, especially clam-shrimp of the order Spinicaudata (a group of phyllopod crustaceans that occur on every continent except Antarctica) fit the criteria of "model organisms" noted above: they are speciose (Martin and Davis, 2001),
exhibit several different mating systems (dioecy, androdioecy, unisexual selfing hermaphrodites) and have a particularly good freshwater fossil record extending back ~400 million years to the Devonian period (Figure 1.5). The Spinicaudata (Figure 1.6) are restricted to freshwater systems, with the majority of modern taxa inhabiting lakes, ponds and ephemeral water bodies (Dumont and Negrea, 2002). Particularly notable for their diapause stage, clam shrimp produce egg-banks (similar to seed-banks) by burying their eggs in the bottom sediment of the pools they inhabit. These eggs are resistant to desiccation and may survive for decades (or longer) before eventual rehydration (raising numerous questions concerning their interactions with the environment in an adaptive landscape model, as well as begging inquiry into their almost bradytelic evolutionary phenotype). Clam shrimp have a distinctive, chitinous, bivalved carapace (Figure 1.6) attached to the back of the cephalon via the dorsal neck organ, which has multiple growth bands. The carapace is not shed during ecdysis so these growth bands record an ontogenetic sequence. The carapace encloses the body of the organism, which is itself tripartite consisting of a cephalon, trunk and telson. The trunk makes up the majority of the organism and each constituent somite has pairs of filamentous, phyllopod appendages, which are used primarily for feeding. The antennae are used for locomotion.
Figure 1.5. Chronogram of the Devonian period with earliest known Spinicaudatan (Conchostracan) fossils.

Figure 1.6. Hermaphrodite *Eulimnadia dahli*. a) Antennae. b) Brood chamber with egg clutch. h) Head. p) Phyllopod appendages. te) Telson. tr) Trunk.
The clam shrimp are a problematic group, taxonomically. Traditionally referred to as the ‘Conchostraca’ containing the orders Spinicaudata, Laevicaudata and Cyclestheridae, the group is now deemed paraphyletic with the Cyclestheridae showing affiliations with the closely related Cladocera (proposed Cladoceramorpha (Olesen, 2007)). The comprehensive work on crustacean taxonomy by (Martin and Davis, 2001) suggests that the Conchostraca be discarded and that the group including the clam shrimp, Notostraca (tadpole shrimp) and Anostraca (fairy shrimp) be referred to as Phyllopoda (Phyllopoda + Cladocera being Branchiopoda; Figure 1.7).

The Spinicaudata are of particular interest as they are diverse, geographically widespread and have a relatively good fossil record. The Spinicaudata are identified mainly via larval morphology (Olesen, 2007) but can easily by distinguished via gross morphology with little training. The order Spinicaudata comprises three families: the Limnadiidae, Cyzidae and Leptestheriidae. The Leptestheriidae are not as diverse or abundant as either Cyzidae or the Limnadiidae and are definitely not as well represented in the fossil record. Contrary to many studies regarding the dynamics of sexual systems, stability and persistence of androdioecy in the Limnadiidae is supported by phylogenetic analyses incorporating inferred ancestral mating systems. (Weeks et al., 2009) constructed a molecular-based phylogeny for populations of different species within Limnadiidae with associated sexual systems for ancestral nodes inferred by the sexual systems at terminal taxa. It is proposed that
Figure 1.7. Branchiopod relationships and sexual systems.
the ancestor of the monophyletic genus *Eulimnadia* (Hoeh et al., 2006) was androdioecious which, when coupled with fossil evidence for *Eulimnadia* being 45-65 million years old (Tasch 1969), provides strong support for the stability of androdioecy.

This observation, by extension, may easily be applied to mutations in clam shrimp lineages leading to female-biased, self-fertilizing hermaphrodites. The emergence of mutations that result in self-fertilizing individuals has been hypothesized to rapidly shift the sexual system to a ‘closed marriage’ (Pannell, 2009) of complete hermaphroditism, the genetic dynamics of which are analogous to those found in asexual systems. In both unisexual systems, the limited variability within clonal/selfing-hermaphroditic lines is predicted to lead to high extinction rates and low speciation rates that, over long time spans, selecting for sexual lineages, especially those with a low probability of producing unisexual mutants (Nunney, 1989).

There are two primary reasons why the Spinicaudata are an excellent candidate for potentially resolving important evolutionary questions concerning reproductive systems. First, the Spinicaudata are reproductively diverse, including dioecious, self-compatible hermaphrodites, androdioecy, parthenogens (asexual) and cyclic parthenogens (Sassaman, 1995). Second, there are numerous species of Spinicaudata, with an estimated ~140 extant species recognized worldwide (Brtek 1997) and many more in the fossil record (Tasch, 1969; Zhang et al., 1976).

Several of the extant Spinicaudata have been particularly useful for studies of breeding system evolution, especially studies of hermaphroditism, androdioecy and
dioecy (Sassaman, 1995; Hoeh et al., 2006; Weeks et al., 2006; Weeks et al., 2009). However, to date, the rich fossil record for these crustaceans has not been utilized in examining long-term questions of breeding system evolution.

In examinations of breeding system evolution, many authors have proposed that androdioecy is a relatively unstable system that should not persist over long periods of time, instead predicting that androdioecy represents a transitional system between dioecy and hermaphroditism (see above and Fig. 1.1). However, the widespread occurrence of androdioecy within the Spinicaudatan family Limnadiidae (Hoeh et al., 2006; Weeks et al., 2009), along with a hypothesized age of the androdioecious genus *Eulimnadia* at between 180 (Weeks et al., 2006) – 45mya (Tasch, 1969), provides evidence to the contrary. It is also important to note that the closely related Notostraca (tadpole shrimp) also exhibit dioecy, androdioecy and hermaphroditism (Sassaman, 1989; Zierold, 2007) and that the divergence of these two groups is estimated to have occurred approximately 330 Ma (Regier et al., 2005).

Another interesting phenomenon noted in extant taxa is the distribution of species that consist solely of self-fertilizing hermaphrodites. The evolution of hermaphrodites from females has occurred six times in known extant taxa and these species are distributed across four genera in two families of Spinicaudata (Weeks et al., 2009; Weeks et al., 2013).

Thus, these branchiopod crustaceans provide an interesting set of taxa with clearly labile breeding systems and a good fossil record, which could prove exceptionally
useful for the study of breeding system evolution if we could infer breeding system from fossils of these crustaceans.

This series of studies in this dissertation aims to provide a synthesis of biological and palaeontological data in the hope of establishing repeatable protocols for investigating the evolution of sexual systems using fossil and extant sources of information. It will also provide a holistic assessment of the quality of fossil data via experimental taphonomy and present the results of applying contemporary comparative techniques to model the evolutionary dynamics of sexual system evolution within a phylogenetic framework. These studies ultimately aim to combine the wealth of information about the evolutionary transitions among various reproductive types gleaned from studies of extant clam shrimp, with the rich representation of clam shrimp throughout the fossil record (from the Devonian to the modern day) to address two widespread hypotheses of reproductive evolutionary theory: 1) that unisexual species should be short lived and less speciose than their dioecious counterparts and that 2) androdioecy is an unstable, transitionary system that should not persist over long periods of time.
CHAPTER II

DECIPHERING THE STRUCTURE AND TAXONOMIC SIGNIFICANCE OF THE ‘CONCHOSTRACAN’ CARAPACE (BRANCHIOPODA: SPINICAUDA, LAEVICAUDA, CYCLESTHERIIDAE)

This work is the result of a four year collaboration between the author, Dr Thomas A. Hegna (University of Western Illinois) & Dr Stephen C. Weeks (University of Akron)

Overview:

The bivalved, chitinous carapace of the paraphyletic group of large branchiopod ‘conchostraca’ (Spinicad, Laevicad, Cyclesthiida) is commonly preserved in terrestrial aquatic deposits and are useful biostratigraphic, palaeoenvironmental and palaeobiological fossils. Despite a long history of palaeontological study and recent interest in their molecular systematics, attempts to reconcile palaeontological and biological systematic schemes have not been undertaken, seriously hampering understanding the evolutionary history of the group. This study assesses long held assumptions concerning the ‘usefulness’ of varying carapace ornamentation in the 'Conchostraca' as a taxonomic tool by surveying living ‘conchostracan’ ornamentation, performing experiments that help us understand the biological origin and taphonomic susceptibility of these structures and comparing patterns found in extant taxa to these
in fossil taxa. These data will provide a foundation for future synthetic research into the evolutionary history of this diverse group of crustacea.

Introduction

‘Conchostracans’ (a paraphyletic group containing the Orders Cyclestheriida, Laevicaudata, and Spinicaudata; Figure 2.1), or clam shrimp, are an often-ignored group of fossils. Traditionally, they have not been a part of any large synthetic studies that have contributed to our understanding of evolution or paleoecology and there have been relatively few specialists working in this group. This has resulted in a great amount of historical confusion in their taxonomy. As a consequence, their taxonomy is often treated very superficially. In fact, some have argued that fossil ‘conchostracans’ are taxonomically useless (Mattox, 1957). The purpose of this paper is to present evidence that strongly refutes these conceptions of ‘conchostracan’ fossils and to provide a sound biological assessment of carapace features traditionally used in assessing phylogenetic relationships within and between extinct taxa. These data will then provide a foundation for future studies regarding the evolutionary history of the group.

‘Conchostracans’ are a group of branchiopod crustaceans that share the ephemeral freshwater habitat of the other “large branchiopods” (i.e. Anostracan “fairy shrimp”, and Notostracan “tadpole shrimp”). They have a distinctive bivalved carapace that originates as a bi-lobed anlagen at the back of the head, thus explaining their common name, ‘clam shrimp’.
Phylogenetically, the ‘Conchostraca’ has been a group in upheaval for much of the past fifty years. Despite the prevalence of the name ‘Conchostraca’, nearly all recent analyses (both molecular and morphological) demonstrate that the group is paraphyletic (possibly polyphyletic, see (Stenderup et al., 2006)). It is composed of three separate monophyletic groups: the Laevicaudata, the Spinicaudata, and the Cyclestherida (Fig. 2.1). Laevicaudatans are characterized by having a short trunk of 10-12 segments, a bivalved carapace that lacks growth lines, and a large head with a distinctive rostrum, or head plate. Spinicaudata have bodies with at least 32 segments, a variable number of limbs, a bivalved carapace with distinct growth lines (actually a form of molt retention, see below), and no headshield. Cyclestherida consists of only one, low-diversity family characterized by a trunk with 16 segments, fused and sessile eyes, a dorsally spinous abdomen, and a globular bivalved carapace with few growth lines (Dumont and Negrea, 2002). Though the taxon ‘Conchostraca’ is today nearly universally acknowledged to be paraphyletic, we will use the term in this paper for ease of reference, with quotation marks indicating its paraphyletic status. The term has a historically well-understood content and circumscribes a group that shares many functional (and taphonomic, see below) features that do not indicate monophyly. The Spinicaudata is the most diverse of the three groups, and its phylogeny has become much better understood in the past several years (Hoeh et al., 2006; Schwentner et al., 2009; Weeks et al., 2009; Xiaoyan et al., 2006). As such, the Spinicaudata receives proportionally more attention in the current assessment.
Of the branchiopods, the ‘conchostracans’ have the most extensive fossil record (going back to the Devonian, see (Novojilov, 1961; Shen, 1978; Brummer, 1980; Shen, 1983; Chen and Morris, 1991)), but it is beset with one major problem—nearly all the fossils consist only of the carapace (i.e., the shells). This requires that the taxonomy for fossil species be based solely on carapace features like shape and growthband ornamentation (Dumont and Negrea, 2002)—in stark contrast with the taxonomy of modern ‘conchostracans’ which, though much more holistic, paradoxically ignores carapace shape and ornamentation (Pereira and García, 2001; Brtek, 2005; Olesen, 2007; Rogers et al., 2012; ). Thus, there is no common morphological language to reconcile the fossil and modern species. Integration of the extant and extinct diversity into a single hypothesis of phylogeny is clearly desirable, but faces major hurdles that are discussed below.

**The Arthropod Cuticle**

The arthropod cuticle represents one of the most successful innovations in evolutionary history: a rigid exoskeleton composed of sheets of the polysaccharide chitin (often architecturally and compositionally reinforced with proteins and biominerals), providing anchorage for musculature, predator defense and desiccation resistance (Gupta, 2011a). The cost of donning such an effective adaptation comes as a need for periodic molting, or "ecdysis." Ecdysis must occur in order for an arthropod to grow, leaving individuals vulnerable to predation for critical periods of time. An array of highly specialized and adaptable features characterize the malleability of the chitin-
reinforced arthropod cuticle (e.g., light but strong wings (Wootton, 1992), mandibles with novel mineral reinforcement (Beklemishev, 1954; Bentov et al., 2012; Mekhanikova et al., 2012; Michels et al., 2012), claws with inner surfaces preferentially reinforced with greater amounts of calcium carbonate (Waugh et al., 2006), and thin, permeable gill membranes (Mary and Krishnan, 1974) which has undoubtedly been a key adaptation in contributing to the diversity and abundance of the phylum since its origin in the Cambrian (Legg et al., 2012a).

The arthropod cuticle is composed of three major layers. The outermost layer, the envelope and the amorphous epicuticle layer below, is composed of waxes, lipids and proteins that play an important role in preventing water loss and saturation (Moussian, 2013). Below the epicuticle is the procuticle. This layer is characteristically laminar in section and often contains more complex helically-stacked Bouligand structures (Cheng et al., 2008) in derived arthropods. In some arthropods, the procuticle is further differentiated into an upper exocuticle and lower endocuticle. However, sufficient structural separation is not always apparent to universally apply this distinction. Within the procuticle, chitin commonly occurs as a composite material. In decapods crustacean for instance, chitin is reinforced with calcium carbonate providing great rigidity and durable structures for defense and predation. The spatial distribution of this reinforcement is finely controlled by the animal, with little reinforcement in areas requiring flexibility (arthrodial membranes, (Hepburn and Chandler, 1976)) and heavy reinforcement in areas requiring strength (Waugh et al., 2006).
The ‘Conchostracan’ Carapace

The ‘conchostracan’ carapace essentially takes the arthropod cuticle blueprint discussed above and folds it back, encasing a very narrow hemocoelic space (Halcrow, 1976). This structuring is apparent in profile sections, with a lowermost internal epicuticle, followed sequentially by the internal procuticle, internal epidermal layer, the central hemocoel, external epidermal layer, outer procuticle and outer epicuticle (Figure 2.2).

In most branchiopods, there is little, if any, easily definable endo/exocuticle within the uniformly laminar procuticle. Pore canals are typically absent, occurring only in the sister taxon Notostraca and possibly the enigmatic Laevicaudata. Additionally, the chemical composition of the clam shrimp carapace itself is unique in that rather than forming a chitin-calcium complex, the carapace of clam shrimp is believed to be chitin-calcium-phosphate (Stigall and Hartman, 2008).

The spinicaudatan and cyclestheriid carapace is particularly interesting in that molts are partially retained during ecdysis. Normally, an arthropod molts its entire exoskeleton during each episode of ecdysis. Spinicaudatans and cyclestheriids selectively retain their carapace during ecdysis, while molting the remainder of their exoskeleton (covering their body, limbs, head and thin inner carapace cuticle; Figure 2.3). The outer surface of the carapace is retained after each molt, with a new, larger carapace nesting underneath, causing distinctive growth lines that represent discrete ontogenetic events. The result of this is that the carapace increases its strength with
each molt. A potential downside of such a strategy is that epibionts are not purged during ecdysis (Kotov and Štifter, 2006). This is in contrast to the complete discarding of exuviae seen in more familiar examples such as crabs, lobsters and other members of the Ecdysozoa. Thus, each adult carapace preserves a complete record of the ontogenetic development of the shape and the ornamentation of the distal edge of the carapace. Each carapace lamella represents a discrete, post-larval instar.

A lack of complete molting is extremely rare, only being documented as an evolutionary feature in the spinicaudatan & cyclesterid clam shrimp, some families of anomopod cladocerans (including some species that retain the cuticle of both the head and carapace; (Kotov and Štifter, 2006; Kotov and Elías-Gutiérrez, 2009)) and the extinct eridostracan ostracods (Olempska, 2011; Olempska, 2004b); although (Fryer, 1999) argues that the molt retention seen in cladocerans is likely not homologous to that seen in the ‘conchostraca’). Molt retention may not be unique to arthropods. Topper et al. (in press) report isolated plates from the ecdysozoan lopopod Onychodictyon sp. which may exhibit molt retention (the authors interpret the fossil as representing the brief period of time prior to molting rather than molt retention—the evidence for either is equivocal).
Figure 2.1. The paraphyletic ‘Conchostraca’ consisting of the Laevicaudata (A), Spinicaudata (B) and Cyclestheriidae (C). Images reproduced with the permission of Joseph Trumpey.

Figure 2.3. Mature *Cyzicus tetracerus* molting. Note the opalescent distal growth ring on the exuviae. This represents the epicuticle of the most recently added growth band. Image taken by, and reproduced with the permission of, Jean-François Cart.
The Carapace in Systematics

Unfortunately, a rift exists between modern and fossil ‘conchostracan’ taxonomy. The taxonomy of fossil taxa is based on frequently preservable features (often those that are more mineralized in life), such as the external carapace (shape, morphology/ontogeny of growth line ornamentation, etc.). Modern Spinicaudatan, Laevicaudatana and Cyclestheridean taxonomy, on the other hand, is almost exclusively based on soft-part morphology (head, limbs, tail); the morphology of the carapace is ignored or mentioned only in passing and never discussed in detail (Dumont and Negrea, 2002). This has resulted in a body of comparative literature where detailed carapace features cannot be surveyed. A recent morphological phylogenetic analysis of the Branchiopoda was only able to include four (of eighty total) characters pertaining to the carapace (Olesen, 2009).

Previous scientists have been skeptical of the utility of carapace characters in phylogenies in the past. For instance, Mattox (Mattox, 1957) considered fossil ‘Conchostracan’ carapaces to lack any useful characters and advocated classing them all into a single genus, *Palestheria*. The basis for this stance was that ornamentation ‘types’ (granulations, striations or punctae) could co-occur within the same specimen throughout its ontogenetic trajectory causing transitions between ornamentation types to occur in an individual valve, therefore rendering them ‘continuous’ and unsuitable for identifying discrete taxa. This opinion was subsequently accepted by paleontologists like (Tasch, 1969), ultimately leading to the emergence of large ‘trash-can’ taxa (e.g.,
‘Estheria’ and ‘Lioestheria’) based on a severely reduced number of descriptive characters. Furthermore, supposed sexual dimorphism (Daday de Dees, 1915; Daday de Dees, 1923; Daday de Dees, 1925; Daday de Dees, 1926) was also cited as a reason to avoid features of the external carapace. The avoidance of utilizing a character suite in a taxonomic framework because it varies is, at its core, bad systematic practice. It is precisely because a character varies that it ultimately becomes phylogenetically informative when used in the correct context.

If we accept this more holistic attitude toward ornamentation patterns in ‘conchostraca’ then the important questions become how does this character vary and what are the underlying causes of such variation? The documentation of multiple types of ornamentation in a single specimen is not a reason to abandon the use of carapace features. On the contrary, it is the discovery of ontogenetic variation recorded in the growth lines of a spinicaudatan, and is therefore another potential source of variation for use in the elucidation of phylogenetic relationships (Stigall Rode et al., 2005).

Further complicating matters is the fact that there are essentially three (largely non-overlapping) systems of fossil ‘conchostracan’ taxonomy. These three systems are due, in part, to language and political barriers—with Chinese (Chen and Shen, 1985; Zhang et al., 1976), Russian (Novojilov, 1961; Novojilov, 1970), and ‘Western’ (Tasch, 1969) classification systems, all of which use a variety of specialist terms in describing and assigning taxa that are not clearly defined (in order to begin unifying language when describing features of the ‘conchostracan’ carapace a small reference vocabulary has
Table 2.1. Terminology used in this study (and others) to describe the morphological features of the spinicaudatan carapace

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carapace</td>
<td>The dorsal bivalve shield, including the set of lamellae (retained via incomplete ecdysis) acquired during the life of the animal</td>
</tr>
<tr>
<td>Lamella</td>
<td>An individual layer of the carapace attributed to an instar or single molting event</td>
</tr>
<tr>
<td>Growth band</td>
<td>An area of a carapace lamella that is exposed, the area between one growth line and the next (or the distal edge)</td>
</tr>
<tr>
<td>Growth line</td>
<td>The distal edge of an individual lamella</td>
</tr>
<tr>
<td>Larval shell</td>
<td>The first carapace lamellum produced during ontogeny. Later underlain by all successive carapace lamella acquired during life. Often referred to as the ‘umbo’ in older literature.</td>
</tr>
<tr>
<td>Hinge</td>
<td>A dorsal fold in the carapace cuticle that allows the valves to move perpendicular to the body axis. Note: the valves are continuous from one side to the next, and are not articulated like the valves of ostracods</td>
</tr>
<tr>
<td>Valve</td>
<td>One side of the carapace</td>
</tr>
<tr>
<td>Pit</td>
<td>Irregular indentations, or steep-sided depression in the carapace exterior &gt;5μm</td>
</tr>
<tr>
<td>Dish/Dimple</td>
<td>Shallow-sided depressions in the carapace exterior. Often the result of light reticulation overlain by epicuticle (i.e. Eulimnadia texana)</td>
</tr>
<tr>
<td>Lirae</td>
<td>Regular, uniform linear ridges, distinct from striations in that they occur perpendicular to the axis of growth</td>
</tr>
<tr>
<td>Node</td>
<td>A positive relief protrusion not associated with the mechanisms involved in ornamentation generation (i.e. seen in Limnadopsis)</td>
</tr>
<tr>
<td>Marginal setae</td>
<td>Setae that develop at the distal margin of the carapace, often break off leaving only the bases (often as punctae) in non-terminal lamellum</td>
</tr>
<tr>
<td>Compound Ornamentation</td>
<td>One or more types of ornamentation combined together (i.e punctae + striations/Lirae in Eocyzicus)</td>
</tr>
<tr>
<td>Ontogenetic trajectory</td>
<td>Refers specifically to the ontogenetic changes that take place on a single line drawn from larval shell to the edge of the carapace (perpendicular to growth lines)</td>
</tr>
<tr>
<td>Striations</td>
<td>Series of densely packed ridges</td>
</tr>
<tr>
<td>Anastomising ridges</td>
<td>Striations that form bifurcating and anastomising ridges usually directional to growth (i.e as seen in Leptestheriidae)</td>
</tr>
<tr>
<td>Punctae</td>
<td>Perforated indentations of small (&lt;5μm) size occurring on the growth band</td>
</tr>
<tr>
<td>Carina</td>
<td>A continuous ridge extending from the larval shell to the distal margin of the carapace, locally disrupting growth band ornamentation</td>
</tr>
<tr>
<td>Isogonal reticulation</td>
<td>Simple reticulate meshwork originating from the underlying arrangement of the epidermal layer where all cells are of approximately the same size and shape (i.e. uniform ornamentation seen in Cyclestheria)</td>
</tr>
<tr>
<td>Polygonal reticulation</td>
<td>More derived reticulation that appears irregular or regular polygons of differing size and shape</td>
</tr>
</tbody>
</table>
been revised and provided herein (Table 2.1)). Despite a long and active paleontological interest in ‘conchostraca’ none of the existing taxonomic systems fully integrate the diversity of extant spinicaudatans. Thus, it is as if we are dealing with the taxonomy of four independent groups. Without beginning to find a way to integrate these disparate classification systems, we will never begin to understand the evolutionary history of spinicaudatans.

This rift must be bridged by attempting to put both fossil and modern spinicaudatans into phylogenetic context in order to begin to understand the evolutionary history of this ancient arthropodan group. Such a ‘marriage’ of modern to fossil clam shrimp taxonomy will also allow more detailed macroevolutionary studies of breeding systems utilizing the ‘choncostracan’ Spinicaudata (Astrop et al., 2012; GALLEGO et al., 2013a; Monferran et al., 2013; Stigall et al., 2013).

To reconcile this divide between modern and fossil taxonomy, we must use the set of character data common between the two: the carapace. But, in order to do that, we must answer this question: Is carapace ornamentation data useful for inferring phylogenetic relationships between different species and lineages?

Carapace ornamentation data has been used in fossil spinicaudatan taxonomy for over 150 years (e.g(Jones, 1862)). Indeed, its use with fossils has only increased since the integration of SEM imagery; virtually every paper on fossil spinicaudatans written in the last ten years has successfully used ornamentation data with SEM images (Li et al., 2009a; Gallego, 2010a; Gallego, et al., 2013a; Kozur and Weems, 2010; Li et al., 2010a;
Monferran et al., 2013; Stigall et al., 2013). Thus, for ‘conchostracan’ paleontologists, carapace ornamentation meets the criteria of a phylogenetically useful character.

But, no one has ever comprehensively examined the carapace ornamentation of modern species and integrated it with their taxonomy. Consequently, the question of whether carapace ornamentation data useful for inferring phylogenetic relationships between different species and lineages remains unanswered for living species. If modern species were to exhibit either an un-interpretable array of ornamentation patterns or if they all exhibited the same pattern, it would become much more difficult to identify fossil species. Indeed, this is precisely what the perceived wisdom about the three ‘conchostracan’ groups has told us for years — namely, that carapace features are taxonomically useless (Mattox, 1957; Tasch, 1969)—with no hard data to support their conclusion.

In order to understand the significance of carapace ornamentation in the evolution of ‘conchostracans’, we must first understand the range of variation and the mechanism(s) generating such variation. Second, we must determine if the variation we observe reflects a phylogenetic pattern or if it is generated by other factors (sexual dimorphism, ecophenotypy, taphonomy, etc.). The studies that follow are part of a multi-faceted approach to provide a better understanding of the biological origin, development, variability and evolution of the clam shrimp carapace in order to build a robust foundation for future integrated biological and paleontological work.
Methods

Scanning electron micrographs were produced using a FEI XL-30 environmental scanning electron microscope at the University of Akron. Additional SEMs were taken by the author at The Paleontological Institute (PIN), Russian Academy of Sciences and Yale University. A total of 31 living species were observed from preserved museum collections and laboratory reared populations. Intra-specific variability in carapace ornamentation was assessed by rearing multiple populations of several species in the lab and observing multiple individuals of each of these species (Cyzicus gynecia, Leptestheria compleximanus, Imnadia yeyetta, Eulimnadia ferriensis). Multiple individuals from several species (Eulimnadia ferriensis, Leptestheria compleximanus, Cyzicus gynecia) were observed in this study and ornamentation was only found to vary within individuals, that is, the same ornamentation occurs within a species but the ‘style’ of that ornamentation will be dependent on the individuals ontogeny, and possibly, pre-mortem valve injury and repair. These observations are largely contradictory to earlier assumptions that the carapace ornamentation was ‘too variable’ to be used as a taxonomic character. On the contrary, observations made in the course of this study suggest that ornamentation is largely invariant within taxa, and (depending on the lineage) may be a systematically useful tool for discriminating taxa at the familial or generic level.

For thick sections, a population of Cyzicus gynecia was reared in the laboratory following the protocol outlined in Weeks & Zucker (1999). At about five weeks,
individuals were removed from the aquaria, refrigerated for one hour to euthanize before being separated from their carapace. The carapace was separated into individual valves prior to fixation. Fixation of individual valves followed the methodology provided by (Brantner et al., 2013). Valves were placed in a 2% glutaraldehyde 0.1 M sodium cacodylate solution for 3 hours before being washed with a 0.1 M sodium cacodylate solution repeatedly every 10 minutes for 60 minutes. Valves were then moved into a 2% OsO₄ 0.1M sodium cacodylate solution for 90 minutes. After washing with deionized water, valves were stained via En Bloc stain using 2% uranyl acetate. The carapace is soaked in this solution for 30 minutes. The solution is then removed and the container is filled with enough water just to cover the surface of the carapace. This container is placed in a larger beaker of acetone, pulled into a vacuum, and then left overnight (approx 13 hrs). The next day, 100% acetone is added and subsequently removed and replaced every 20-30 minutes for five hours. An acetone/plastic mix in a 90:10 ratio is added to the carapace, covered with foil, and then placed under a hood overnight. This is followed by the addition of 100% plastic, after which specimens were then heated in an oven for 15 minutes. Afterwards, the plastic is removed and a fresh 100% plastic is added. After heating further for 15 minutes, the carapace is pulled out and left at room temperature. Carapaces are then placed in the correct molds, covered with 100% plastic, and placed into a 60 degree C oven for 48 hours. Once the fixation and embedding was completed, thick-sections were produced using a glass-knifed microtome.
Taphonomy

Decay

Desiccation experiments were performed using *Eulimnadia* sp. reared in the lab. *Eulimnadia* is a comparatively weakly cuticularized species of spinicaudatan, observations on its taphonomy are not generalizable to all species (i.e., the more robustly cuticularized genus *Cyzicus*). As specimens died naturally, their corpses were removed from the tank, and laid on their side and placed on a glass slide (proximal to the same lamp heating the tank of living *Eulimnadia* sp.).

Transport experiments

In order to test for the possible confounding effects of post-mortem transport on the ‘Conchostracan’ carapace, high energy disturbance and travel over varying distances was simulated using the extant Spinicaudatan *Cyzicus gynecia*. Individuals were euthanized by refrigeration at 4°C for 24 hours before starting the experiment. Four groups of three individuals each were used as replicates to test the effect of differing amounts of decay prior to transport. These groups were a) ‘fresh’ individuals to be subjected to transport shortly after death and three groups that were left to decay in a small amount of tank water for b) one week, c) two weeks and d) four weeks post-mortem. A small 500ml rock tumbler was used to simulate transport. The barrel of the tumbler was filled with a mixture of aquarium sand and water at a 1:2 ratio,
respectively. The circumference of the barrel was measured at 25cm and the time for the tumbler to complete one rotation was measured at 1 second, thus producing a distance transported/time simulation of approximately 90 meters per hour. Individuals were transported distances of 100m, 500m and one kilometer. Carapaces were then imaged via light microscope and SEM.

**Ontogeny, Sexual Dimorphism & Ecophenotypy**

Both laboratory reared and wild (preserved) samples were observed in this study and males and females were studied where possible. The thorough investigation of ecophenotypic morphological change was beyond the scope of this study and requires a dedicated repeated measures ecological investigation.

**Valve Injury and Repair**

In order to observe the process of valve repair after injury, young but fully grown individuals of *C. gynecia* were temporarily removed from their tank and the margin of their carapace was either ‘cut’ with dissection scissors or ‘poked’ with a dissection needle to induce injury. Trauma to the epidermal layer beneath the cuticle of the carapace was immediately apparent but seemed of little concern to individual clam shrimp. These individuals were returned to a ‘recovery tank’ and allowed to continue to
live for another week before being euthanized by refrigeration and the valves removed for observation via light microscopy and SEM (Figure 2.2.)

Results & Discussion

SEM investigation of Spinicaudatan families, along with related taxa, revealed a wide array of carapace ornamentation (Figures 2.4, 2.5 & 2.6). A common feature seen in the Spinicaudata, Laevicaudata, Cyclestheriidae and also in some Cladocera is the occurrence of a small & dense reticulate patterning on the lower layers of the procuticle (Figure 2.4). This is likely the result of how the cells in the epidermal layers are stacked. Subsequently, when the beginnings of new cuticle are produced, the laminae in contact with the epidermal layer will reflect the cellular arrangement to some degree. Interestingly, this was also suggested by Tasch in his 1969 treatise as the origin of carapace ornamentation. This arrangement also accounts for the existence of a single, underlying reticulate layer in otherwise lightly or un-ornamented taxa such as Limnadopsis occidentalis (Figure 2.4E), Eulimnadia (Figure 2.5, 2.6 & 2.7) and Imnadia yeyetta(Figure 2.4F).

In lightly mineralized taxa, such as Cycletheriidae, Eulimnadia and most Cladocera, there are very few procuticular laminae and either no or very discrete (some Cladocera) reticulae. Mineralized ‘swellings’ (like those seen in the Leptestheriidae and Cyzicidae) that result in the more elaborate ornamentation, also seen in fossil taxa, subsequently conceal or distort underlying procuticle reticulation. However, larger
secondary reticulate patterns occur in several groups, likely resulting from ornamentation originating from the intra-cuticular layer (Figure 2.2) rather than the reticulation of the underlying procuticle (see Figure 2.4A). This indicates that the reticulate patterning exhibited by lower levels of the procuticle may be visible as surface ornamentation in lightly mineralized and thinner carapaces (i.e., Limnadiidae); very few cuticular layers and a thin epicuticle are ‘draped’ across the reticulate network, often resulting in regular dish-like depressions in taxa with a thinner carapace cuticle (Fig 2.4A,D). Reticulate cuticle sculpturing is also known from some groups of insects and other Crustacea, most notably the Ostracoda, where several excellent studies have elucidated the cellular origin of such patterning (Okada, 1981; Okada, 1982).
Figure 2.4. Examples of reticulate patterning that occur in branchiopod carapaces. A) *Cyclestheria hislopi* (Cyclestherida) (Ep: epicuticle, Pr: procuticle). B) *Cyzicus moresi* (Cyzicidae) C) *Daphnia pusilla* (Daphniidae) (taken by and reproduced here with the permission of Alexey Kotov). D) *Lynceus biformis*. E) inner procuticle (Laevicaudata) (iPr) exposed via breakage in *Limnadopsis occidentalis* (Limnadiidae) (iEp: inner epicuticle). F) *Imnadia yeyetta* (Limnadiidae)
Figure 2.5. Carapace ornamentation in the Cyzicidae and Leptestheriidae. A) *Cyzicus mexicanus*. B) *Caenestheriella belfragei*. C) Injured and subsequently repaired portion of *Cyzicus gynecia*. D) *Maghrebestheria morrocana*. E) *Leptestheria compleximanus*. F) *Eocyzicus parooensis*. 
Figure 2.7. Ontogenetic changes in carapace ornamentation in various Spinicaudatan taxa. Line drawing illustrates location of sampling transect.
SEM data collected on the carapace ornamentation patterns of living spinicaudatans shows that the observed patterns are distinctive in different families and that similarity is correlated with relatedness amongst examined species (Figures 2.4, 2.5, 2.6 & 2.7).

**Range of variation**

Species belonging to Limnadiidae vary from having effaced growth lines with no discernible ornamentation (producing a smooth carapace surface) to bearing shallow, dish-shaped depressions that likely result from distortion of faint isogonal reticulation overlain with epicuticle (Figure 2.6). Species belonging to Cyzicidae often have more elaborate ornamentation, such as large, irregular reticulate patterning, deep, steep-sided punctae, deep irregular pits, or combinations of these features (Figure 2.5). Species belonging to Leptestheriidae have combinations of irregular reticulation (restricted to younger growth bands) and raised, anastomosing ridges (Figure 2.5).

Similar results were observed for the other conchostracan suborders outside of the Spinicaudata: Laevicaudata and Cyclestheriida (Figure 2.4A, D). Both of these groups exhibited either no ornamentation or isogonal reticulation. Patterns observed in *Cyclestheria hislopi* suggest that ornamentation patterns are stereotyped within this particular species across multiple continents (North America, Asia, and Australia). This matches observations of the patterns in lab-reared specimens of *Cyzicus gynecia*, *Leptestheria compleximanus* and *Eulimnadia ferriensis*—ornamentation patterns do not vary significantly within modern species.

**Taphonomy**
Important contributions to arthropod taphonomy have been made by other workers (Allison, 1986a; Briggs, 1995; Orr and Briggs, 1999a; Orr et al., 2008). However, because of differences between different arthropod taxa, it is useful to consider results only from conchostracans rather than generalizing from other arthropod taxa. In particular, few workers have closely examined the patterns in decay and disarticulation in weakly or non-mineralized arthropods (Orr et al., 1998; Orr and Briggs, 1999b; Briggs, 2003; Orr et al., 2008).

**Decay**

Excellent treatments in Orr & Briggs (1999) and Orr et al. (2008) contain observations on the decay and disarticulation of conchostracans (model taxon: *Eulimnadia texana*). They observed that the external cuticle (exoskeleton) was the most resistant tissue of the animal, but there was considerable variation in exactly how resistant different parts of the exoskeleton were to decay. We made similar observations during our decay experiments (Figure 2.8). The carapace was the most resistant part of the exoskeleton, but other parts, such as the second antennae, mandibles, gut, telson and caudal furca, also showed significant resistance to decay. The robust adductor muscle connecting the valves effectively prevents the valves from assuming a ‘butterfly’ posture except after near-complete decay of the viscera and some degree of turbulence in the water. As the more labile internal tissues decayed away, the more resistant portions of the exoskeleton became prone to disarticulation upon disturbance. After 30 days, virtually all that was left was the exoskeleton.
**Transport**

The carapace of *C. gynecia* proved extremely resistant to transportation and decay. No changes in surface ornamentation were observed across transported distances or time decayed prior to transport. In fact, specimens decayed for four weeks and transported one kilometer even retained setae at the margins of distal growth bands. The softer integument of the Spinicaudatan body began to dis-articulate after one week and separated from the carapace prior to transportation after four weeks; only the ‘fresh’ specimens remained completely intact over all distances (Figure 2.9). All specimens decayed for one week or longer experienced complete destruction of the soft body integument after 100 meters of transport. From these results it may be concluded that the carapace not only maintains its structural integrity, but also the integrity of its ornamentation patterns post-mortem and even during transport (Astrop and Hegna, 2011).

**Ontogeny, Sexual dimorphism & Ecophenotypy**

Ontogenic variation happens in all species. However, the advantage here is that spinicaudatans and cyclestheriians preserve that record of ontogenetic change in their carapace, which can subsequently be observed and accounted for. What we observe are taxonomically specific ornamental ‘trajectories’ (Figure 2.7) across the carapace though ontogeny.

Dimorphism in ornamentation was not observed for any of the species examined in this study. Slight variation in shape and size is alluded to by some authors but were
likely referring to shape dimorphism, not ornamentation dimorphism. Ecophenotypy has been alluded to by other authors (Tassi et al., 2013) without evidence.
Figure 2.8. Specimens of Cyzicus gynecia decayed for one week (A) and four weeks (B) prior to transport experiment.

Figure 2.9. Examples of C. gynecia decayed prior to transport (A) and subsequently subjected to transport simulation (B) decayed in dry environment to simulate desiccation, minor damage to peripheral growth ring. C) decayed in aqueous environment – no discernible damage.
**Injuries, Teratologies & Epibionts**

Living species of ‘conchostracans’ can develop a range of conditions, masking the ornamentation of the shell, ranging from epizoans to carapace injuries (Tasch, 1961; TIA, pers. obs.). The preservation potential of epizoans is low, but there is circumstantial evidence for them in the fossil record and were observed (and subsequently removed) in this study when preparing *Limnadopsis occidentalis*.

Injuries are less ambiguous. Injuries exhibiting some degree of re-healing are not uncommon, and have been observed in material from China, Argentina (Figure 2.10D) and Africa in particular (fossil valves exhibiting injury and repair were also noted by Tasch, 1961). Some healed wounds in the carapace are located on the ventral margin, consistent with tears that could happen to females from the males’ claspers. The manner of healing that spinicaudatans display is closely related to their mode of growth. The margin of the carapace acts as a template for the new layer of carapace cuticle, with growth occurring everywhere along the margin of the carapace. When an injury occurs, like the cuts or a tears inflicted upon individuals in the course of this study, the margins of the injury grow like the other margins of the carapace during the next molt (Figure 2.10A,B,C). Small injuries (less than a growth line in length) can be easily stitched together by this growth within one molt cycle (this is
Figure 2.10. A) Light microscopy and B,C) SEM images of repair in a damaged valve of *C. gynecia*. Note the gradual resumption of the original carapace shape, the effect of the injury becomes less severe with each molt, but a clear record of the recovery is retained. The ornamentation on the growth band at the site of injury is underdeveloped and deformed directional to the direction of growth. D) Valve

Figure 2.11. Inverted carapace teratology exhibited by few specimens of *Imnadia yeyetta* reared in the lab.
what is typically observed in the fossil record). Larger tears may never stitch together, and instead the margins of the injury may grow to form overlapping lobes (observed in the lab).

Whatever the severity of the injury, it is recorded in the ornamentation of subsequent growth bands. The cause of this stems from the way that arthropods grow. When arthropods undergo ecdysis, they resorb the lower layers of their cuticle, and create a new layer underneath—essentially using the old cuticle as a template. When the old cuticle is cast off (as in most arthropods), the new layer expands before hardening (Richards, 1958). The process is the same for spinicaudatans, except for the fact that they do not cast off the old carapace. The injured area is a template for subsequent growth. By studying injury and repair in ‘conchostracan’ carapaces, we can better understand the implications of the mode of growth and the significance of dorsal ornamentation patterns.

Is wounding a confounding factor for recognizing ornamentation in the fossil record? Most injuries occur on only one side of the shell, have the superficial appearance of a tear or rip, and exhibit a strong asymmetry. These factors make it unlikely that an injury could be conflated with a true ornamentation pattern in a large sample.

Living spinicaudatans will rarely develop a carapace deformity where each valve of the carapace develops concave-outward (Figure 2.11). This deformity does not impact the carapace ornamentation, only the shape of the carapace. This type of
deformity lacks the ‘nesting’ seen in desiccated valves and has never been observed in the fossil record.

Another interesting observation, seen on all five purposefully injured specimens of *C. gynecia*, was that upon injury, a narrow growth ring occurs immediately after the physical trauma was inflicted (Figure 2.12A). This may be a stress response, as laboratory-reared samples also molt soon after removal from their parent tank.

Of further interest are similar patterns of differential growth ring width and coloration in the fossil record (Figure 2.12B,C,D). Although the differential coloring of growth bands in fossils may represent differences in the biogenic composition of growth rings in the original animal, they do not record any original elemental data.

This difference in the mode of regular molt cycle as a response to injury may lead to an ‘immature,’ less mineralized and developed growth ring being left in the middle of an injured and repaired carapace. The lack of visible injury, but presence of differential preservational color of the fossil taxa in Figure 2.12, could plausibly be the result of this ‘stress-molting’ in response to fluctuating/unfavorable environmental conditions (similar to those experienced when lab-reared specimens are removed from their tank). If so, this would make this phenomena of palaeoecological significance and worthy of further palaeobiological study.

Interestingly, connections between the ‘finger-nail-clipping’-like exuviae that result from the shed of the outer layer of the last growth ring that were often seen floating on top of aquaria containing live populations in the laboratory were also
identified as fossils in collections from the UK, Argentina and China (Figure 2.13). This is valuable palaeobiological evidence that a fossil population had been stable enough to molt repeatedly in-situ and should be of note to future studies involving fossil Spinicaudata.
Figure 2.12. Differential coloration of growth bands in A) injured *C. gynecia* B) *Halysestheria yui* C) *Estheria midedendorfi* and D) *Estheria forbesi*

Figure 2.13. A) molting *Cyzicus tetracerus* (courtesy of jean-françois cart), arrow indicates opalescent shed of outer layer of last growth band which is more mineralized than the remainder of the exuviae. B) Stained remanents of decayed exuviae in *Eulimnadia* sp., only the outer cuticle of the last growth band remains. Preserved growth band molts in C) *Eosolimnadiopsis santacruensis* & D) *Estheria midedendorfi*
Conclusions

The distribution of generalized ornamentations observed in this study across the ‘Conchostraca’ can be seen in Figure 2.14.

The occurrence of isogonal reticulation in members of the Cladocera (Figure 2.4C), Spinicaudata (Figure 2.4E,F), Laevicaudata (Figure 2.4D) and Cyclestheriida (Figure 2.4A) suggests that this may be a basal feature for the group. Indeed, the isogonal reticulation observed seems to originate from the arrangement of cells in the epidermal layer and likely represents a ‘first-step’ in cuticle mineralization/sclerotization. Interestingly, the enigmatic Early Devonian Ulugekemia exhibits isogonal reticulation across the carapace (Fig 2.10D, also see (Gallego and Melchor, 2000)).

Elaboration of ornamentation occurs most frequently within the Spinicaudata (although a fair range of ornamentation types exist in the cladocera, their fossil record is relatively poor and are not the focus of this assessment). The degree of elaboration appears to be linked to the robustness of the carapace. Those taxa that have thinner, transparent and likely less mineralized carapaces, such as the Cyclestheriidae (2.4A) and the Spinicaudatan family Limnadiidae, have no discernible ornamentation, isogonal reticulation or a faint, low-relief isogonal reticulation obscured by the epicuticle. The Limnadiidae are also recognizable by gross carapace morphological features, such as a large, sub-circular umbonal region, relatively few growth lines, no marginal setae on growth bands and an angular antero-dorsal margin. Subsequently, fossil forms that also share both carapace morphology (large larval shell, limnadiiform carapace outline,
proportionally wide growth bands) and ornamentation features include the genera *Megasitum* from the Devonian of Siberia (Figure 2.15A,B), *Yunmenglimnadia* and *Perilimnadia* from the Palaeogene of China (Chen and Shen, 1981) and *Falsisca* from the Permian of Brazil (Ferreira-Oliveira and Rohn, 2008). These taxa (fossil and extant) are all members of the superfamily Vertexioidea (Zhang et al., 1976) and, due to shared carapace morphology and reduced/absent ornamentation, it seems likely that modern Limnadiids arose from a Vertexiid ancestor.

The Leptestheriidae are a systematically transient group, and despite being erected as a family based on morphological features, they consistently emerge within the Cyzicidae in molecular analyses (e.g., (Schwentner et al., 2009)). The presence of large, irregular reticulation in the umbonal region of the Leptestheriid *Maghrebestheria* is similar to the irregular reticulation seen in many Cyzicid species. Therefore this type of ornamentation is likely a shared taxonomic feature of these two groups. The Leptestheriidae differ from the Cyzicidae in gross carapace morphology (elongate carapace with marginal setae and slight recurvature of the dorsal carapace margin) and in their dendritic, anastomising ridge-type ornamentation. These anastomising ridges where observed in detail via TEM by (Rieder et al., 1984) and found to be ‘inflated’ regions between the epicuticle and procuticle composed of a ‘granular’ material (likely corresponding to the intra-cuticular space observed in this study; Figure 2.2). Due to the position and morphology of this feature it is possible that these features may represent mineralization resulting in ornamentation that is not seen in taxa with a thinner, less robust carapace.
The phylogenetic placement of *Eocyzicus* as sister to *Leptestheria* in multiple phylogenetic analyses (Schwentner et al., 2009, Weeks et al., 2013) is a relationship supported by ornamentation patterns observed in this analysis in that in addition to small, uniformly placed punctae, *Eocyzicus* exhibits wide, transverse anastomizing ridges (somewhat similar to the condition seen in the Leptestheriidae) that overlay the punctae and become more pronounced distally (Figure 2.7).
Figure 2.14. Stylized ornamentation patterns mapped onto a simple phylogeny (sensu Schwentner et al., 2009, Weeks et al., 2013). *This diagram by no means attempts to encompass all the variation observed in ornamentation*, but rather highlights particular ornamental trends specific to taxa. Those taxa with two ornamental types may exhibit either or a combination of both.
Figure 2.15. A,B) The Upper Permian vertexiid *Megasitum harmonicum* from Kazakhstan; note the limnadiid outline, large larval shell, lack of discernible ornamentation (wrinkles match desiccation patterns observed in this study) and few, wide growth bands; carapace characteristics similar to many living representatives of the superfamily Lioesthereioidea in the extant Limnadiidae. C,D) *Ulugekemia borisi* from the Middle Devonian of Siberia; note similarity of isogonal reticulation to that seen in modern *Lynceus* and *Cylcestheria*. E,F) Ornamentation of the Jurassic eosestheriid *Carapacestheria disgragaris*; note similarity to modern *Cyziciidae* taxa.
As a result, it would be reasonable to assume that these two groups are, in fact, sister taxa and that *Eocyzicus* either requires reassignment to the Leptestheriidae or a dedicated taxonomic review in order to assess the applicability of a new familial rank for taxa assignable to *Eocyzicus*. Ornamentation in both the Leptestheriidae and *Eocyzicus* observed in this study lead the author to agree with the assumption of (Zhang et al., 1976): the Leptestheriidae (and in my opinion, *Eocyzicus*) likely originated from a Loxomegaglyptid ancestor (within the Eoestherioidea;(Zhang et al., 1976)), as fossil members of this clade also exhibit combinations of irregular reticulation and minute punctae overlain by anastomising ridges, striations or lirae (Figures 2.7, 2.15) [e.g., *Paraleptestheria menglaensis*, (Chen and Shen, 1980) *Tremembeglypta saadi* (Gallego and Mesquita, 2000), *Shizhuestheria truncata* (Li et al., 2009a).

In the Cyzicidae, irregular reticulation and elaborations of this reticulation are dominant (Figures 2.5, 2.7, 2.15). It is likely this irregular reticulation is of a shared developmental origin to similar ornamentation seen in Leptestheriidae (Figure 2.5E) and other fossil taxa and may be indicative of affinities to a larger ‘Cyzicid’ clade, likely the Euestheriidae that originates in the superfamily Eosetherioidea (Zhang et al., 1976)). ‘Inflammation’ of the irregular reticulation (similar to that seen in the dendritic lirae of *L.compleximanus* (Rieder et al., 1984)) may result in deep, irregular pits, such as those seen in *Caenestheria belfragei*, or wide depressions, as seen in the distal growth bands of *Cyzicus gynecia.*
Transitions between irregular reticulate and liarate ornamentation types are often observed in fossil taxa, and as such suggests possible shared ornamentation characteristics between the Leptestheriidae, Cyzicidae and fossil families such as Eosestheriidae (Chen et al., 2007; Gallego, 2010a), suggesting a possible phylogenetic relationship (Figure 2.5E, 2.14).

This study shows extant orders and families of ‘conchostraca’ may be identified without the use of soft parts by referencing both gross carapace morphology and ornamentation. Specific ornamentation complexes appear to be useful in identifying taxa at the generic level and in the groups with more robust, mineralized carapaces, may be useful at the generic and species level. However, such detail is beyond the scope of this investigation. This would be an excellent area for further study.

The current study provides the first comprehensive survey of extant ‘conchostracan’ carapace ornamentation in an effort to provide biological context to an often utilized but poorly understood palaeontological character. It is clear from observations made herein that not only do informative, inter-specific differences in ornamentation occur, but that such variation is useful for identifying fossil taxa at different taxonomic levels. The current findings additionally inform inferences about the origins of extant groups, something that has, until now, not been possible. Previous claims that there is ‘too much variation’ may be discarded in light of dedicated study and the diversity of ornamentation warrants further, integrated studies by both branchiopod palaeontologists and neontologists.
CHAPTER III

QUANTIFYING THE COMPOSITIONAL & MATERIAL PROPERTIES OF THE SPINICAUDATAN CARAPACE – INTEGRATING MATERIAL SCIENCE AND EXPERIMENTAL TAPHONOMY

This section of this chapter represents the output of a collaborative project with Alyssa Stark (UA Integrated Bioscience), Vassav Sahni (UA Polymer Science) and Todd Blackledge (UA Biology) that is currently being prepared as manuscript for submission.

Overview

Spinicaudatan ‘clam shrimp’ are freshwater branchiopod crustaceans that occur worldwide in lakes and temporary pools. Spinicaudata are easily recognized due to their weakly mineralized bivalved carapace. This carapace is unique among arthropods because it is subject to only partial molting. During ecdysis (molting), the outer surface of the carapace is not shed, resulting in the organisms' retention of a complete ontogenetic record, comprising distinct growth-rings representing each molt. Along with an unusual chemical composition, this results in the clam shrimp presenting an interesting biological material not seen anywhere else: a multi-laminar calcium-phosphate-chitin complex that survives numerous destructive taphonomic processes
(including transport, decay, compaction, and desiccation) to become the almost exclusive component of the clam shrimp within the fossil record. This 380 million year fossil record is poorly understood and represented almost exclusively by the cuticle of the carapace. Understanding the material properties and chemical composition of this structure may not only aid in a better understanding of the evolutionary history of this group but also facilitate efforts to develop novel materials that retain functional properties even in unfavourable aquatic conditions. Therefore, this study aims to provide quantitative information about the composition and mechanics of this unique and interesting biological material.

Introduction

The Arthropoda are the most diverse, successful and adaptable animals on Earth. Originating in the Cambrian some 520 million years ago (Legg et al., 2012b) arthropods rose to be essential components of global ecosystems and are now ubiquitous in aqueous, aerial and terrestrial environments. The quality of the fossil record for arthropods ranges from exquisitely preserved, amber-encased specimens (e.g. (Barden and Grimaldi, 2013)) to isolated, partial elements of the exoskeleton. Factors influencing the entrance of arthropod remains into the fossil record are of key importance to correctly interpreting the evolutionary history of the group. Understanding the biotic and abiotic parameters that allow fossilization is the primary role of the science of taphonomy, and palaeobiologists have long experimented with organismal remains to
understand how processes such as decay, transport, predation and desiccation may ultimately shape the preservational record of life on earth via the process of fossilization (e.g. (Allison, 1986b; Briggs, 1995; Behrensmeyer et al., 2000; Krause Jr et al., 2011)).

The arthropod cuticle is a key adaptation that provides both structural support and a barrier between an organism’s internal biology and the harsh surrounding environment. This cuticle comprises several layers all containing the polysaccharide chitin: a thin outer epicuticle, a mineralized exocuticle and a less rigid endocuticle (Gupta, 2011b). The cuticle is almost exclusively the only component of arthropods represented in the fossil record. Hardening of pure forms of chitin via sclerotization occurs in many arthropods, but chitin commonly occurs as a composite material, which is the case with most crustacea. In crustacea, chitin is combined with calcium carbonate into laminae in a unique helical pattern (Bouligand structure, see Cheng et al., 2008), providing great rigidity for protection.

‘Clam shrimp’ (Order Spinicaudata) are seemingly inconspicuous crustaceans that inhabit temporary freshwater habitats world-wide and are easily recognized as adults. This carapace is unique among invertebrates in that molts are partially retained during ecdysis. The outer surface of the carapace is retained after each molt causing distinctive growth lines that represent discrete ontogenetic events. This results in the carapace forming a ‘layered’ structure, superficially similar to that seen in molluscan bivalves. Such retention is in contrast to the complete discarding of exuviae seen in more familiar examples such as crabs, lobsters and other members of the Ecdysozoa.
Additionally, the chemical composition of the clam shrimp carapace itself is unique: rather than forming a chitin-calcium complex, the carapace of clam shrimp is believed to be primarily chitin-phosphate (Stigall and Hartman, 2008), a product of their freshwater environment which is calcium deficient. It is unclear, however, whether there are differences in the level of mineralization of the carapace between species and if these differences correlate with differences in the material properties. By extension, such differences (if they exist) could modify the likelihood of being preserved in the fossil record.

In comparison, the arthropod cuticle is known to play a significant role in the preservation potential of these invertebrates (Edgecombe and Legg, 2013) and is the most likely reason for the excellent fossil record of the phylum; we would expect the same to be true for the clam shrimp. Clam shrimp are of particular interest in palaeobiological and geological studies because they have an excellent fossil record extending back to the Devonian (380mya) and their natural ecology confines them to freshwater, making them useful as an indicator of historical freshwater environments. Coupled with their accretional carapace growth, they may also record information about environmental stresses, fluctuations and seasonality in successive growth ‘rings’. More recently, several studies have explored the possibility of carapace morphology reflecting dimorphism within fossil clam shrimp populations, in turn allowing the diagnoses of particular sexual systems in geologic time (Astrop et al., 2012; Gallego et al., 2013b; Monferran et al., 2013; Stigall et al., 2013). Understanding taphonomic influences of
preservation within different lineages of clam shrimp is of great importance to understanding the reliability of data used for such studies.

The lack of mechanical and chemical characterization of this unique material results in limited use of this arthropod in palaeobiological studies. We hypothesize that family-level differences in the composition, and thus mechanical properties of the clam shrimp carapace, affect susceptibility to taphonomic processes and by extension are an important source of preservational bias that need to be considered when utilizing the fossil record of this order. For example, it is a reasonable assumption that weaker, less mineralized carapaces may break with the heavy mechanical forces associated with the fossilization process, whereas those that are ‘tougher’ may be more resilient and therefore better represented in this group’s long history. To further illustrate this, when comparing relative numbers of fossils collected we can see that some groups within this order seem to "appear" and "disappear" throughout evolutionary time (see Limnadiidae in Figure 3.2). This observation is highly suspicious and may have more to do with the preservation of these small organisms and the ability of their mineralized shells to resist mechanical forces during the fossilization process, rather than completely independent evolutionary events.

To assess this possibility of "preservational bias," taphonomic data derived from carapaces of living clam shrimp must be collected and compared. Comparisons of living materials to fossilized materials is rarely done, yet this our only chance to assess how differences in material properties can influence the probabilities of fossilization possibly
resulting in highly selective and intrinsically biased lineage representation in the fossil
record. The best candidates for such an extant/extinct comparisons are species of "living
fossils" that appear relatively unmodified in fossil and modern species.

In this study, we broadly sampled the order Spinicaudata by choosing representatives of
the three extant families (Cyzicidae, Leptestheriidae and the Limnadiidae). Both the
Leptestheriid and Limnadiid species used in this study show varying degrees of
dimorphism in carapace shape due to their sex system (dioecy for Leptestheria and
either hermaphroditism, androdioecy or dioecy for the Limnadiidae); however the
Cyzicid species used is exclusively hermaphroditic therefore exhibiting strict
monomorphism. We measured material properties, such as force to fracture, strength,
and work to fracture in each species as well as tested for a difference in dimorphs for L.
*compleximanus*. We also quantified the chemical components of the carapace,
hypothesizing that those richer in both calcium and phosphate (i.e., more mineralized)
would be stronger and more resistant to fracture.
Figure 3.1. Clam shrimp representatives. A) *Leptestheria compleximanus*. B) *Eulimnadia ferriensis*. C) *Cyzicus gynecia*. Scale bar equals 2mm.

Figure 3.2. Generic diversity of Branchiopod ‘clam shrimp’ over geologic time. Solid lines = extant, dashed = extinct. Note the periodic disappearance of Limnadiidae from the record, the persistence of the Cyzicidae and the recent occurrence of Leptestheriidae.
The evolutionary success and ecological persistence of the clam shrimp is likely tied to this unique biological material; however it is unclear how this material behaves under loading and what this could mean for the preservation potential of the group. We aim to resolve this discrepancy by assessing key material properties of the clam shrimp carapace, as well as characterizing its chemical components, providing useful information for both biomaterial design and application as well as inform further zoological and palaeobiological research in this interesting group of crustaceans.

**Methods**

Three species of clam shrimp were used for compositional assessment and materials testing. These three species were chosen to represent the three extant families within the clam shrimp (*Cyzicus gynecia, Leptestheria compleximanus, Eulimnadia ferriensis*; Figure 3.1). All populations were reared in the laboratory following the protocol outlined in (Weeks and Zucker, 1999). Live samples were refrigerated for sedation and subsequently removed from their carapaces. Valves were stored at 4°C for no longer than three days while trials where in progress. Samples used for elemental assessment using energy dispersive x-ray spectrography (EDAX) were immediately removed from their carapaces and quickly dried via absorption before embedding in epoxy.

EDAX was performed using a Princeton Gamma Tech energy dispersive X-ray spectrometry (EDS) system within an FEI XL-30 environmental scanning electron
microscope with a Ge detector. Uncoated samples were embedded in epoxy resin on a glass slide before being ground in order to expose a transverse section through the carapace. Samples were washed with alcohol and dried before being placed in the SEM chamber at 90 ± 2 kPa. EDAX analyses were performed using a voltage of 25 keV. Two individuals of *L. compleximanus*, *C. gynecia* and one of *E. ferriensis* were used.

Elemental composition of polished sample surfaces was quantified based on number of ‘counts’: hits of particular frequencies associated with particular elements on the x-ray detector. Elemental proportions were then calculated relative to highest count. ZAF is an intensity correction technique that aids in accuracy of quantification where Z accounts for the ‘atomic number effect’ which describes the premature loss of an electron prior to production of x-rays; A accounts for the ‘Absorption effect’, which is the probability that an x-ray will transfer energy to another electron while exiting the sample; and F accounts for fluorescence: if/when an x-ray interacts with another atom, it is left in the excited state emitting its own x-rays. This ‘secondary fluorescence’ was recognized and accounted for. Quantitative information was obtained using ZAF Quantification (Standardless) via the EDAX Genesis software package V4.52.

We measured the material properties of individual clam shrimp shells using a similar methodology to a punch test or a punch-and-die test (Aranwela et al., 1999; Choong et al., 1992; Sanson et al., 2001). The punch test measures values such as punch strength and work to punch (detailed below in Table 3.1). In a punch test, a sample is loaded so that the lowering of the ‘punch’ (in this case a 0.3 mm wide glass rod) will
result a single pressure point normal to the sample. The punch is lowered at a controlled and consistent rate until the sample fractures (also known as ‘failure’). To avoid friction associated with other materials tests (i.e., shear test) punches are small enough to fit through the die (the ‘die’ in this case is a stainless steel nut with a 2.5mm aperture), which holds the sample, without touching the die as it is lowered (see Figure 3.3 for a schematic of the experimental set-up).

Due to the inherent challenges of both biological materials testing and punch tests in particular, some suggest this test method should not be adopted (Aranwela et al., 1999; Edwards et al., 2000; Vincent, 1992). However the Spinicaudatan carapace cannot be ‘worked’ to specified parameters like larger, more robust biological materials (bone, wood, etc.) and were constrained by the limits of a small, thin and fragile sample.

As the Spinicaudata are aquatic, their shells are highly sensitive to desiccation. Deformation often occurred (curling in thinner samples) and when tested, material properties were qualitatively different after only a few minutes in air. Our use of the punch test addressed these initial problems as samples can be quickly mounted as whole shells and tested before extensive drying or damage occurs.

We also chose the punch test as it investigates material and structural properties (Evans and Sanson, 2005), both of which are highly relevant for considering how taphonomic processes affect the quality of an organism’s fossil record.

Finally, the curved nature of our samples provided additional complexity which seemed best controlled by a punch test (see methodology below). Several material
properties are commonly reported when using a punch test and these are outlined in Table 3.1 (Edwards et al., 2000; Evans and Sanson, 2005; Sanson et al., 2001). Force and displacement are measures used to calculate specific material properties. For example in Figure 3.4 a force-displacement curve is shown where the maximum force and maximum displacement is circled.
Table 3.1: Measured and calculated mechanical properties for the punch test, adapted from (Edwards et al., 2000; Evans and Sanson, 2005; Sanson et al., 2001). F is the maximum force measured during testing, T is the thickness of the sample, A is the area of punch and D is the displacement of the punch mounted in the moving crosshead of the test machine.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Force to Punch (N)</th>
<th>Toughness (J)</th>
<th>Punch Strength (N m-2)</th>
<th>Specific Punch Strength (N m-2 m-1)</th>
<th>Work to Punch (J m-2)</th>
<th>Specific Work to Punch (J m-2 m-1)</th>
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<tbody>
<tr>
<td>Calculation</td>
<td>Maximum force recorded prior to fracture</td>
<td>FXD Area under force-displacement curve</td>
<td>F/A (F/A)/T or (F/A)XD/T or W/T</td>
<td>(F/A)XD or R/A or S/T</td>
<td>Absolute work done to punch through sample per unit thickness</td>
<td></td>
</tr>
<tr>
<td>Definition</td>
<td>Raw force to fracture sample</td>
<td>Resistance to crack propagation</td>
<td>Strength of sample at point of testing</td>
<td>Specific work done to punch through sample per unit thickness</td>
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<td></td>
</tr>
</tbody>
</table>

Abbreviations: F = Force; R = Roughness; S = Specific; SS = Specific Strength; W = Work; SW = Specific Work.
The area under the force-displacement curve (grey) is then reported as general ‘toughness’. Actual toughness is not easily measured with biological materials (Freeman and Lemen, 2007), as terms such as toughness, strength, and elasticity have precise parameters in a materials science context that may not be intuitive in a colloquial context. Using these measurements and measured values of the sample or punch dimensions, we were able to calculate the listed material property values in each of the three species studied (see representative force-displacement curves for each species in Figure 3.6).

In this experiment a small punch area (A = 0.03mm$^2$) and large punch and die clearance (1.15mm) was used (contrary to the small clearance suggested by Aranwela et al., 1999). The small area and large clearance was used primarily due to the curved nature of our samples. A large punch would have been more strongly affected by the curvature of the shells (which is not consistent across species and is not easy to measure due to limitations in time to desiccate and fragility of the samples). By selecting a small punch the effect of curvature was minimized, as was heterogeneity of the sampling site.

Ten valves (shells) of *C. gynecia*, *L. compleximanus* and *E. ferriensis* were used for materials testing. The body of each clam shrimp was removed from the carapace which was subsequently separated along the dorsal hinge to provide two valves. However not all valves were used due to premature damage during dissection or material testing. Therefore, of the ten valves for each species, six different individuals contributed valves to the *C. gynecia* sample group and eight different individuals contributed to the *E.*
*ferriensis* sample group. Fourteen valves were used in the dioecious (male/female) species *L. compleximanus*. Of the fourteen valves from *L. compleximanus*, six valves from three different females and eight valves from five different males were used. Immediately before testing, sample valves were removed from vials filled with water and blotted dry to remove excess water. Samples that had been removed from water for over five minutes were not used in the data analysis due to clear structural changes observed in the valve (i.e., curling) and subsequent inflated material property values.

Samples were then mounted on a Nano Bionix tensile tester (Agilent Technology) and positioned for the punch test. In order to load the sample to failure at the punch site, we suspended the samples on a nylon lock nut (#6-32) with a 2.5mm internal diameter. Ethyl cyanoacrylate glue ("superglue") was brushed lightly around the top rim of the nut and allowed to cure slightly to avoid wicking of glue onto the sample (which was observed in earlier trials of the experiment). After curing, the sample was placed on the glued edge of the nut and pressed lightly into place along the circumference of the nut. All samples were larger than the nut diameter to ensure consistent attachment of the sample to the testing apparatus and to control for variations in size of the individual. Although there was some size variation within and across species, the standard nut diameter controlled for variations in shell size by limiting the sample testing area to 4.91mm² for all samples. Prior to removing the sample valve, the base of the nut was glued firmly to a standard push pin mounted in the bottom grip of the Nano Bionix tensile tester (see Figure 3.4 for the pin-shell set-up). This grip is attached to a force sensor with a force and displacement resolution of 1-2µN and 1µm respectively. In the
top movable crosshead grip, the glass rod was positioned central to the sample (Figure 3.4). The top crosshead apparatus with the fixed glass rod was then lowered at a fixed speed of 1.00E-02 µm/per second. As the rod contacted and pressed the sample, displacement occurred and force was recorded using Testworks 4.0 software (MTS Corp.).

Samples were punctured to failure. Force and displacement values were used for data analysis. In early tests, the force-displacement curve showed a distinctive ‘jump’ in values that we associated with the glass rod sliding down the inside of the concave shell. While the rod was positioned above the most concave portion of the shell, it was not possible to confirm ideal placement of the rod by eye alone. We determined that an aberrantly fast change in force indicated sliding of the rod down the concave wall of the sample which represents incorrect placement of the rod and all samples that had this "jump" in force were removed from analysis. Jumps ranged from about 0.6mN to about 3.5mN. This allowed us to control for the puncture location in all samples as force curves that show a smooth displacement, without jumps, signified that the rod was in the most concave portion of the shell in all samples used for analysis.

Total area under the force-displacement curve was integrated using a sigmoid function to fit the data and is reported as "toughness". An example of the fitting function overlaid on the raw data values is shown in Figure 3.4. Due to the fragility of the samples and rapid rate of desiccation, we were unable to measure thickness of each sample prior to testing. In order to estimate sample thickness we averaged measured
thickness of ten additional valves from six individuals from *E. ferriensis* and five individuals from *C. gynecia*. Samples for *L. compleximanus* were more limited and we were unable to measure a standard thickness value for this group, therefore properties normalized by thickness could only be estimated for *L. compleximanus*. All ten valves for each group (*E. ferriensis* and *C. gynecia*) were harvested on a similar timescale as those used for material analysis and were separated and refrigerated in a similar manner before thickness measurements were completed.
Figure 3.3. (Left) Schematic of the experimental set-up showing the clam shrimp shell suspended across a nylon lock nut. A glass rod was attached to a movable cross head grip which was lowered at a speed of 1.00E-02 µm/per second until it punctured the shell to failure. The resulting load on the sample prior to failure was recorded by the bottom force sensor. To allow for complete puncture of the shell, it was positioned and glued to lie across a nylon lock nut. The lock nut was attached to the bottom force sensor grip by a standard push pin. (Right) Photograph of one carapace sample mounted on the pin-nut testing stage.

Figure 3.4. Example fitted curve. Data were fit using a sigmoid function and area under the fitted function (grey) was used to determine material 'toughness' (Force X Displacement). Black cross bars represent the fit and dots are the measured data points. The point of maximum extension and maximum load is denoted with a circle.
Statistical Analysis

Force and displacement data were analyzed using Igor Pro software version 6.2.2.2 (WaveMetrics, Inc.). An analysis of variance (ANOVA) and a Tukey HSD post hoc test of all species combinations for each measurement and material property were performed. We also performed a student’s t-test to test for a difference between sexes in *L. compleximanus*. Statistical analyses were performed with JMP version 10 (SAS Institute Inc.). All errors are reported as mean ± 1 s.e.m.

Results

Results of energy dispersive x-ray spectrographic analysis of all three taxa are shown in Figure 3.5. Regions of elemental signal are clearly localized to the thin section of the clam shrimp carapace (as indicated by color). These results confirm that the clam shrimp carapace includes a calcium-phosphate complex (Ca, P) that most likely represents mineralization of the chitinous component (C, O) of the cuticle. Trace amounts of zinc, sulfur and other elements also occurred in very low quantities. A weak signal for silicon was also reported but this is likely an artifact of the abrasive used in the preparation of resin-embedded specimens. Only relevant elemental percentages are reported in Table 3.2. The percentage of calcium and oxygen seen in the EDAX analysis (which corresponds to the chitinous component of the carapace), is relatively similar in all three species. Quantitative differences are clear, however, in the mineralized component of the carapace where *C. gynecia* has a relatively high calcium and
phosphate percentage, and both *E. feriensis* and *L. compleximanus* appear to have a considerably less mineralized carapace (lower calcium/phosphate percentage; Table 3.2). Interestingly, the lowest calcium/phosphate signal comes from *E. feriensis* which has a sodium component (the only one observed) that is equivalent in magnitude to the calcium-phosphate signal in *L. compleximanus* (about 3-4%). Further investigation using multiple specimens from this species and contemporaneous taxa within the family Limnadiidae is required to deduce whether or not this is a particular anomaly in this case or a feature of the species or clade.
Table 3.2. Elemental composition of six individual Spinicaudatan carapace valves. Two specimens for *L. compleximanus* and *C. gynecia* and one specimen for *E. ferriensis* were used to investigate intra-specific variation.

<table>
<thead>
<tr>
<th>Species</th>
<th>C %</th>
<th>O %</th>
<th>Na %</th>
<th>P %</th>
<th>Ca %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leptestheria compleximanus</em></td>
<td>67.6</td>
<td>27.52</td>
<td>0</td>
<td>1.94</td>
<td>2.94</td>
</tr>
<tr>
<td><em>Leptestheria compleximanus</em></td>
<td>78.87</td>
<td>16.49</td>
<td>0</td>
<td>1.56</td>
<td>3.09</td>
</tr>
<tr>
<td><em>Cyzicus gynecia</em></td>
<td>65.6</td>
<td>17.45</td>
<td>0</td>
<td>5.34</td>
<td>11.62</td>
</tr>
<tr>
<td><em>Cyzicus gynecia</em></td>
<td>54.38</td>
<td>24.24</td>
<td>0</td>
<td>7.35</td>
<td>14.03</td>
</tr>
<tr>
<td><em>Eulimnadia ferriensis</em></td>
<td>54.30</td>
<td>29.16</td>
<td>3.15</td>
<td>0.23</td>
<td>1.24</td>
</tr>
</tbody>
</table>
Materials Testing

The overall ANOVA models, which compare all three species in each of the measured material properties, show that there is a significant difference between the three groups in all of our measured material properties (Table 3.3). This result is also reflected in the “toughness” force/displacement curves for the species tested. Specifically, when testing each species pairing, *L. compleximanus* was statistically lower in all recorded measurements than both *C. gynecia* and *E. ferriensis* in all measured properties. *C. gynecia* and *E. ferriensis* did not differ significantly in any of the measured properties except when standardized by thickness of the sample (SS and SW), which is not surprising as the punch test is strongly dependent on sample thickness (Choong et al., 1992) meaning that both have achieved similar material properties of the carapace via different methods and that thickening of the cuticle is only one route to a ‘robust’ carapace. These results are complicated by the measurement of sample thickness. This was done on a subset of samples which were not used in testing due to the fragility of the samples and their ability to desiccate rapidly. This was also done for the whole valve and not for the individual punch site (the individual punch site proved too small an area for suitable measurement). For both *C. gynecia* and *E. ferriensis* we measured 10 valves each and took the average to represent the thickness of their respective species groups. Although sample thickness was estimated in this way, we do not believe it has a significant impact on our results as the differences between the groups were very strong and thickness was measured similarly across species.
Figure 3.5. EDAX/sectioning. Scanning electron micrographs and subsequent element maps of the carapace cross-sections. R=Resin matrix, C=Carapace section.

Figure 3.6. Representative force-displacement curves for all three species (Light grey = *Cyzicus gynecia*; Dark grey = *Eulimnadia feriensis*; Black = *Leptestheria compleximanus*).
In contrast, it was not possible to measure fresh samples of *L. compleximanus* due to the failure to produce live populations from previously fruitful lab samples (it is suspected laboratory populations may have been ‘over-sampled’ effectively depleting viable breeding individuals). Thus, slightly decayed carapaces were used, and the report therefore represents an average thickness from only 4 individuals that were kept in a different manner than the other two species. When we compared fresh and slightly decayed samples in *C. gynecia* and *E. ferriensis* we found that decay seemed to increase thickness. Therefore the report of estimated thickness for this group, and subsequently material properties that take into account sample thickness (SS and SW), are incorrect and likely lower than the reported value. Interestingly, when including *L. compleximanus* in the ANOVA for these values (SS and SW) it appears *L. compleximanus* was significantly lower than both *C. gynecia* and *E. ferriensis*. Thus, although thickness was measured with samples that may have been thicker than those tested in experiments, these inflated values were still sufficient to show a significant difference in all measured properties between *L. compleximanus* and the other two groups. In fact, it is expected that this difference could be even stronger if it had been possible to test fresh samples. Due to this *L. compleximanus* will not be eliminated from further discussions as it is believed the estimated measurements are sufficient for comparison in this study. Significance values in Table 3.3 are from the ANOVA of all three species for this reason. Interestingly no differences were found in any of the measured material properties based on sex in *L. compleximanus* (*p > 0.5* for all). While all tests between species had
sufficient statistical power, the power comparing sexes was very low, likely due to similarity of the means and standard deviation of the data around the mean.
Table 3.3: Measured and calculated mechanical properties from the punch test for three species of clam shrimp. Thickness (\(x\)) is not of each sample tested but of a subset. Brackets indicate where values are estimated based on thicknesses measured from poorly preserved samples. Toughness (\(†\)) is defined as the area under the force-displacement curve. P-values are reported for each property from an ANOVA which tests for differences between all three species. Error is reported as \(\pm 1\) s.e.m.

<table>
<thead>
<tr>
<th>Species</th>
<th>Thickness (x) (m) (\times 10^5)</th>
<th>Force to Punch (N) (\times 10^3)</th>
<th>Toughness ((\text{J}) \times 10^3)</th>
<th>Punch Strength (N m(^{-2})) (\times 10^7)</th>
<th>Specific Punch Strength (N m(^{-2}) m(^{-1})) (\times 10^6)</th>
<th>Work to Punch (J m(^{-2})) (\times 10^3)</th>
<th>Specific Work to Punch (J m(^{-2}) m(^{-1})) (\times 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cyzicus gynecia</em></td>
<td>1.810±0.1 76</td>
<td>111.5069 ± 6.2512</td>
<td>8.491±0.6 0760</td>
<td>37.17±2.08</td>
<td>20.54±1.15</td>
<td>28.30±2.03</td>
<td>15.64±1.12</td>
</tr>
<tr>
<td><em>Eulimnadia ferriensis</em></td>
<td>0.830±0.0 67</td>
<td>91.8806±5.9219</td>
<td>7.3944±0.6 6970</td>
<td>30.63±1.97</td>
<td>36.90±2.38</td>
<td>24.65±2.23</td>
<td>29.70±2.69</td>
</tr>
<tr>
<td><em>Leptestheria compleximanus</em></td>
<td>[1.800±0.0 187]</td>
<td>63.3521±5.7653</td>
<td>4.7120±0.5 4260</td>
<td>21.12±1.92</td>
<td>[11.73±1.07]</td>
<td>15.71±1.18</td>
<td>[8.73±1.00]</td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0002*</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Discussion

The ultrastructure of the clam shrimp carapace is a multi-layered cuticle composed of successive layers of the endocuticle and exocuticle retained during the partial molt (Rieder et al., 1984; Martin, 1991; Olempska, 2004a). The accreted, multi-laminar ‘macro-structure’ of the clam shrimp carapace appears to be relatively resilient to taphonomic processes (destructive processes such as decay, desiccation and compaction influence the likelihood of organismal remains entering the fossil record) compared to the non-mineralized cuticle of the organism inside (Krishnan, 1958). This analysis of the clam shrimp carapace clearly shows differences in both chemical composition and specific material properties between the three extant families of clam shrimp.

Results from elemental analysis show that the proportion of mineralized cuticle is highest in the *C. gynecia*, containing some 16-21% calcium-phosphate, considerably more than the ~5% seen in representatives of the family Leptestheriidae (*L. compleximanus*) and ~2% in the representative of the family Limnadiidae (*E. feriensis*). Results from the materials testing, however, show that *C. gynecia* and *E. feriensis* are no different from each other in all material properties except those that take into account the distinct difference in valve thickness. *L. compleximanus*, on the other hand, is significantly lower in all measured material properties.

The combined results of the chemical and material analysis are interesting and somewhat unexpected. The hypothesis that more mineralized valves would require
more force to fracture, be "tougher" (crack propagation), stronger (crack initiation) and require more work to fracture was not the case. While the highly mineralized *C. gynecia* reported high values for the material properties measured, the weakly mineralized *E. feriensis* was surprisingly no different from *C. gynecia* in all properties except those that take into account thickness of the valve (SS and SW). These results immediately cause us to reject our hypothesis and suggest that mineralization does not directly influence the ‘robustness’ of the overall material and shape of the valve (where thickness of the material is not accounted for). In fact, it is perhaps the low mineralization of *E. feriensis* that allowed for higher recorded material property values, which were within the range of those measured for the highly mineralized *C. gynecia*. For instance, the force-displacement curves for *C. gynecia* and *E. feriensis* (Figure 3.6) clearly show that while maximum force to fracture is about equivalent, the displacement of the sample prior to failure is larger for *E. feriensis* than *C. gynecia*. Thus, the energy to fracture the sample is slightly larger, causing *E. feriensis* and *C. gynecia* to behave similarly via two different approaches: high mineralization and stiffness (steep slope of the force-displacement curve) vs. higher extensibility (longer displacement or ‘stretching’ in colloquial terms) of the sample prior to fracture respectively. The result of these two approaches causes these two species with different material compositions to have similar strength and "toughness" or energy required to fracture.

It is important to note here that these initial properties (F, R, S and W) do not take into account valve thickness, which is much lower in *E. feriensis* than *C. gynecia*. When we take into account thickness, *E. feriensis* reports lower values than *C. gynecia*
for SS and SW. So the raw material itself, controlled for differences in thickness, is different; however the overall structure of the valves and their failure behavior is not different. These findings suggest that these two species can achieve similar material properties by two very different means (high mineralization and high thickness (i.e. Cyzicidae) vs. low mineralization and low thickness (i.e. Limnadiidae)).

*Leptestheira complexiamnus*, on the other hand, does not reach the high maximum force values seen in *C. gynecia* and *E. feriensis* nor does it reach high displacement values characteristic of *E. feriensis* (Figure 3.6). Therefore, this species performs lower than both *C. gynecia* and *E. feriensis* in all materials tests. Interestingly this is despite being the intermediately mineralized example of the three that we expected to be only slightly less thick than *C. gynecia*.

The direct comparison between *C. gynecia* and *L. compleximanus*, which we estimated to have similar thickness yet different levels of mineralization, suggests that material properties can be directly related to mineralization. However, mineralization requirements can be overcome by producing a thin, less mineralized but more extensible carapace like in *E. feriensis*. Although it is unclear what makes *E. feriensis* extensible but also able to sustain a high force prior to fracture (F), the carapace from this species was the only to report sodium in the composition analysis report. To our knowledge, sodium has not been reported as a component of the clam shrimp carapace and could have important implications for its material properties. Interestingly the Na-Ca-P total percentage from the elemental analysis of *E. feriensis* was similar to the Ca-P
total percentage for *L. compleximanus*, suggesting the possibility of alternate methods for mineralization in clam shrimp.

The clam shrimp carapace readily enters the fossil record, where conditions allow, and seems much more resistant to taphonomic processes than the soft parts of branchiopod crustaceans, as is evidenced from the thousands of instances of carapace preservation and the relatively few instances of fossilized clam shrimp soft tissue (Zhang et al., 1990). When considering the fossil record of proposed members of these lineages, there are far more fossil taxa with proposed affiliations to the extant Cyzidae than there are to the extant Limnadiidae (Novojilov, 1961; Tasch, 1969), despite both families having a similar diversity in the present (Brendonck et al., 2008). At first glance this may be directly due to the preservation potential of the highly mineralized and thick carapace of the Cyzicidae. It should be noted, however, that the palaeontological systematics of clam shrimp are in need of revision (Tasch, 1969; Novojilov, 1970; Zhang et al., 1976). Therefore, current assignment of fossil taxa at the familial level should be tempered by this consideration.

To avoid potentially biased systematics, the materials testing performed in this analysis allowed us to take a unique empirical look at how mechanical forces associated with naturally occurring, destructive taphonomic processes (e.g., post-mortem transportation, abrasion, predation and scavenging) may affect the entrance of clam shrimp remains into the fossil record. More specifically, if major differences in material properties existed between clam shrimp taxa, it would not be unreasonable to assume
subsequent differences in the proportional representation of associated taxa in the fossil record.

The results of this analysis found the material properties of the carapace of *L. compleximanus* to be consistently weaker in all measurements taken. In the fossil record we see that *Leptestheriidae* (Loxomegaglyptidae lineage; see chapter IV) are represented poorly, consistent with their weak carapace when compared to the *Limnadiidae* (Vertexioidea lineage) and *Cyzicidae* (Euestherioidea lineage). In contrast, the lack of significant differences between *E. feriensis* and *C. gynecia*, with respect to resistance to the mechanical forces generated in this experiment, insinuates that these two groups are equally as likely to resist destructive taphonomic effects despite substantial differences in their carapace composition.

By extension, one would not expect mechanical forces associated with destructive taphonomic processes to produce severe taxonomic bias in the fossil record of either the families Cyzicidae, Limnadiidae or related ancestral groups assuming similar carapace compositions in the past. This is not the case, however, as the presence of Cyzicidae (and their ancestral Euestherioidea lineage) is consistent throughout the fossil record whereas Limnadiidae is patchily distributed, "appearing" and "disappearing" throughout time (see Figure 3.2). In this case, perhaps the low level of mineralization (compared to the Cyzicidae) contributes more to the very poor fossil record for this family than the equally robust carapace (when considering overall structure and material components). This would suggest that members of the
Limnadiidae are not inherently less resistant to destructive taphonomic effects, such as post-mortem transport and abrasion, scavenging, or predation, but rather are less likely to promote post-depositional fossilization process, such as permineralization, which would help them become more prevalent in the fossil record (Briggs, 2003). When considering phylogenetic relatedness, we find that the family Leptestheriidae which have relatively ‘weak’ carapaces are more closely related to the more robust Cyzicidae (*C. gynecia*). Although the thinner shelled representative of the Limnadiidae (*E. feriensis*) exhibit similar material properties of the carapace compared to the Cyzicidae, they are more distantly related than the Cyzicidae is to the Leptestheriidae. This lack of phylogenetic bias in material performance suggests there is another factor driving the mechanical properties of these three groups.

One possible factor may be ecology. The Leptestheriidae are ecologically different from the other two families in that they are active burrowers, spending the majority of their lives slowly moving through shallow layers of sediment in temporary pools (Martin and Cash-Clark, 1993). This different mode of life may contribute to different physical requirements on the carapace as an anatomical feature and these different functional constraints could then be reflected in the material properties of the carapace. Likewise, both Cyzicidae (*C. gynecia*) and Limnadiidae (*E. feriensis*) share a similar ecological niche, which is mainly movement through the water column (unlike Leptestheriidae), suggesting that although they have different strategies for achieving similar material properties, ecological requirements are strong enough to require both groups to develop a carapace that is overall quite strong and resistant to fracture.
Conclusions

The results of this study indicate that although the thinner carapace of the Limnadiidae (represented by *E. feriensis*) is less mineralized, it is more extensible, conferring a different dimension of material strength without the need for costly investment in mineral deposition. This may be due to ontogenetic differences, in that Limnadiids are often characterized by their quick growth to sexual maturity. Many species in the family are pioneer colonizers that often exhibit varying degrees of hermaphroditism (likely for "reproductive assurance"; Weeks (2009).). Therefore, quick growth and shorter molt cycles would certainly benefit from a less demanding carapace composition and a reduced need for periods of ‘tanning’ (periods of time during inter-molt phase that are dedicated to cuticle hardening). The Cyziciidae, on the other hand, molt at more infrequent intervals for longer periods of time and often inhabit less ephemeral water bodies. The longer lived, slower growing Cyziciidae do not exhibit the ‘rush’ to sexual maturity seen in the Limnadiidae. Thus, it is likely that the Cyziciidae put more investment into the mineralization of their carapace as a defensive structure rather than rushing to reproduce in a boom-and-bust fashion as seen in the Limnadiidae.

Interestingly, each of the representative species tested here had similar overall material properties (when not accounting for sample thickness) and also exhibit similar ecology (suspension in the water column). Thus differences in mineralization may be more directly tied to the need to grow quickly and with less mineral requirements to
outcompete or be the primary successors of new environments. These differences may
drive a different means of achieving strong protective shells.

The less mineralized, extensible Limnadiid carapace and the thicker, more
mineralized carapace of the Cyzicidae are both likely to resist the physical stresses
associated with post-mortem transport of biological remains in aqueous environments,
implying that pre-depositional processes may not play a large role in producing
taxonomic bias in the fossil record of either group. However, the relative weakness of
the Leptestheriid carapace may explain their near-absence from the fossil record.

Further integrated studies testing different aspects of how this carapace material
behaves are required for both a better understanding of the Spinicaudatan carapace as
a biological structure and how such a structure behaves when subjected to the
destructive forces of taphonomic processes other than those mentioned here.
Specifically, assessing post-depositional effects such as permineralization and
dissolution would be useful. Regardless, this study provides a unique approach to
understanding the forces involved in organismal remains entering the fossil record and
how different parameters of the ‘taphonomic window’ may vary for different taxa
depending on their biological composition. Using modern analogues and heuristic
integrative approaches to understand how organic remains of different taxa may enter
the fossil record is an extremely useful and an underutilized approach that has the
potential to open up areas of palaeobiological and biological material science that were
previously unreachable.
CHAPTER IV

PHYLOGENETIC RELATIONSHIPS BETWEEN LIVING AND FOSSIL SPINICAUDATAN TAXA: A REVIEW

Overview

In order to begin to understand relationships between living and fossil groups of Spinicaudatan clam shrimp, it is important to review historical and contemporary attempts at evaluating the evolutionary history of the group. A comprehensive review of previous phylogenetic and paleontological work is presented here, alongside the most inclusive molecular analysis of the Spinidcaudata to date, in an attempt to bridge the gap between neontological and fossil works and to present a foundation upon which future studies may contribute to the understanding of Spinicaudatan evolutionary history. This chapter is broken into four discrete sections dealing with various aspects of Spinicaudatan neontology and paleontology. First, a review of the current state of branchiopod systematic is presented, followed by a comprehensive molecular phylogenetic analysis of the Spinicaudata. The third section reviews contemporary efforts in the paleontological study of fossil Spinicaudata with familial and super-familial descriptions of major fossil groups. Finally, an effort to establish biologically sound hypotheses of relatedness between fossil and living lineages of clam shrimp is
presented, which will also allow biological patterns (such as sexual system evolution) to be investigated across the groups over geologic time.

**Branchiopod Systematics: An Overview**

Branchiopod phylogenetics have been the subject of much study in recent years because they represent an ancient group of arthropod crustaceans and an early transition of arthropods from marine to freshwater environments. The Branchiopoda are the most diverse class of crustacea and may be the most morphologically disparate (Martin, 1992). Monophyly of the group is widely supported by both contemporary morphological (Olesen, 2007; Olesen, 2002) and modern molecular analyses (Richter et al., 2007; Spears and Abele, 2000). The position of the Branchiopoda within the Crustacea, however, is debated and even the crustacea are now widely believed to be at least paraphyletic with respect to the hexapoda, resulting in the increasingly invoked Pancrustacea clade (Aleshin et al., 2009; Giribet et al., 2001; Oakley et al., 2013; Regier et al., 2005). A variety of sister group relationships have been suggested for the Branchiopoda in contemporary phylogenetic analyses, notably the Hexapoda (Aleshin et al., 2009), (although this analysis excludes remipeda/cephalocarida), Xenocarida (Remipeda/Cephalocarida (Giribet et al., 2001) and Copepoda (Koenemann et al., 2010; Rota-Stabelli et al., 2013).
A recent comprehensive analysis by (Oakley et al., 2013) provides an extensive arthropod phylogeny constructed from both molecular, transcriptome and morphological data in which branchiopods fall into a new ‘epic’ arthropod clade called the Allotriocarida. This clade contains the Cephalocarida as sister to the Branchiopoda, a clade which in turn is sister to Remipeda and Hexapoda (Figure 4.1).
Figure 4.1. Pancrustacean relationships sensu Oakley et al.,

Multicrustacea

Cephalocarida

Branchiopoda

Hexapoda

Ostracoda

Figure 4.2. Proposed phylogenetic relationships within the diplostracan clade sensu Olesen 2007 (A), Spears & Abele 2000 (B), Olesen 1998 (C) & Stenderup et al., 2006 (D).
There have been many attempts at resolving the phylogenetic relationships within the Branchiopoda over the past 20 years (Figure 4.2). Early attempts to deduce the topology of the Branchiopod tree utilized morphological characteristics, as well as ontogenetic traits (larval traits; Fig. 4.2A), and produced a ladderized topology placing the Anostraca basal to the Phyllopoda (Figure 4.3). This topology also renders the Diplostraca (Figure 4.3), Onychocaudata (Spinicaudata + Cyclestheriida + Cladocera, sensu Olesen & Richter 2013) and Cladoceramorpha monophyletic and the ‘Conchostraca’ (Spinicaudata, Laevicaudata & Cyclestheriida) paraphyletic. With the advent of modern molecular systematic techniques, this topology saw much re-arrangement (e.g., Figure 4.2B,C,D) and led to much debate regarding branchiopodan phylogenetic relationships (see (Fryer, 1999; Fryer, 2002)). Despite this debate, the development of molecular techniques in the latter part of the 20th century, and dedicated and thorough multi-locus analyses of the Branchiopoda within larger pan-crustacean frameworks, converge upon similar topologies (e.g. (Oakley et al., 2013; Olesen and Richter, 2013; von Reumont et al., 2012). The advantages of using molecular techniques to inform phylogenetic relationships over morphological methods have been historically contentious (Swofford, 1991), but using molecular information in such a way does not suffer the intrinsic bias associated with coding morphologic characters. While being far from a panacea in phylogenetics, utilizing molecular data does allow for a more objective array of evolutionary informative characters to be easily accessed and analyzed, the morphologic information can then be reviewed in light of these analyses. The differing phylogenetic hypotheses depicted in figure 4.2 serve to illustrate the
conflicting ideas about branchiopod relationships. Of particular interest is the ‘wandering’ position of the Laevicaudata, appearing as sister to the Notostraca (Figure 4.2B) and Cladocera (Figure 4.2C) despite being viewed colloquially as part of the paraphyletic ‘Chonchostraca’, which united the large bivalved branchiopods (Spinicaudata, Laevicaudata and Cyclestherida). The addition of molecular data to the existing morphological dataset in Stenderup et al (2000) produces a more intuitive set of relationships (Figure 4.2C), closer to that of Olesen’s 2007 molecular-based study, but with an aberrantly placed Notostraca as sister to the Cladoceramorpha. With Olesen’s (2007) (Figure 4.2A) molecular-based hypothesis now supported by a number of independent works (von Reumont et al., 2012; Oakley et al., 2013; Olesen and Richter, 2013), it appears that the morphological characters traditionally used for the systematics of branchiopods in the past were either misleading or misinterpreted.

The Spinicaudata, as a family, have received markedly less phylogenetic treatment, most of which has been traditionally morphological (Olesen, 1998; Olesen, 2002; Olesen, 2009). They have only recently begun to receive dedicated molecular study (Hoeh et al., 2006; Schwentner et al., 2009; Weeks et al., 2008). With new studies on specific Spinicaudatan genera appearing more frequently (Schwentner et al., 2009; Schwentner et al., 2011; Schwentner et al., 2013), more taxa have become available from which to elucidate phylogenetic relationships.

In order to gain a sound footing on which to consider the relationships between extant and fossil Spinicaudatan groups, a robust and inclusive phylogenetic hypothesis
Figure 4.3. Higher-order branchiopod groups (sensu Olesen 2007, von Reumont et al. 2012, Oakley et al. 2013)
for living species is needed. Assumptions that different fossil lineages gave rise to modern families (Chen and Shen, 1985; Novojilov, 1970; Zhang et al., 1976) need to be assessed in order to provide a phylogenetic context in which to evaluate the occurrence and persistence of different sexual systems over geologic time.

The section below describes the construction of the most comprehensive Spinicaudatan phylogenetic hypotheses to date and the implications of the proposed relationships. This is followed by a brief overview of the history of ‘conchostracan’ paleontology, which in turn leads to an open discussion regarding incongruences and agreement between paleontological and neontological evolutionary hypotheses of the Spinicaudata.

Phylogenetic Reconstruction

28s, 16s ribosomal and EF1α nuclear sequences were retrieved from GenBank for 42 spinicaudatan species and 10 Notostracan species, along with the cephalocarid crustacean *Hutchinsoniella macracantha* for use as a deep-rooted outgroup (Table 4.1). Data were aligned using CLUSTAL W 1.82 (Thompson et al., 1994) via the online server CIPRES Science Gateway (Miller et al., 2010). Alignments were assessed and trimmed by eye in Mesquite (Maddison and Maddison, 2011) to congruent sequences of 788 base pairs for 28s, 432 base pairs for 16s, and 769 base pairs for EF1α. Optimum evolutionary models were selected using the jModelTest 2 program (Darriba et al., 2012). For Bayesian Inference (BI), all runs were performed using MrBayes v.3.2.1 (Huelsenbeck
and Ronquist, 2001) at 10,000,000 generations (mcmc) for each run with 100,000
generations discarded as burn-in. Standard settings with four chains were used for both
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Table 4.1. GenBank accession numbers for all sequences used in the phylogenetic analyses. Missing data symbolized by ~.
parallel runs in MrBayes. Each chain was sampled every 1000 generations. Each run was examined to ensure likelihood stability during burn-in and consistency between runs.

Results

Analyses of the three nucleotide sequences by jmodeltest resulted in an HKY model with a discrete gamma-invariant-site model being suggested for all three partitions. The maximum clade credibility produced by the Bayesian analysis included a monophyletic Notostracan outgroup, and monophyletic Limnadiidae and Leptestheriidae within the Spinicaudata (Fig. 4.4). Of interest is the paraphyly the Cyzicidae where the genus *Eocyzicus* is recovered as sister to the Leptestheriidae. The proposed sister relationship between *Eocyzicus* and the Leptestheriidae appears strongly supported whereas the relationship between this clade and the *Cyzicus* (C. gynecia, C. tetracerus, C. californicus) / *Caenestheria* (C. lutraria)/*Caenestheriella* (C. setosa, C. gifuensis) remains questionable. As in previous analyses (Schwentner et al., 2009) the genera *Limnadopsis* and *Paralimnadia* form a monophyletic endemic Australian clade.
Figure 4.4. Phylogenetic reconstruction via Bayesian inference of the Spinicaudata based on 16s, 28s & EF1α sequence data. Numbers above branches represent posterior probability estimates. Australian flag denoted endemic Australian *Limnopsis/Paralimnadia* clade.
The History of Spinicaudatan Paleontology

Fossil spinicaudatans are represented by nearly 30 families (Zhang et al., 1976) and have a record reliably extending to the Devonian (Givetian-Eifelian stages 382-393mya (Novojilov, 1970). Fossil Spinicaudatans are widely used for continental biostratigraphy (Chen, 1994; Gallego and Martins-Neto, 2006; Kozur and Weems, 2007; Kozur and Weems, 2010), paleogeography (Tasch, 1987) and are considered important paleoenvironmental indicators of ephemeral freshwater environments (Frank, 1988; Webb and Bell, 1979). Despite the fact that fossil spinicaudatans are generally thought to be much the same as their living counterparts (Dumont and Negrea, 2002), there are entire extinct superfamilies (i.e., Leaioidea) which have no modern representatives and carapace ornamentation patterns that have no modern analogue (Gallego, 2010a). The lack of scientific treatment utilizing both living and extinct Spinicaudata has resulted in severely patchy and incongruent ideas about their evolutionary history. The following section attempts to provide a brief, somewhat colloquial, history of Spinicaudatan paleontology and a review of the merits of particularly influential works in the area.

During the establishment of the science of paleontology in the early 1800’s, most specimens of fossil Spinicaudatan valves were regarded as actually representing the remains of molluscan Lamellibranchia. Publication of ‘A Monograph of Fossil Estheriæ’ (skillfully compiled by (Jones, 1862)Rupert T Jones) confirms the suspicions of other renowned paleontologists, such as Charles Lyell and Louis Agassiz, that these bivalved
fossils with a flattened profile and peculiar micro-sculpture were, in fact, the remains of bivalved ‘entomostraca’ (crustacea). Jones spent the next forty years of his paleontological career regularly publishing on ‘fossil Estheriæ’ from Canada (Jones and Kirkby, 1884), Brazil (Jones, 1897), Australia (Jones, 1870) and Siberia (Jones, 1901), as well as revising finds made in the continental USA (Jones, 1863; Jones, 1891; Jones and Woodward, 1893). These works paved the way for future research to build upon his early efforts in investigating the diversity and history of the group (e.g., (Bock, 1946; Bock, 1953a; Copeland, 1957; Copeland, 1962).

After the emergence of the phylogenetic concept in the late nineteenth century, paleontological works began to include hypotheses concerning relationships between groups of taxa. Following the discovery that fossil Spinicaudata could be of use in continental biostratigraphy and the prospecting of natural resources, such as oil and coal, major paleontological work concerning fossil Spinicaudata conducted in the former USSR, China and the USA began to appear in the 1950’s. Unfortunately, because of geopolitical boundaries, very little collaboration occurred between major researchers across the globe. The historical effect of this lack of communication is the existence of several detailed taxonomic schemes for taxa in different parts of the world.

Today, three major bodies of work by three prolific paleontologists are acknowledged as representing our collective contemporary knowledge of fossil Spinicaudata. The Russian paleontologist Nestor Novojilov (НОВОЖИЛОВ) produced a plethora of articles and books concerning the systematics of fossil Spinicaudata from
Siberia, Mongolia and parts of China. While much of this work is meticulous and wonderfully illustrated, there seem to be many taxa that have been erected based on single specimens, many of which are from identical localities. Indeed, when I had the chance to observe Novojilov's collections at the Moscow Palaeontological Institute (PIN) in February 2013, I saw that many of the taxa erected in this manner were likely synonymous to other taxa described at the same locality. When I questioned PIN staff about the apparent 'over-splitting' I was informed that it was likely an "historical artifact": the former USSR put pressure on scientists to originate taxa within the USSR in order to bolster the nationalist ideal of Russia being the 'crucible of creation'. Pressure from the Soviet regime to produce utilitarian research for prospecting natural resources was also exceptionally intense and may have led to splitting fossil populations into separate taxa when, in fact, paleontologists may have been observing intra-specific variation, such as sexual dimorphism or ontogenetic change.

Excellent examples of this splitting are the sixteen species of the Devonian genus *Megasitum* described from the Volga region by (Novojilov, 1970) all of which show little evidence of being systematically different from each other in terms of attributable characteristics (Figure 4.5). Although these historical anecdotes may be of little empirical value, they are nonetheless important when considering future attempts to synthesize a global taxonomic framework for fossil Spinicaudata.

The current observations are not meant to infer that Novojilov's work was of diminished scientific worth. To the contrary, the scale of his collections is a testament to
his renowned commitment to paleontology, and the specimens from Novojilov's collection are important additions to the current study.
Figure 4.5. a sample of species in the genus *Megasitum* from the Volga region of the former USSR (adapted from (Novojilov, 1970)): i) *Megasitum lundongaense*, ii) *M. kaljugense*, iii) *M. lundongaense*, iv) *M. kaljugense*, v) *M. jurmangaense*, vi) *M. sotainium*, vii) *M. vanum*
The major western effort to produce a systematic framework for the study of fossil branchiopods, and specifically the Spinicaudata, was undertaken by Paul Tasch in the 1950’s-1980’s. Tasch’s research culminated in two important books: *Fossil Conchostraca of the Southern Hemisphere* (Tasch, 1987) and the *Branchiopoda* section of the *Treatise on Invertebrate Paleontology* (Tasch, 1969). Tasch’s work is still the most comprehensive treatment of the fossil record of the Spinicaudata available in the English language, although many of the systematic relationships inferred within fossil groups and between fossil and extant groups made in these works are in need of revision in the light of more recent molecular work. A large quantity of the specimens collected by Tasch (particularly fossil Spinicaudata from Antarctica) is now housed in the collections of the Smithsonian museum in Washington, D.C. This collection is unfortunately somewhat neglected and in need of dedicated curation.

The most substantial body of work concerning the paleontological record of the Spinicaudata has been carried out over the past 50 years by paleontologists based at the Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences. Early work by professors Chen Pei-ji, Shen Yan-bin and Zhang Wentang culminated in the comprehensive book *Fossil Conchostraca of China* (Zhang et al., 1976), a book which describes and provides figures of every fossil species known from China at the time of publication. This work is exhaustively comprehensive and includes chapters on extant Spinicaudatan biology and morphology, systematic revisions, ornamentation evolution and hundreds of microscope images. Although parts of the Chinese literature were translated during the current study, it is clear that the field of ‘Conchostracan’
Paleontology would benefit greatly from an English translation of *Fossil Conchostraca of China* (Zhang et al., 1976).

The works of (Novojilov, 1970; Tasch, 1969) and (Zhang et al., 1976) are outstanding, independent attempts to summarize Spinicaudatan paleontology knowledge at the time of their publication. Since these major works, despite an increase in active scientists studying fossil Spinicaudata (Figure 4.6), there has been no effort to unify and revise these works, further deepening the divide between paleontological and neontological research of the group. Though not within the scope of this study, a revision of fossil taxa in the order, and an attempt to resolve relationships between living and fossil groups, would be immensely beneficial to future studies.
Figure 4.6) The number of active Spinicaudatan researchers through time by geographic region based on publications. Red lines mark peaks in new research.
The phylogenetic hypothesis of relationships between fossil and living groups presented in (Zhang et al., 1976) has been reproduced here for clarity (Figure 4.8). These evolutionary relationships were proposed by Chinese paleontologists based on stratigraphic occurrence and morphological features of the carapace, such as the position of the umbo (and larval shell), the number, size and frequency of growth lines, the shape of the carapace (Figure 4.7), the presence and nature of carinae in the extinct family Laeiidae and, arguably most importantly, the ornamentation that occurs on the growth bands (covered comprehensively in Chapter II). Despite the lack of a cladistic methodology, this hypothesis (Fig. 4.8) is the product of experts in the field of ‘Choncostracan’ paleontology, and as such is of intrinsic value. Therefore, I will be using Figure 4.7 as a foundation upon which to discuss fossil and extant Spinicaudatan relationships.
Familial Descriptions

The following descriptions of fossil Spinicaudatan families are based on translation from the original (Zhang et al., 1976) Chinese text, additional published literature (where cited) and my own observations of global fossil Spinicaudatan collections.

**Superfamily**: Vertexioidea (Zhang et al., 1976) (Lioestherioidea in (Chen and Shen, 1985))

**Palaeolimnadiopsidae and Ipsilonidae**

Palaeolimnadiopsids appear in the Devonian of the former USSR and are primarily distinguished by the recurvature of the growth lines to form a point at the dorso-ventral marginal junction of the carapace (Novojilov, 1961). This can be seen in mature growth lines prior to the carapace margin but is often not pronounced until late-stage growth. This character, presumably, is what led to the description of their unique shape as ‘limnadiiform’ (Limnadopsiform in Figure 4.6). (Zhang et al., 1976) propose a direct relationship between the Palaeolimnadiopsidae and the living genera *Limnadopsis* based on this character alone. This assumption is discussed in more detail later.

Fossils of Palaeolimnadiopsids have often been reported to display ‘mesh’ or reticulate type ornamentations. Indeed, the palaeolimnadiopsid genera observed in these studies exhibited irregular reticulate to irregular liariate ornamentation. The Ipsilonidae were erected by (Novojilov, 1958) and are considerably less well defined.
Both families range from the Devonian to the Cretaceous. The erection of such a high-level taxon on so few (and likely homoplastic) features is dubious at best. Indeed (Straškraba, 1965) directly criticizes Novojilov's proposal at erecting Palaeolimniopdidae on such features, an opinion which I share. I believe that Palaeolimnadiopsidae and Ipsilonidae are likely polyphyletic and consist of taxa from multiple other families grouped together via homoplasy.
Figure 4.7. Stylized carapace shapes utilized in previous literature (Cycladiform, Telliniform & Limnadopsiform) and in this study (Limnadiform, Cyzciform)

Figure 4.8 - Recreation of the phylogenetic relationships between fossil and living taxa proposed by Zhang et al., 1976. Dashed lines indicate uncertain affinities.
Figure 4.9. Representatives of some fossil families A) *Cyzicus* (*Euestheria*) *crustapatulus* - Euestheriidae B) *Triassoglypta* sp. Loxomagaglyptidae C) *Leaia gondwanella* - Laeiidae D) *Estheria middendorffi* another Euestheriid E) *Lioestheria malacaraensis* - Fushonograp'idae F) *Palaeolimnadia sp.* - *Palaeolimnadiidae*. All scale bars = 1mm.
Vertexiidae

The Vertexiidae first appear in the Carboniferous and are primarily identified by ‘tumor-like’ ‘protrusions’ on the carapace. This family includes the more extravagantly spiked forms such as *Vertexia touricornis* (Novojilov) (the presence and magnitude of these elaborate carapace decorations is a point of contention as some researchers have claimed to not be able to see the features described by Novojilov, coupled with the mechanical operation of opening a bivalved carapace with large dorsal protrusions being biologically complicated, begs for this group to receive serious revision) as well as forms that exhibit rounded nodes or lumps on the larval shell (it is my opinion, after observation of type specimens, that many of these features are likely the result of either compaction of the three dimensional carapace or sclerotized soft parts, such as mandibles, contributing to local relief of the fossil). Observing the diverse array of carapace morphologies and ornamentation between genera of this group (e.g., *Megasitum, Vertexia, Pemphilimnadiopsis*), it is likely that the erection of an entire family based upon the presence of carapace protuberances to be improper taxonomic practice and that the Vertexiidae likely comprise a para/polyphyletic group of taxa with a homoplastic condition of carapace ‘protuberances’.

Palaeolimnadiidae (Figure 4.9F)

The main features of the Palaeolimnadiidae are a large larval shell, a smooth carapace with a flat or arched dorsal margin, few wide growth bands and either absent or slight reticulate ornamentation. (Zhang et al., 1976) suggest that this lineage began in
the Carboniferous from an unknown form, likely allied with the vertexiidae, which is not an unreasonable assumption given the similarity in gross morphology of palaeolimnadiopsids and vertexiid genera such as *Megasitum*. Again, this family contains species erected on the presence of ‘warty protrusions’ but not assigned to the vertexiidae based on disparate size highlighting some taxonomic inconsistency in character priority (the taxon in question, here ‘*Bulbilimnadia*’, has been observed by myself and Oscar Gallego of CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) Argentina; I believed these nodes on the larval shell to be interference from mineralized soft parts until shown new specimens that seemed to show that, in this case at least, the protuberances do indeed originate on the carapace). It appears that in addition to being a valid and important fossil family, the Palaeolimnadiidae likely represent some of the earliest distinguishable members of the ‘Limnadioidea’ (Novojilov, 1970), a larger clade that is also proposed to contain other limnadiid-like taxa such as *Cornia, Megasitum* and *Echinolimnadia*, as well as the modern Limnadiiidae.

**Perilimnadiidae**

First recorded from the Permian of Siberia, the Perilimnadiidae are very similar to the Palaeolimnadiida but often exhibit larger larval shells and preserved ‘shell glands’ (adductor muscle attachment scars) that can also easily be seen through the thin carapace of modern limnadiids. Small ‘mesh’ ornamentation is mentioned in the original description of the family (Zhang et al., 1976) but is never depicted. It is suggested that
the Perilimnadiidae originated from a palaeolimnadiid ancestor and that the Tertiary perilimnadiid *Yunmenglimnadia* may belong to a branch that ultimately led to the extant genera *Eulimnadia*, *Limnadia*, *Paralimnadia* (sensu (Rogers et al., 2012)), *Metalimnadia*, *Imnadia*, *Calalimnadia*, *Australimnadia* and *Afrolimnadia*. This phylogenetic assumption seems reasonable given the similar gross morphology and simple (if present), light isogonal reticulation of the Perilimnadiidae, that is also present as small, faint, regular dish-like depressions in living members of the Limnadiidae. I believe the Perilimnadiidae to represent an intermediate group in a larger ‘Limnadoïd’ clade with Palaeolimnadiidae being Paleozoic/Mesozoic representatives, Perilimnadiidae being Mesozoic/Cenozoic representatives and the Limnadiidae being Cenozoic/Modern representatives.
The Vertexioidea contain probable ancestors of the modern Limnadiidae (and by extension, the Limnadiidae themselves) along with more enigmatic taxa that likely need serious revision, such as the Vertexiidae. It does not seem unreasonable, given the biologically sound establishment of the Palaeolimnadiidae and Perilimnadiidae, that the erection of a superfamily to contain all fossil and living limnadiid-like taxa can be established while excluding the probably polyphyletic groups, Limnadiopsidae and Vertexiidae. Indeed, Novojilov attempted something akin to this with the erection of the ‘Limnadoidea’ (Novojilov, 1970) and, in this case, Novojilov's taxonomic scheme may be more sensible.

**Superfamily: Lioestherioidea (Eosestherioidea (Chen and Shen, 1985))**

Lioestheriidae, Euestheriidae, Orthothemosiidae and Aquilonoglyptidae

The Orthothemosiidae and Aquilonoglyptidae are minor taxa that likely belong to either the Lioestheriidae or Euestheriidae. The Euestheriidae first occur at the base of the Devonian and are thought to be both the ancestral lineage to which modern Cyzicidae belong and are also hypothesized to have given rise to the families Loxomegaglyptidae and Eosestheriidae which ultimately led to the extant family Leptestheriidae. The Lioestheriidae and Euestheriidae are comparatively small in size with sub-ovate carapaces of cyziciform, telliniform or cycladiform shape (Fig. 4.6). The larval shell is positioned anteriorly and often rises above the dorsal margin but is by no means a guaranteed feature. Larval shells are typically small and followed by regular, tightly spaced growth bands. A broad telliniform carapace is typical for this group and is
not seen in living taxa. The ornamentation observed in these groups appears as irregular reticulations and irregular liarae with much variation in either type.

The Lioestheriidae and Euestheriidae are extremely diverse groups containing the majority of taxa that are attributed to the superfamily Lioestherioidea (Eosetherioidea in (Chen and Shen, 1985)). This is likely an artifact of the families having broad, confusing and often overlapping definitions. In my opinion, these groups are in need of being revised in order to split or subdivide these large groups into more manageable operational taxonomic units. The exact relationship between, and division of, the Lioestheriidae and Euestheriidae remains somewhat unclear and is in need of dedicated study. An example of the Fushunograptidae, a more recent addition to this group, can be seen in Figure 4.9E, along with two examples of Euestheriids (Figure 4.9A&D)

Loxomegaglyptidae, Eosestheriidae and Diestheriidae

The Loxomegaglyptidae range from the Late Permian to the tertiary and are hypothesized to have led to the modern family Leptestheriidae based on carapace ornamentation, such as raised anastomizing ridges deriving from complex irregular polygonal reticulation. The Loxomegaglyptidae are typically telliniform, cycladiform or cyziciform in shape (Fig. 4.6) but differ from the Lioestheriidae/Euestheriidae in having a sub-ovate, sometimes elongate, outline that may taper toward the dorsal margin.

The diverse, widely distributed Eosestheriidae and the sino-endemic Diestheriidae are likely related to the Loxomegaglyptidae but first occur in the late
Jurassic. The Eosestheriidae often exhibit somewhat similar ornamentation to the Loxomegaglyptidae, but also exhibit granular areas of ornamentation on early growth bands followed by inflated, irregular liarae sometimes underlain with dense, regular perforation-like punctae. This style of ornamentation was also observed in the recent genus Eocyzicus (see Figure 2.7 in Chapter 2). It is likely, when regarding morphological features and carapace ornamentation, that the Loxomegaglyptidae/Eosestheriidae are ancestral to both the extant Leptestheriidae and the genus *Eocyzicus*. The style of ‘swollen’ anastomizing ridges derived from irregular polygonal reticulation and underlying perforated punctae are diagnostic of fossil taxa in this group and have been observed in living taxa *Leptestheria compleximanus*, *Maghrebestheria morrocana* and *Eocyzicus parooensis*.

The Lioestherioidea (Eosestherioidea (Chen and Shen, 1985)) are likely the most diverse and long lived superfamily of Spinicaudata with fossils dating to the early to mid-Devonian and several extant taxa being distributed globally (Leptestheriidae & Cyzicidae). It is of my opinion that the Lioestherioidea present an excellent source of information for the study of the evolution of carapace ornamentation. Such studies, utilizing modern paleontological techniques, would be of great value in understanding the macroevolutionary history of the group as well as providing important data for future phylogenetic, biostratigraphic and palaeoenvironmental works.
**Superfamily**: Estheriteoidea

Asmussidae and ‘Orthesteriidae’ (Fushunograptidae Wang in (Hong et al., 1974) and the Chinese ‘Halyestheriidae’ complex

The Asmussidae represent some of the earliest Devonian Spinicaudatan fossils and are typically cyziciform/telliniform in shape (Fig. 4.7) with central or sub-centrally positioned small larval shells which are followed by many growth bands. The Asmussidae are thought to represent ‘primitive’ forms of ‘choncostracan’ valves and often do not look like typical carapace remains. The ‘Orthesteriidae’, now more widely referred to as the Fushonograptidae (and referred to as such from this point), are hypothesized in (Zhang et al., 1976)(Fig. 4.8) to have originated from an Asmussid ancestor, although the reasoning behind this is not apparent. The Fushonograptidae contain mostly taxa endemic to China and likely gave rise to several families there (Jilinestheriidae, Halysestheriidae and Dimorphostracidae; referred to here as the ‘Halyestheriidae’ complex) as well as showing relationships to the more widespread Estheriteidae (more recent discoveries have revealed a Fushonograptid presence in South America e.g. (Monferran et al., 2013)). These taxa exhibit predominantly telliniform carapace shapes (Fig. 4.6) with small, sub-quadrate larval shells positioned mid-anteriorly along the dorsal margin. Most have tightly packed, evenly spaced sub-quadrate growth bands that are ornamented with hachure-type ornamentation (as in hachure lines that show relief in cartography) which consists of straight, uniform and
thin radial lairae that may span the entire growth band or less that may originate from irregular polygonal reticulation.

Afrograptidae

Although the Afrograptidae (Novojilov, 1957) are depicted as having an unknown affiliation in the original diagram by (Zhang et al., 1976). They were erected as a superfamily by (Shen, 2003) to include forms constrained by beading on the growth bands, resulting in radial corrugations in some taxa. The taxonomic affiliations of this family have received dedicated attention in studies by (Gallego and Caldas, 1990) and (Shen, 2003).

More recent works have referred to the Estheriteoidea in regards to a “Eosestherioidea-Estheriteoidea group” (Jones & Chen 2000, Gallego & Martins-Neto 2006) suggesting an affinity between the Lioestherioidea (Eosestherioidea (Chen and Shen, 1985)) and Estheritioidea. This is not unreasonable given gross morphological similarities but would benefit from clarification via dedicated study.

Superfamily: Leaioida

Leaiidae (Monoleiolophidae, Praeleaiidae)

The Leaioida (‘Leaiina’ (Zhang et al., 1976)) are represented in the taxonomic scheme by three families: the Leaiidae, Monoleiolophidae and the Praeleaiidae (Fig. 4.7). However, the latter two likely belong within the Leaiidae as proposed by (Tasch,
The Leioidea are easily recognized as the only group bearing pronounced radial carinae (keeled ‘ribs’ that run somewhat perpendicular to the angle of growth). Hypothesized to orginate as nodes in the larval shell, these carinae become more pronounced and often curved as the Leaiid grew.

The laeiids represent some of the oldest known ‘clam shrimp’ dating as far back as the Givetian (387.7 ± 0.8 mya). No preserved soft parts of this entirely extinct superfamily have been found so the assumption that they are even Spinicaudatan is a bit tenuous. However, given the gross morphology of the carapace, a Spinicaudatan affiliation is not unreasonable. The evolution of radial carinae is not restricted to the Leioidea and is seen in members of the Afrograptidae, suggested to be of similar ontogenetic origin as some umbonal ‘nodes’ seen in some Palaeolimndiid specimens and, interestingly, has been reported in the recent Siberian Cyziciid *Baikalolkhonia tatianae* (Galazy and Naganawa, 2010; Naganawa, 1999), however, it remains poorly described and understudied.

Conclusions

Since the publication of Zhang et al. (1976), a somewhat controversial revision (Chen and Shen, 1985) removed the Liosetheria from the Lioestherioidea (the remaining clade being renamed Eoestherioidea) and assigned as basal to the Vertexiidae. This new vertexiid clade with a Lioestheriid root was subsequently renamed “Lioestherioidea”. Unsurprisingly, this has resulted in some serious taxonomic confusion and should be
addressed in a dedicated revision of fossil Spinicaudatan systematics. The reasoning behind this move remains unclear but should be addressed in future studies.

To avoid confusion, the more widely referred (Zhang et al., 1976) scheme is used in this study. This enables the retention of more phylogenetically sensible ancestral clades for the Limnadiidae (Vertexioidea) and Leptestheriidae/Cyzicidae (Lioestherioidea).

A major problem arising in the (Zhang et al., 1976) phylogenetic hypothesis is the polyphyly of the Limnadiidae caused by the placement of modern Limnadopsis as derived from Palaeolimnadiopsis (Figure 4.7). The erection of the Palaeolimnadiopseidae by (Defretin-Le Franc, 1965) was based entirely on recurvature of the dorsal margin (and distal growth bands). This feature has been observed by the author in living Limnadopsis, Leptestheria and fossil Laeia. Considering the presence of this character in multiple lineages, it seems unwise that this feature alone should be used to sustain an entire family. It seems more likely that this feature is homoplasic and has evolved multiple times.
Figure 4.10 A) *Challaolimnadiopsis mendozaensis* from the Trissic of Argentina (Shen et al., 2001) B) Ornamentation of *C. mendozaensis* C) Recent *Limnadopsis occidentalis* from central Australia D) Pustulate ornamentation of *L. occidentalis* (note epiphyte/detritus covering in top left of SEM)
Further, the ornamentation often documented for fossils of Palaeolimnadiopseidae range from polygonal-reticulate to dendritic liarae. Typically these are ornamentation types not associated with the ancient limnadiid lineage Vertexioidea, but rather with the lineage Lioestherioidea that contains the ancestors of the Cyzicidae and Leptestheriidae (recent taxa exhibiting ornamentation of this type). Additional ornamental evidence that the Palaeolimnadiopseidae are not only unsuitable candidates for precursors of modern Limnadopsis is shown in Figure 4.10. The ornamentation of *Limnadopsis occidentalis* (Figure 4.10D) is the first known documentation of ornamentation in this genus. As is plainly visible, the ornamentation itself is simple yet unique: small pustules arising sporadically from an otherwise smooth surface. The smooth surface from which this ornamentation arises is somewhat similar to the smooth, reduced ornamentation seen in other Limnadiids. Combine this evidence with the close, Australian endemic sister relationship to the much more traditionally limnadiform Paralimnadia (Figure 4.3) and it seems quite possible that modern *Limnadopsis* is not related to fossil taxa that show dorsal recurvature, and thus is in fact a recent occurrence of such a feature in an isolated endemic group.

The lack of a modern analog and conflicting evidence from carapace ornamentation that seems to suggest at least some Palaeolimnadiopseidae may have close affinities to the Lioestherioidea should be thoroughly investigated in future palaeobiological studies. Clarification of this systematic problem would help consolidate efforts of resolving paleontological – neontological conflicts in Spinicaudatan systematics.
A second, less severe taxonomic issue concerning the Vertexiidae is that of the Perilimnadiidae/Palaeolimnadiidae. Both are distinguished using similar features and species could arguably be assigned to either group. Given the diversity seen in the carapace shape of modern Limnadiids (see *Eulimnadia, Limnadopsis,* and *Paralimnadia*), it seems likely that older representatives of the group were similarly diverse. Revision of the Perilimnadiidae and Palaeolimnadiidae, while not urgent, would also benefit future palaeobiological studies.

The major features in the evolution of the Spinicaudatan carapace and their implication for the evolutionary history of the group is summarized concisely in Figure 4.11. It appears that an underlying isogonal reticulation is the basal condition of carapace ornamentation in the Spinicaudata. This isogonal reticulation reflects a basic reprinting of the underlying epidermal layer's cellular structure. Deviations from this basal condition, in terms of augmentation of the carapace cuticular structure, occur at multiple points in the history of the group (the details of which are covered extensively in Chapter II). The particular ‘nodes’ that lead (via ontogeny) to the distinct radial carinae seen in the Laeioidea appear early in the group's history (Figure 4.11ii) and are restricted to that group, although superficially similar features appear in some families of Estheritiioidea. The majority of variation in carapace ornamentation occurs in the Estheriteoidea and Lioestherioidea lineages, both of which share an ancestral root with the modern Cyzicidae and Leptestheriidae. The evolution of what I believe to be the inflated intra-cuticular region between the procuticular layers (see Figure 2.2 in Chapter II) and not seen in other groups occurred early in the history of this lineage (Figure
This development allowed further, complex variation of ornamentation to be derived from an otherwise simple underlying pattern. It is also possible that this process allowed the deposition of minerals in the construction of such ornamentation leading to the thicker, more mineralized carapaces seen in the Cyzicidae. The occurrence of punctate and broad dendritic ansatomising, ridge-type ornamentations typical of living *Eocyzicus* and Leptestheriidae are likely derived from a Loxomegaglyptid/Eosetheriid ancestor (Figure 4.11iii). Interestingly the relatively recent geographic isolation of the Limnadiid clade in Australia (Figure 4.11a) has led to a surprising amount of endemism in the diverse genus *Paralimnadia* (the monospecific Australimnadia) and, of course, the enigmatic *Limnadopsis* which is markedly disparate in gross carapace morphology in comparison to other Limnadiids. The large carapace size, dorsal spines, posterior recurvature and pronounced ornamentation are features typically associated with living and fossil members of the Lioestherioidea, Estheritioidea and Vertexiidae. The pustulate ornamentation seen in *L. occidentalis* (Figure 4.11iv), while unique to the genus and relatively simple, is considerably more ‘elaborate’ when compared to other Limnadiid genera.

By integrating molecular and paleontological evidence, it is shown here that inferring evolutionary relationships between living and fossil taxa in character depauperate groups is not only possible, but illuminates unsuspected, long-standing taxonomic incongruities. The systematic treatments and suggestions in this chapter by no means represent the scale or depth of dedicated study that the fossil Spinicaudata deserve and are meant only to serve the immediate demands of the hypotheses being
tested. Further international collaborative research in this field will hopefully lead to a universal revision of the group that will allow larger, macroevolutionary questions to be addressed across global collections.
Figure 4.11. Evolution of major carapace feature in the Spinicaudata. i) inflation and mineralization of intra-cuticular area resulting in the more elaborate ornamentation seen throughout the Lioestherioidea. ii) The appearance of radial carina is restricted to the extinct (†) Laeiidae. iii) Occurrence of punctate and broad dendritic ansatomising, ridge-type ornamentation typical of fossil Loxomegaglyptidae. iv) Recent evolution of dorsal recurvature and pustulate ornamentation in living Limnadopsis. a) Hypothesized separation of Australia and Antarctica some ~30 million years ago.
The following research was published under the following title: Astrop, T., L. Park, B. Brown, and S. Weeks. 2012. Sexual discrimination at work: Spinicaudatan ‘Clam Shrimp’ (Crustacea: Branchiopoda) as a model organism for the study of sexual system evolution in Palaeontologica Electronica 15:2

Overview

Biological interactions are rarely preserved in the fossil record and where they do occur, are often difficult to discern. Therefore, the evolution of sexual systems over geologic time in animals has been difficult to investigate. The reproductively labile Spinicaudata (‘clam shrimp’) are a model clade for the study of sexual systems, containing dioecious (males and females), androdioecious (males and hermaphrodites) and self-fertilizing hermaphrodites. Herein we present a methodology in which mating systems can be inferred by the quantification of carapace shape differences attributable
to sexual dimorphism in fossil specimens. We develop our methodology by comparing the carapaces of six species from two families of extant Spinicaudatans using eigenshape analyses. Sexual dimorphism was successfully quantified using morphometric techniques combined with discriminant analyses, correctly identifying males and females/hermaphrodites 92% of the time in extant taxa. Thirty-four specimens of the Jurassic clam shrimp *Carapacotheria disgragaris* were analyzed utilizing the methods developed with extant species. From these fossil data, we were able to detect two distinct carapace shapes and were able to assign 100% of individuals to either shape. The mean carapace shapes of the fossil specimens fit well with the average outlines for males and females in the extant species, enabling us to calculate a sex ratio of 51:49 males:females and thereby assign the sexual system of dioecy. This study begins to successfully utilize the fossil record of the Spinicaudata to elucidate ancient sexual systems, which will likely have far reaching implications for our understanding of the evolutionary dynamics of sexual systems over geologic timescales.

**Introduction**

Within evolutionary biology, one of the most perplexing questions is: why are there so many methods of reproduction (Barrett, 2002b; Charlesworth, 2006; Jarne and Auld, 2006; Kondrashov, 1993b; Vallejo-Marín et al., 2010; Williams, 1975)? The more common reproductive forms include asexuality (clonal females only), hermaphroditism (all individuals are both male and female) and dioecy (separate males and females). The list of less common forms is enormous, including cyclic parthenogenesis (asexuality
punctuated by periods of dioecy), sequential hermaphroditism (one sex first then switching to the other sex), gynodioecy (females and hermaphrodites) and androdioecy (males and hermaphrodites), to name a few.

One of the most frustrating aspects of studying the evolution of reproductive systems is that we have been unable, to date, 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 (sex, feeding, symbiosis, etc.) in the fossil record is an exceedingly rare phenomenon. When evidence of these interactions is present within the fossil record, they are almost exclusively restricted to examples of epibiosis (Brande, 1982; Fernandez-Leborans, 2010), predator-prey interactions (Kelley et al. 2003) or plant-insect relationships (Labandeira, 1998; Pott et al., 2008). Although the importance to evolutionary biology of understanding the long-term evolution of reproductive systems cannot be overstated, information concerning the mating systems of extinct taxa remain almost completely unknown (Sassaman, 1995) except for rare instances (Klimov et al., 2011).

“Clam shrimp” (Spinicaudata) are a group of primitive crustaceans within the Class Branchiopoda; their fossil record is very good and extant taxa exhibit a diverse array of reproductive systems. They are identifiable as adults due to their distinctive bivalved carapace from which they derive their common name (Figure 5.1). Extant clam shrimp are geographically widespread, occurring on every continent except Antarctica.
(although there is an excellent Antarctic fossil record for these crustaceans, (Tasch, 1987)) with a particularly good freshwater fossil record extending back 400Ma to the Devonian period (Novojilov, 1961; Shen, 1978; Tasch, 1969). Restricted to freshwater, the majority of modern taxa inhabit lakes, ponds and ephemeral water bodies (Dumont and Negrea, 2002; Weeks et al., 2009). Like other branchiopods, they exhibit naupliar larval stages and have a variable number of body segments (Frank, 1988).
Figure 5.1. Hermaphrodite *Eulimnadia dahli*. 1) Antennae. 2) Telson. 3) Trunk. 4) Phyllopod appendages. 5) Brood chamber with egg clutch. 6) Head.
The clam shrimp are a problematic group, taxonomically (see Chapter IV). Traditionally referred to as the ‘Conchostraca’ containing the orders Spinicaudata, Laevicaudata and Cyclesterideridae, the group is now deemed paraphyletic with the Cyclesterideridae showing affiliations with the closely related Cladocera (proposed Cladoceramorpha; (Olesen, 2007)). The comprehensive work on crustacean taxonomy by (Martin and Davis, 2001) suggests that the Conchostraca should be discarded and that the group, including the clam shrimp, Notostraca (tadpole shrimp) and Anostraca (fairy shrimp) be referred to as Phyllopoda (Phyllopoda + Cladoceramorpha being the Branchiopoda).

The Spinicaudata are of particular interest as they are diverse, geographically widespread and have an excellent fossil record, as noted above. The order Spinicaudata comprises three extant families: the Limnadiidae, Cyzidae and Leptestheriidae. The Leptestheriidae are not as diverse or abundant as either the Cyzidae or Limnadiidae and are definitely not as well represented in the fossil record.

The Spinicaudata contain species that exhibit dioecious, androdioecious and hermaphroditic mating systems (Sassaman 1995, Weeks et al.2009, Bratner 2011) spread across the three constituent families, providing an excellent opportunity to study the evolution of sexual systems over geologic time. Many authors have proposed that androdioecy is a relatively unstable system that should not persist over long periods of time (Charlesworth, 1984; Lloyd, 1975; Wolf and Takebayashi, 2004) and is transitional between dioecy and hermaphroditism. The widespread occurrence of androdioecy
within the family Limnadiidae (Weeks et al., 2009; Weeks et al., 2006), along with a hypothesized age of the androdioecious genus *Eulimnadia* at between 180 (Weeks et al., 2006) – 45mya (Tasch, 1969), would seem to be evidence to the contrary. It is also important to note that the closely related Notostraca also exhibit dioecy, androdioecy and hermaphroditism (Sassaman, 1989; Zierold, 2007) and that the divergence of these two groups is estimated to have occurred approximately 330Ma (Regier et al., 2005). Thus, these branchiopod crustaceans provide an interesting set of taxa with clearly labile breeding systems and a good fossil record, which could prove exceptionally useful for the study of breeding system evolution.

Assessing fossil sex ratios is a likely candidate for assigning fossil reproductive systems. Sex ratio has been described as the ‘*only information concerning reproductive systems able to enter the fossil record*’ in fossil ostracodes (Abe, 1990). Sex ratio information has been successfully used to determine reproductive systems in extant clam shrimp (Weeks et al., 2008). Ongoing taphonomic studies also indicate no bias in preservation potential between sexes, meaning extension of this method into the fossil record should yield reliable information (Astrop and Hegna unpublished data, Chapters II & III). Sex ratio is thus a promising trait for assessing fossil reproductive modes.

Many palaeontological works have suggested that discrete variation in clam shrimp carapace shape is indeed evidence of sexual dimorphism (Table 5.1). The bimodal distribution of these data in many cases, and the similar number of growth rings in the two morphs, supports the idea that these two categories represent sexual
dimorphism rather than variation attributable to differences in ontogenetic stages of
development (Brown et al., In press).
Table 5.1. Suggested instances of dimorphism in fossil clam shrimp inferred via carapace outline or dimensions.

<table>
<thead>
<tr>
<th>Species</th>
<th>Superfamily</th>
<th>Age</th>
<th>Locality</th>
<th>Source</th>
</tr>
</thead>
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<tr>
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<td>Cyzoidea</td>
<td>U.Carb-L.Perm</td>
<td>ANTARCTICA - Shackleton Glacier</td>
<td>Babcock et al. et al. 2002</td>
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<tr>
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<td>Limnadioidea</td>
<td>U.Cret</td>
<td>BRAZIL - Potiguar Basin</td>
<td>Lana and Carvalho 2002</td>
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<td>Endolimnadiopsis rusconii</td>
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<td>U.Tria</td>
<td>ARGENTINA - Mendoza Province</td>
<td>Gallego 2005</td>
</tr>
<tr>
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<td>Eosestherioidea</td>
<td>M.Jura</td>
<td>CHINA - Sichuan Basin</td>
<td>Li, Hirano et al. et al. 2009</td>
</tr>
<tr>
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<td>Leaioidea</td>
<td>E.Carb</td>
<td>AUSTRALIA</td>
<td>Jones and Chen - 2000</td>
</tr>
<tr>
<td>Cratostracus? Cheni</td>
<td>Estherioidea</td>
<td>L.Cret - Aptian</td>
<td>CHINA - western Liaoning</td>
<td>Li and Batten 2004</td>
</tr>
<tr>
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<td>E.Cret</td>
<td>CHINA - eastern Jilin</td>
<td>Li et al. et al. 2007</td>
</tr>
<tr>
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<td>U.Cret</td>
<td>CHINA - Jilin Province</td>
<td>Li et al. et al. 2009b</td>
</tr>
<tr>
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<td>Perm</td>
<td>USA - Kansas</td>
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<tr>
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<td>Eosestherioidea</td>
<td>Tria</td>
<td>ARGENTINA - Vera Formation</td>
<td>Gallego 2010</td>
</tr>
<tr>
<td>Abrestheria rotunda</td>
<td>Eosestherioidea</td>
<td>L.Cret</td>
<td>CHINA - Northern Hebei</td>
<td>Li et al. et al. 2006</td>
</tr>
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<td>L.Jura</td>
<td></td>
<td>ANARTICA - Shackleton Glacier</td>
<td>Shen 1994</td>
</tr>
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</table>
The carapace of the Spinicaudata has not been subjected to a contemporary, comprehensive study and observations that have been made are often incomplete or conflicting (Chen and Hudson, 1991; Martens, 1983; Martens, 1985; Olempska, 2004a; Rieder et al., 1984; Wang, 1989). This has resulted in a poor understanding of a unique anatomical feature.

What is known is that the carapace is constructed via multiple lamellae from successive growth stages and that these lamellae are found in three discrete layers: a thin epicuticle on top of a thicker exocuticle and protocuticle (Martin, 1992). The carapace itself is weakly biomineralized with a chitin and calcium-phosphate complex (Stigall and Hartman 2008, Chapters II & III).

The Spinicaudatan carapace is unique in that partial molting during ecdysis produces discrete growth lines that record the ontogenetic history of each individual. Although the growth lines represent discrete ontogenetic stages, the formation of the lines appears to be influenced by their growth environment rather than a consistent set of lines set down in a specific time sequence (Weeks et al., 1997). The exact mechanisms involved in the partial molting in clam shrimp are not understood but are thought to differ from the molt retention seen in some ostracodes, such as the Cytherellidae (Jones, 2003) and Eridostraca (Olempska, 2011). A dedicated contemporary investigation of the Spinicaudatan carapace is needed in order to understand the structure and function of this important diagnostic feature.
The quantification of carapace shape using contemporary morphometric techniques, as implemented in this study, builds on historical suggestions of preserved dimorphism in clam shrimp in order to diagnose both sex ratio and, by extension, the reproductive system. A more detailed examination of carapaces in relation to sexual dimorphism allows a new approach to fossil “Conchostracan” studies and the interpretation of mating systems in fossil species (via sex ratio comparisons and/or generic identities – see below). This new approach is of great importance for studies of breeding system evolution.

Methods

The Morphometric ‘revolution’ (Rohlf and Marcus, 1993) and the subsequent development of modern, computer-based techniques (see (Adams et al., 2004; Laffont et al., 2011) has enabled the investigation of morphological variability by quantifying features traditionally assigned qualitative values based on primary homology assessments between and within taxonomic groups (de Pinna, 1991).

Eigenshape analysis (MacLeod, 1999) is a powerful contemporary technique employed in the ordination of curved outlines (Lohman, 1983; Lohmann and Schweitzer, 1990). It has been effectively applied in previous biological, paleontological and paleoenvironmental analyses (Astrop, 2011; Krieger et al., 2007; MacLeod, 2002; Wilson et al., 2013) and has also been successful in detecting sexual dimorphism in fossil Ostracoda (Elewa, 2003). Standard eigenshape analysis is suitable for the exploration of variation in the Spinicaudatan carapace because multiple, discrete, homologous
features (i.e., landmarks in geometric morphometric analyses; Bookstein 1986) are absent. The changes in carapace shape (associated with the maintenance of a brood chamber, development of claspers and other anatomical differences between the sexes) are not only suspected to be significant, but in light of previous applications, likely to be detected through analysis of outlines. Changes in the shape of carapace outlines are described as changes to individual margins. The location of margins and major anatomical features of a clam shrimp can be seen in Figure 5.1.

Eigenshape analyses (sensu (MacLeod, 1999) operate via the conversion of the digitized outline of an individual specimen into equidistant, Cartesian (x-y) coordinates. The digitized co-ordinates are then transformed into a shape function as angular deviations (phi function: φ; (Zahn and Roskies, 1972) from the previous step (coordinate) in order to describe the shape of the curve (sequestering size from the analysis). 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.

This study utilizes three independent datasets. The first consists of four species from two genera within the family Limnadiidae: the dioecious species *Limnadia badia*
(Wolf 1911) and *Limnadia stanleyana* (King 1855), along with the androdioecious species *Eulimnadia dahlia* (Sars 1896) and *Eulimnadia texana* (Packard 1871).

Fifteen males and 15 females/hermaphrodites were selected from populations reared in the laboratory, producing a dataset of n=120. This data set was designed to investigate the strength and extent of any dimorphic ‘signal’ in carapace shape. A second, smaller dataset was assembled to assess the phylogenetic consistency of shape dimorphism within the group by using the Australian endemic species *Limnadopsis occidentalis* (Timms 2009) and a representative of the family Cyzidae, *Cyzicus mexicanus* (Claus 1860).

In order to apply the above protocol to extinct taxa, it was important to develop a methodology to validate the existence of ‘morphotypes’ within a fossil sample. Therefore a third dataset of the Lower Jurassic (Ferrar Group, Toarcian Stage), Estheriid *Carapacestheria disgragaris* (Tasch, 1987) was assembled. Fossil specimens were originally collected from the Shackleton Glacier region, Carapace Nunatak, South Victoria Land, Antarctica (Stigall et al. 2008). The sedimentary interbeds of Kirkpatrick Basalt deposits from which these fossils are derived represent high palaeolatitude lakes where algal biofilms enabled the preservation of weakly biomineralized lake flora and fauna (Stigall and Hartman, 2008) (For detailed geologic, stratigraphic and palaeontological information see (Stigall Rode et al., 2005; Stigall and Hartman, 2008; Tasch, 1969).
All specimens were imaged using a Jenoptik Progres C5 digital camera attached to a binocular microscope and desktop computer with Jenoptik Capture Pro software. The outlines of individual carapaces were digitized using tpsDig v2.10 (Rohlf, 2006) and then subjected to an eigenshape analysis. Digitized outline data were saved as .tps files which were then processed using modified versions of the Eigenshape v2.6 and Guide to models v0.7 Mathematica notebooks available via the morpho-tools 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. The eigenshapes produced by the analysis describe two-dimensional axes of shape change that can be used to construct morphospaces that specimens may be projected into, allowing trends in shape variation to be observed. All specimens used in this study are adult, exhibiting multiple growth lines, claspers (in males) and egg clutches in the brood chamber (in females/hermaphrodites) (Figure 5.2).
Figure 5.2. *Limnadia badia* female (1) and male (2). Carapace dimorphism prominent on dorsal margin associated with presence/absence of brood chamber. *Limnadopsis occidentalis* female (3) and male (4) displaying similar carapace dimorphism to that seen in *L. badia*, despite gross morphologic difference. Female (5) and male (6) *Cyzicus mexicanus*. The dimorphism in *Cyzicus* is more subtle than that seen within the Limnadiidae, the female (5) being slightly more elongate with a flattened postero-ventral margin. ‘Female’ morphotype (7) and ‘male’ morphotype (8) of *Carapacetheria disgragaris*. All scale bars equal 1mm.
A total of 120 adult specimens were imaged (left valve only) to create the first dataset. Populations of *L. badia, L. stanleyana, E. texana* and *E. dahli* were reared in the laboratory following the protocols outlined in (Weeks and Zucker, 1999). Of these taxa, 15 of each sex (male/female for *Limnadia*, male/hermaphrodite for *Eulimnadia*) were processed. Preserved specimens of *L. occidentals* and *C. mexicanus* were used to create the smaller, second dataset, while 34 well preserved specimens of *C. disgragaris* from palaeontological collections at Ohio University and the Natural History Museum, London (Figure 5.2 7 & 8) were identified to create the third, fossil dataset. All specimens were imaged facing left and outlines digitized to 500 equidistant points beginning from the junction of the anterior and dorsal margins of the carapace. A composite .tps file of outline data for all 120 specimens was constructed using the free tpsUtil v1.44 (Rohlf, 2009) software. These data were then subjected to a standard eigenshape analysis. The outlines were treated as closed curves and were mean centered and standardized to remove size, scale and rotation from the analysis (see (Slice, 2005). All statistical analyses (ANOVA, Clustering, Discriminant) where performed using PAST v2.14 (Hammer et al., 2001).

**Results**

Standard eigenshape analysis of all 120 outlines produced 119 eigenshapes, the first four of which account for ~30% of the observed variance captured by the analysis (Figure 5.3).
Figure 5.3. Modes of shape change represented by eigenshape (ES) axes 1-4. Eigenshapes produced via standard eigenshape analysis (*sensu* MacLeod 1999) of digitized outlines of 120 total specimens of *E. dahli, E. texana, L. badia and L. stanleyana*. Dark-light shading indicates representative shapes with low to high scores on each of the four axes.
By observing the trends in shape change captured by each eigenshape axis, it is possible to describe corresponding biological change seen in the extant clam shrimp datasets. The first eigenshape axis (Figure 5.3) exhibits considerable change in the anterior portion of the dorsal margin which corresponds to the presence/absence of the brood chamber in egg-bearing individuals and males, respectively. This axis also captures change in angularity of the junction between the posterior and dorsal margins. The second eigenshape axis is related to the relative position of the brood chamber. Eigenshape axis three captures change in the posterior and ventral margins while eigenshape four represents the ‘roundedness’ of the dorsal margin.

A plot of the first two eigenshapes clearly shows a divergent trend between the two sexes along either axis (Figure 5.4). Despite some clear overlap, the two groups are well defined when this dataset is assessed using a hierarchical clustering approach (Ward’s method; Figure 5.5). The distinction between the two sexes was highly significant (Table 5.2). The fact that the overlap on the first two eigenshape axes did not translate to higher levels of mis-scoring (Fig 5.5) and exceptionally high levels of distinction between the two sexes (Table 5.2) underscores the importance of the second two eigenshapes at further distinguishing the two sexes. A MANOVA of this dataset using the first four eigenshapes reports a significant difference between scores for both sexes.

The raw eigenscores for the first four eigenshapes produced were utilized in discriminant analyses that successfully identified 97% of all specimens based on sex
Figure 5.4. A plot of 120 extant specimens (E. dahli, E. texana, L. badia and L. stanleyana) by sex (red = female/hermaphrodite, blue = male) on the first and second eigenshape axes. Mean shapes for males (blue) and females/hermaphrodites (red) are shown both separately and overlapping to highlight dimorphism.
The five misclassified individuals are likely outliers in terms of erroneous shape capture, mis-identifications or possibly juveniles. Mis-classified individuals occurred in both genera. Carapace shape can also be used to discriminate between taxa. This has been shown in previous studies using combinations of linear measurements and area-estimations (Hethke et al., 2010; Zierold, 2007) as well as landmark-based methods (Stoyan et al., 1994). However, these studies did not take into account carapace shape dimorphism. Using the eigenshape method described here, it is possible to correctly identify species for 92.3% (115 of 120 properly classified) of the measured individuals (Fig. 5.5).

Using these same techniques to analyze smaller datasets (n= 20-25) of the phylogenetically disparate clam shrimp *Cyzicus mexicanus* (Figure 5.2 5 & 6) and morphologically disparate *Limnadopsis occidentalis* (Figure 5.2 3 & 4), sexes were also successfully discriminated 76% and 73% of the time, respectively (Table 5.2). MANOVA’s performed on the first four eigenshape scores of either data sets (Table 5.2) revealed that the between-group difference in carapace shape is not significant for *Cyzicus* (p=0.151; Figure 5.6) and weakly significant for *Limnadopsis* (p=0.0498; Figure 5.7) in these samples. Although these data exhibit less clear distinction between the sexes (Figures 5.6 & 5.7) than the first data set (Figures 5.4 & 5.5), it is likely that the smaller sample size and more subtle carapace dimorphism influenced the ability of the analyses to both capture the shape of and discern the differences between the sexes present, highlighting an important consideration for future work.
Table 5.2. Species used in this study with associated MANOVA significance values (p*) of between-sex differences and discriminant scores (DS) as a percentage classified correctly. The first four eigenshapes produced by analysis of the separate datasets were used in the MANOVA and discriminant analyses. † Denotes fossil taxa.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>M</th>
<th>F</th>
<th>MANOVA (p*)</th>
<th>DS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. badia, L. stanleyana, E. texana, E. dahlia</td>
<td>60</td>
<td>60</td>
<td>&lt;0.0001</td>
<td>97</td>
</tr>
<tr>
<td>L. occidentalis</td>
<td>11</td>
<td>11</td>
<td>0.0498</td>
<td>73</td>
</tr>
<tr>
<td>L. badia</td>
<td>15</td>
<td>15</td>
<td>&lt;0.0001</td>
<td>100</td>
</tr>
<tr>
<td>E. texana</td>
<td>15</td>
<td>15</td>
<td>&lt;0.0001</td>
<td>100</td>
</tr>
<tr>
<td>C. mexicanus</td>
<td>9</td>
<td>15</td>
<td>0.151</td>
<td>76</td>
</tr>
<tr>
<td>C. disgragaris †</td>
<td>18</td>
<td>16</td>
<td>&lt;0.0001</td>
<td>97</td>
</tr>
</tbody>
</table>
Figure 5.5. Hierarchical cluster analysis (Ward’s method) of all 120 extant specimens using eigenshape axes 1-4. Note distinct clustering of two groups with little error. Terminal nodes labeled with species/sex abbreviations. Blue=Male, Red=Female/Hermaphrodite
Figure 5.6. Plot of *C. mexicanus* on eigenshape axes 1 & 2 with dendrogram of hierarchical cluster analysis (Ward’s method) using the first four eigenshapes. Mean shapes for males (blue) and females (red) are shown separately and overlapping to highlight dimorphism.

Figure 5.7. Plot of *L. occidentalis* on eigenshape axes 1 and 2 with dendrogram of hierarchical cluster analysis (Ward’s method) using the first four eigenshapes. Mean shapes for males (blue) and females (red) shown separate and overlapping to highlight dimorphism.
Initial observations of *C. disgragaris* revealed two distinct carapace shapes: one elongate (Figures 5.2.8) and one more rounded (Fig. 5.2.7). These shapes are reflected in both the outline of the carapace (Figure 5.8) and the growth lines preserved. Eigenshape analysis of the fossil dataset produced four major vectors of shape change that accounted for ~30% of the observed variance. In order to test the initial observations of two discreet morphotypes within *C. disgragaris*, a hierarchical cluster analysis was performed (using the first four eigenshapes and applying Ward’s clustering method). The hierarchical cluster seen in Figure 5.8 shows branch lengths based on distance, clearly illustrating the existence of two distinct groups. Discriminant analysis of these two groups (proposed by the hierarchical cluster analysis approach) using the first four eigenshapes, resulted in 97% success of classifying individual fossils into these two groups (Table 5.2).

In order to test the effectiveness of the hierarchical clustering approach, extant androdioecious *E. texana* (Figure 5.9) and dioecious *L. badia* (Figure 5.10), where the sex of individuals is known *a priori*, were tested using the same protocol described above. Hierarchical clustering produced two-group assignments that successfully assigned sex 97 or 100% of the time, respectively. MANOVAs using the first four eigenshapes of either data set reveals that between group differences are highly significant (Table 5.2).
Figure 5.8. Plot of C. disgragaris on eigenshape axes 1 and 2. Mean shapes for morphotype 1 ‘male’ (blue) and morphotype 2 ‘female’ (red) highlight possible dimorphism with associated Hierarchical cluster analysis (Ward’s method) using eigenshape axes 1-4. The designation of specimens to either two morphotypes is based on the distinct two group clustering.
Figure 5.9. Plot of *E. texana* males and self-fertilizing hermaphrodites on eigenshape axes 1 & 2 with dendrogram of hierarchical cluster analysis (Ward’s method) using the first four eigenshapes.

Figure 5.10. Plot of *L. badia* males and females on eigenshape axes 1 & 2 with dendrogram of hierarchical cluster analysis (Ward’s method) using the first four eigenshapes.
Discussion

The long-term evolutionary forces driving the diversity and maintenance of breeding systems have traditionally been addressed by theory alone (Charlesworth, 1984; Charlesworth, 2006; Charnov, 1982; Kondrashov, 1993a; Nunney, 1989; Pannell, 2009; Williams, 1975) due to the lack of detailed historical information and absence of breeding system assignments in the fossil record. This study successfully quantifies and interprets sex-specific carapace morphology for the first time in fossil Spinicaudata and allows informative biological inferences to be made from fossil taxa.

We have shown that carapace shape does indeed carry a strong, sex-based signal, as had been previously suggested but not rigorously tested (Bock, 1953b; Gallego, 2005; Gallego, 2010b; Li et al., 2009a; Li et al., 2009b; Li et al., 2010b; Oleynikov, 1969; Tasch, 1969; Tintori and Brambilla, 1992; Zierold, 2007). Traditional attempts at utilizing morphometric measurements to quantify carapace shape via linear measurements (Babcock et al., 2002; Jones and Chen, 2000; Machado et al., 1999; Oleynikov, 1969; Tintori and Brambilla, 1992; Zierold, 2007) and, in one case, landmark based methods (Stoyan et al. 1994), have produced mixed results that likely do not best represent the gross dimorphic carapace morphology. Although dimorphism had been previously noted in *C. disgragaris* by (Tasch, 1987), (Shen, 1994) deemed dimorphism “more difficult to determine” due to the distorting effects associated with fossilization despite being able to provide some distinction between proposed morphotypes (similar to those described via eigenshape analysis in this study) using linear height/length...
measurements of the valves. Although some degree of tectonism in the form of folding is reported in the Kirkpatrick Basalt from where specimens of *C. disgragaris* were collected (Stigall and Hartman, 2008), the specimens selected for this study showed no signs of warping or distortion of any kind. Ongoing taphonomic studies (Astrop and Hegna, unpublished data) have revealed that the Spinicaudatan carapace is resistant to extensive aqueous post-mortem transport. Also of import is that after deposition, remains of Spinicaudatans are surrounded by sediment and subsequently prevented from laterally expanding during compaction, as found by (Briggs and Williams, 1981).

The usefulness of the current approach is our ability to use these methods on both extant and fossil clam shrimp and underscores the ability of the detailed eigenshape methodology to correctly distinguish sexes in species where sex is known *a priori*. The observations that these methods clearly extract sexual type in extant species, the clear similarity of shape between the fossil and extant male/female (hermaphrodite) carapaces, as well as the strong signal of two morphotypes in the fossil data show these methods can be successfully used to assess individual sex in fossil clam shrimp carapaces. Using individual gender determination, we can further distinguish population sex ratios in fossilized death assemblages.

The results from the extant taxa highlight that morphological dimorphism, in the form of carapace shape, occurs throughout the Spinicaudata. Differences in the mode of dimorphism between disparate taxa is likely the result of differing anatomical locations of the egg-clutch/brood chamber (dorsal in the Limnadiidae, lateral in the Cyzidae and
Leptestheriidae), and possibly the size and position of claspers in males of all taxa. Upon application of the eigenshape method to a fossil taxon, it is clear that it is indeed possible to detect dimorphism in extinct clam shrimp and subsequently, it is reported here that the sex ratio between the two morphotypes of *C. disgragaris* 51:49 male:female. (Weeks et al., 2008; Weeks et al., 2009) have noted that sex ratio can be used to assign breeding system in extant clam shrimp species: 0% male samples are self-fertilizing hermaphrodites, 20-30% males are androdioecious, and 50+% males are dioecious. It is unlikely that the bimodal variance observed in both the clustering and scatter plot (Figure 5.8) is the result of ontogenetic variation as all specimens exhibit several growth lines, which do not occur in juveniles. It is also unlikely that the two ‘morphotypes’ are the result of the preservation of co-occurring species: most modern clam shrimp populations are monospecific, but where clam shrimp species do occur together they are morphologically and phylogenetically disparate (Dumont and Negrea, 2002; Orr and Briggs, 1999a). Given that these two morphotypes are consistent with shape differences between the sexes observed in extant clam shrimp taxa (Figure 5.2), the morphotypes may be considered male and female (Figure 5.9; compare also with average shapes in Figures 5.10 and 5.11). Thus, it is likely that *C. disgragaris* was a dioecious clam shrimp species.

In addition, it is also important to note that due to their thin, chitinous carapace and weakly sclerotized soft integument, clam shrimp may be susceptible to pre-depositional (transport, predation, desiccation) and post-depositional (decay, compaction) processes, and as such, these sources of variation may distort carapace
shape in particular ways. In order to account for these sources of variation, a dedicated
taphonomic study was conducted (see Chapter III) that allows us to characterize how
such variables influence the entry of clam shrimp into the fossil record and elucidate
how to recognize and account for these sources of taphonomic variation.

The successful elucidation of sex-specific carapace shape, and by extension sex
ratios, in this study shows that contemporary morphometric analyses of fossil clam
shrimp are capable of providing the quantitative information required to assign
breeding system to extinct clam shrimp. The extraction of breeding system from fossils
can now allow tests of hypotheses concerning the long-term evolution and maintenance
of breeding systems in these interesting, and reproductively labile, crustaceans.
Overview

Within evolutionary biology, one of the most perplexing questions is "why are there so many methods of reproduction?". The array of reproductive mechanisms is extensive. The existence of so many methods of reproduction in the natural world remains difficult to explain. Contemporary theories assume that sexual reproduction should allow long-term survival as the 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, to date, been unable to utilize information locked within the fossil record to assess reproductive 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) in the fossil record is exceedingly rare. This study represents an integrated approach to studying evolutionary transitions among sexual systems using the enigmatic branchiopod crustacean order Spinicaudata. Using both information from
extant taxa uncovered by a plethora of biological and ecological studies and the rich representation of the Spinicaudata throughout the fossil record (from the Devonian to the modern day) I am able to address two, previously untested, canonical hypotheses of reproductive evolutionary theory: that unisexual species should be short lived and less speciose than their outcrossing counterparts and that androdioecy is an unstable, transitionary system that should not persist over long periods of time.

Introduction

The current biological canon explaining the ubiquity and persistence of sexual reproduction in the animal kingdom is that outcrossing increases genotypic diversity and by extension confers a genetic lability thought necessary for organisms to adapt to changing environments (Nunney, 1989). The natural extension of this hypothesis is that unisexual lineages should be evolutionarily short-lived and less speciose than their outcrossing sexual counterparts (Fisher, 1930; Muller, 1964; Muller, 1932; Weismann, 1889), as they are forbidden access to such latent diversity.

Since the mid 1970’s, a plethora of theories have sought to provide a short-term genetic and/or ecological benefit to sexual reproduction (reviewed in Bell 1982; Kondrashov, 1993), with the majority of these theories being abandoned for lack of support (Otto 2009). This theoretical conflict is brought into sharp focus by extant speciose clades that exhibit high degrees of unisexuality, such as Spinicaudatan ‘clam-shrimp’(Weeks et al. 2009), ostracods (Schön et al., 2009), Orabatid mites (Norton et al., 1988) and bdelloid rotifers (Arkhipova and Meselson, 2000; Fontaneto et al., 2007;
Fontaneto et al., 2012; Welch et al., 2004). The lack of definitive support for short-term benefits to outcrossing sexual reproduction has logically led to the re-examination of long-term benefits as a mechanism to maintain sexual reproduction (Nunney, 1989).

From its inception, the study of evolution has progressed by a historical collaboration between studies in paleontology and evolutionary biology (see Smocovitis, 1992 for review). However, one major area of evolutionary biology, the evolution of mating systems, has been difficult (to nearly impossible) to assess in the fossil record. The difficulty of assessing the sex of fossils is widespread, and in those taxa that do 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, making assessments of reproductive evolution impossible. Because of this, tests of the long-term benefits of sexual reproduction in multicellular organisms (Fisher, 1930; Muller, 1964; Muller, 1932; Weismann, 1889) have also been impossible.

The current study uses new, integrative methods to reliably assess the long-term stability of reproductive modes in an obscure taxon of crustaceans that exhibit great lability of reproductive mode in modern taxa: branchiopod crustaceans in the order Spinicaudata (Figure 6.1). The Spinicaudata have become a model system for studies of reproductive evolution (Sassaman, 1995; Weeks et al., 2013; Weeks et al., 2009; Weeks et al., 2006a) largely because they exhibit a diversity of reproductive systems: dioecy (males + females), androdioecy (males + hermaphrodites) and selfing hermaphroditism (Brantner et al., 2013; Sassaman, 1995; Weeks et al., 2013). Selfing hermaphroditism in
spinicaudatans has independently evolved a minimum of four times from dioecious ancestors (Weeks et al., 2013). Why would any lineage repeatedly give up the well studied benefits of dioecious sexual reproduction? Androdioecy has persisted within this group for at least 25 million years (Weeks et al. 2006b), the longest recorded for any animal or plant taxon (Weeks, 2012). Combining the rich background information on extant Spinicaudata with new palaeontological approaches that can extract sex ratio estimates to ascertain reproductive systems in fossil populations of Spinicaudata (Astrop et al., 2012) allows us a unique opportunity to test two long standing hypotheses regarding the evolution of sexual systems: 1) that unisexual species should be short lived and less speciose than their outcrossing counterparts (Nunney, 1989) and 2) that androdioecy is an unstable, transitionary system that should not persist over long periods of time (Charlesworth, 1984; Lloyd, 1975; Pannell, 1997; Wolf and Takebayashi, 2004).
Figure 6.1. The Limnadiid Spinicaudatan *Calalimnadia mahei*. **B**: Brood Chamber with eggs, **C**: Bivalved carapace, **H**: Head, **N**: Neck organ responsible for production of carapace ‘fold’, **P**: Phyllopodus thoracic limbs, **Tr**: Trunk, **Te**: Telson.
Preliminary studies of both extant (Astrop et al., 2012) and fossil (Gallego et al., 2013; Monferran et al., 2013; Stigall et al., 2013) taxa have shown that morphometric techniques can be used to distinguish male, female and hermaphroditic clam shrimp on the basis of shape differences in their carapace. The current study extends these shape comparisons using a large collection of fossil clam shrimp taxa from across the world to characterize sex-specific shape differences among various fossil clam shrimp lineages. These methods are then combined to assess sex ratios in fossil clam shrimp. Because sex ratios are indicative of mating system type in modern taxa (Weeks et al., 2008), assessing these sex ratios in fossil clam shrimp allows, for the first time, assignment of mating systems to fossil species in a reproductively diverse taxon. The results of these analyses allow us to directly address the two evolutionary predictions noted above and to test theories of breeding system evolution that, to date, have remained elusive to empirical assessment.

Methods

Sampling for this study was conducted at multiple museums and repositories across the world (Table 1). 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 the previous chapter (Chapter V) and 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. The digitized co-ordinates are then transformed into a shape function as angular deviations (phi function: \( \phi \); (Zahn and Roskies, 1972)) from the previous step (coordinate) in order to describe the shape of the curve (sequestering size from the analysis; Figure 6.1). 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 1; this variable is manually discarded and the 2\(^{nd}\) 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 used to construct morphospaces that specimens may be projected
into, 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.
Table 6.1. Number of fossil species represented by adequate numbers to be of use in this study from visited institutions. 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.

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<td>NIGPAS</td>
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<td>AMNH</td>
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Figure 6.2. Illustrations depicting the conversion of Cartesian x-y coordinates to phi values. (Left) Standardized outline of random specimen of the extant Spinicaudatan *Eulimnadia texana*, red dot indicates start point. (Right) Phi (φ) plot. X = coordinate position, Y = phi value. 500 equidistant points are used in this example for clarity.

Figure 6.3. A simple bar plot comparing the variance captured by the first eight eigenshapes in analyses interpolating 500 equidistant points (black) and 10 equidistant points (red) in the same dataset of *Eulimnadia texana*. 
Some improvements were made to the protocol outlined in (Astrop et al., 2012) during the course of this study. It was found that using 500 equidistant points (to derive 499 respective phi co-ordinates) in fossil taxa allowed a lot of taphonomic interference and human error to enter the analysis ‘diluting’ the strength of the signal of dimorphism. As the Spinicaudatan carapace is essentially ‘ovoid’ in nature, with no discrete parts, it can be reduced to a very simple set of deviations in that shape. By performing eigenshape analysis on a sample data set of the dimorphic Limnadiid *E. texana*, interpolating both 500 & 10 points, it was possible to examine the effectiveness of either number of sample points. Reducing number of interpolated points to ten allowed more affine or ‘global’ variation to remain dominant (while non-affine shape changes, such as individual taphonomic variance between specimens, becomes ‘drowned out’; Figure 6.3). Cluster analysis of the the data based solely on eigenshape 1 produced similar groupings, the difference being that ES1 produced using 10 interpolated points resulted in one mis-identification (male assigned to hermaphrodite cluster) while 500 points resulted in two.

As fossil data is intrinsically more ‘noisy’ than the extant data (taphonomy, orientation in rock matrix), reduction of the outline of a specimen to exclude such noise and retain the more uniform shape trends associated with dimorphism within a population is desirable.
Evolutionary Context

In order to provide a framework for interpreting the evolutionary dynamics of sexual systems over geologic time, hypothesized relationships between extinct taxa are based on (Zhang et al., 1976). Where extant taxa are included, the phylogenetic reconstruction in Chapter IV and observations of carapace ornamentation made in Chapter II. It should be emphasized that the relationships inferred by (Zhang et al., 1976) are not cladistic in nature but do represent the culminated knowledge of some of the most experienced fossil spinicaudatan researchers of the 20th century.

Trees were constructed manually in Mesquite (V2.75) based on existing literature (e.g. (Chen and Hudson, 1991; Novojilov, 1961; Zhang et al., 1976) 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 implemented, which allowed for time-scaling of branches in the tree and to test for any serious issues in assuming data collected is 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

A total of 29 species of fossil Spinicaudata were represented by suitable sample size to be of use in these analyses (Table 1). Individual fossil specimens from collections were deemed viable if there was little to no visible taphonomic interference of the outline of the preserved carapace valve. Small 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 the higher eigenshapes. A total of 1,098 specimens from 29 species were analyzed using the morphometric protocol outlined above (Table 6.2).

In order to establish the presence of different morphotypes, an important question that was not considered in previous studies (Astrop et al., 2012) must be addressed: what would monomorphism (species composed of entirely self fertilizing hermaphrodites) look like under this morphometric protocol?

To understand how different quantities of variation in shape affect the identification of discrete shape groups (sexes or ‘morphotypes’), a sample of the extant androdioecious Limnadiid *Eulimnadia texana* was analyzed as a dimorphic population (15 males, 15 hermaphrodites) and a purely hermaphroditic population (only the 15 hermaphrodites). The first two eigenshape axes contained 82% of the variation for the male and hermaphrodite dataset and 57% of the variation observed in the
hermaphrodite-only dataset. A hierarchical cluster analyses of these respective datasets (Figure 6.4) clearly shows two groups separated by long branch lengths (relative to disparity between either clusters eigenshape scores) in the dimorphic dataset (Fig. 3B) while in the monomorphic dataset (Fig. 3A) branch lengths are considerably lower and showed no distinct groupings. Therefore, if monomorphism were to occur in fossil taxa it would be expected to display a similar pattern using this methodology.
<table>
<thead>
<tr>
<th>Species</th>
<th>Familial affiliation (ASTROP indicates suggested change)</th>
<th>Collection</th>
<th>Specimen #</th>
<th>Age</th>
<th>Useful Eigenvalues captured</th>
<th>N-2</th>
<th>N-M1</th>
<th>N-M2</th>
<th>%M2</th>
<th>Predicated sexual system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carapaceotheria disparagris</td>
<td>Eosiecheridae (Shen 1990)</td>
<td>NHM London, Ohlo</td>
<td>NHM 32569-81</td>
<td>Jurassic</td>
<td>1.2</td>
<td>72%</td>
<td>84</td>
<td>17</td>
<td>50.0</td>
<td>Dioecy</td>
</tr>
<tr>
<td>Martiniaestheria (Lioestheria)</td>
<td>Antrocerotheridae (Lalley)</td>
<td>Argentina</td>
<td>Uncurated</td>
<td>Lower Cretaceous</td>
<td>1.2,3</td>
<td>49%</td>
<td>15</td>
<td>7</td>
<td>46.7</td>
<td>Dioecy</td>
</tr>
<tr>
<td>Challaiolomadiopsis mendozaensis</td>
<td>Eosiecheridae (Sensi Zang et al., 1976)</td>
<td>Argentina</td>
<td>Uncurated</td>
<td>Triassic</td>
<td>1.2,3</td>
<td>72%</td>
<td>14</td>
<td>7</td>
<td>50.0</td>
<td>Dioecy</td>
</tr>
<tr>
<td>Wolfestheria albotaurina</td>
<td>Fushunopitidae (Wang 1974)</td>
<td>Argentina</td>
<td>Uncurated</td>
<td>Upper Jurassic</td>
<td>1.2,3</td>
<td>36%</td>
<td>33</td>
<td>14</td>
<td>42.4</td>
<td>Dioecy</td>
</tr>
<tr>
<td>Menasaotheria wuchenni</td>
<td>Eosiecheridae (Zhang et al., 1976)</td>
<td>Argentina</td>
<td>Uncurated</td>
<td>Lower Upper</td>
<td>2.3</td>
<td>20%</td>
<td>23</td>
<td>9</td>
<td>39.1</td>
<td>Dioecy</td>
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<tr>
<td>Leviaiastheria readwoodi</td>
<td>Leviaidae (Raymond 1946)</td>
<td>SMNH</td>
<td>usnm426155</td>
<td>Mid-Upper</td>
<td>1.2</td>
<td>34%</td>
<td>16</td>
<td>6</td>
<td>37.5</td>
<td>Dioecy</td>
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<tr>
<td>Estheria forbesi [all]</td>
<td>Eosiecheridae (Sensi Zang et al., 1976)</td>
<td>NHM London</td>
<td>NHM 44349 - 11883, ARG</td>
<td>Triassic</td>
<td>1.2</td>
<td>59%</td>
<td>116</td>
<td>63</td>
<td>54.3</td>
<td>Dioecy</td>
</tr>
<tr>
<td>Cyclicus (Eusecheria) crustapatus</td>
<td>Eosiecheridae ASTROP</td>
<td>SMNH</td>
<td>usnm427000-06 &amp;</td>
<td>Lower Jurassic</td>
<td>1.2</td>
<td>30%</td>
<td>33</td>
<td>10</td>
<td>30.3</td>
<td>Androdioec</td>
</tr>
<tr>
<td>Eosiecheridae ludowenseensis</td>
<td>Eosiecheridae (Sensi Zang et al., 1976)</td>
<td>Argentina</td>
<td>Uncurated</td>
<td>Jurassic</td>
<td>1.2</td>
<td>67%</td>
<td>48</td>
<td>17</td>
<td>35.4</td>
<td>Androdioec</td>
</tr>
<tr>
<td>Lioestheria malacarriensis</td>
<td>Fushunopitidae (Lalley et al. 2011)</td>
<td>Argentina, SMNH</td>
<td>usnm427580</td>
<td>Jurassic</td>
<td>1.2,3,4</td>
<td>90%</td>
<td>55</td>
<td>38</td>
<td>65.1</td>
<td>Androdioec</td>
</tr>
<tr>
<td>Eusiecheria taichi</td>
<td>Eosiecheridae (Moffett et al., 2013)</td>
<td>Argentina</td>
<td>5718</td>
<td>middle Late</td>
<td>1.2,3,4</td>
<td>91%</td>
<td>20</td>
<td>9</td>
<td>45.0</td>
<td>dioecy</td>
</tr>
<tr>
<td>Eosiecheria bocagenensis</td>
<td>Eosiecheridae ASTROP</td>
<td>NHM London</td>
<td>NHM 1961 - 135007</td>
<td>Upper-Triassic</td>
<td>1.2</td>
<td>68%</td>
<td>38</td>
<td>13</td>
<td>34.2</td>
<td>Androdioec</td>
</tr>
<tr>
<td>Eusiecheria bocagenensis [?]</td>
<td>Eosiecheridae ASTROP</td>
<td>Argentina</td>
<td>Uncurated</td>
<td>middle Late</td>
<td>1.2</td>
<td>71%</td>
<td>38</td>
<td>13</td>
<td>34.2</td>
<td>Androdioec</td>
</tr>
<tr>
<td>Eusiecheria bocagenensis</td>
<td>Eosiecheridae ASTROP</td>
<td>Argentina</td>
<td>Uncurated</td>
<td>Upper-Triassic</td>
<td>1.2</td>
<td>54%</td>
<td>61</td>
<td>33</td>
<td>54.1</td>
<td>dioecy</td>
</tr>
<tr>
<td>Trinobacca sp. 3</td>
<td>Lioestheridae (Raymond 1946)</td>
<td>Argentina</td>
<td>Uncurated</td>
<td>Late Triassic</td>
<td>1.2,3</td>
<td>82%</td>
<td>28</td>
<td>11</td>
<td>39.3</td>
<td>dioecy</td>
</tr>
<tr>
<td>Eusiecheria middenendorfi</td>
<td>Eosiecheridae ASTROP</td>
<td>NHM London</td>
<td>NHM 1962 - uncataloged</td>
<td>Upper Cretaceous</td>
<td>1.2</td>
<td>62%</td>
<td>34</td>
<td>11</td>
<td>32.4</td>
<td>Androdioec</td>
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<tr>
<td>Leiaiastheria leviidae</td>
<td>Leviaidae (Raymond 1946)</td>
<td>NHM London</td>
<td>NHM 3088-3114</td>
<td>Lower</td>
<td>1.2</td>
<td>61%</td>
<td>30</td>
<td>10</td>
<td>33.3</td>
<td>Androdioec</td>
</tr>
<tr>
<td>Cyclicus (Eusecheria) fornasaurabalis</td>
<td>Eosiecheridae (Sensi Zang et al., 1976)</td>
<td>SMNH</td>
<td>usnm426198</td>
<td>Lower Jurassic</td>
<td>1.2</td>
<td>68%</td>
<td>13</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Cyclicus (Eusecheria) crustabundis</td>
<td>Eosiecheridae (Sensi Zang et al., 1976)</td>
<td>SMNH</td>
<td>usnm427001-4785</td>
<td>Lower Jurassic</td>
<td>1.2</td>
<td>59%</td>
<td>17</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Cyclicus (Eusecheria) antarcticus</td>
<td>Eosiecheridae (Sensi Zang et al., 1976)</td>
<td>SMNH</td>
<td>usnm426177</td>
<td>Lower Jurassic</td>
<td>1.2</td>
<td>58%</td>
<td>17</td>
<td>8</td>
<td>47.1</td>
<td>9</td>
</tr>
<tr>
<td>Perliniellidae sp.</td>
<td>Perliniellidae (Sensi Zang et al., 1976)</td>
<td>AMNH</td>
<td>Tray L290-C0</td>
<td>Upper Permian</td>
<td>1.2</td>
<td>59%</td>
<td>28</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Hemicycloidea melichthiolaocida</td>
<td>Leviaidae (Raymond 1946)</td>
<td>AMNH</td>
<td>Tray L290-C0</td>
<td>Upper Permian</td>
<td>1.2</td>
<td>62%</td>
<td>45</td>
<td>12</td>
<td>26.7</td>
<td>33</td>
</tr>
<tr>
<td>Eusiecheria (Lioestheria) branchocarca</td>
<td>Eosiecheridae ASTROP</td>
<td>AMNH</td>
<td>Tray L290-C0</td>
<td>Cretaceous</td>
<td>1.2</td>
<td>58%</td>
<td>41</td>
<td>21</td>
<td>51.2</td>
<td>20</td>
</tr>
<tr>
<td>Eusiecheria sinomi</td>
<td>Eusiecheridae ASTROP</td>
<td>PIN</td>
<td>Uncurated</td>
<td>Upper</td>
<td>1.2</td>
<td>73%</td>
<td>17</td>
<td>7</td>
<td>41.2</td>
<td>10</td>
</tr>
<tr>
<td>Limnalia volucia</td>
<td>Paleolimnadiidae (Sensi Zang et al., 1956)</td>
<td>PIN</td>
<td>2141/1</td>
<td>Upper Permian</td>
<td>1.2</td>
<td>61%</td>
<td>19</td>
<td>9</td>
<td>47.4</td>
<td>10</td>
</tr>
<tr>
<td>Eusiecheria laupingensis</td>
<td>Eosiecheridae (Zhang et al., 1976)</td>
<td>NHM PAS</td>
<td>97438-37</td>
<td>Early Cretaceous</td>
<td>1.2</td>
<td>76%</td>
<td>37</td>
<td>17</td>
<td>54.1</td>
<td>Dioecy</td>
</tr>
<tr>
<td>Neosiecheria changmaensis</td>
<td>Dixieoridae (Chen 1976)</td>
<td>NHM PAS</td>
<td>45564-45566</td>
<td>Early Cretaceous</td>
<td>1.2</td>
<td>71%</td>
<td>60</td>
<td>28</td>
<td>46.7</td>
<td>32</td>
</tr>
<tr>
<td>Dicytostheria elongataovata</td>
<td>Halysiecheridae (Zhang et al., 1976)</td>
<td>NHM PAS</td>
<td>Uncurated</td>
<td>Upper Cretaceous</td>
<td>1.2</td>
<td>69%</td>
<td>99</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Halysiecheria hui</td>
<td>Halysiecheridae (Zhang et al., 1976)</td>
<td>NHM PAS</td>
<td>Uncurated</td>
<td>Upper Cretaceous</td>
<td>1.2</td>
<td>63%</td>
<td>69</td>
<td>33</td>
<td>47.8</td>
<td>36</td>
</tr>
</tbody>
</table>
Of the species examined, four (~14%) taxa (Dictyestheria elongate/ovata, Palaeolimnadia sp., Cyzicus (Euestheria) formavariabalis and Cyzicus (Euestheria) crustabundis) produced multiple (>3) discrete clusters or many small clusters with long branch lengths (see one example in Figure 6.5A).

Observing the branching patterns in the extant (Figure 6.4) examples and fossil examples (Figure 6.5), similarities and differences appear. The fossil taxon Lioestheria malacaraensis (Figure 6.5B) clearly shares a strong basal dichotomy in shape variation of a magnitude similar to that seen in the dimorphic dataset of the extant Eulimnadia texana (Figure 6.4B). Conversely the Palaeolimnadia sp. (Figure 6.5A) data exhibits no clear clusters reminiscent of the monomorphic dataset of E. texana (Figure 6.4A). 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 can be an effective way of discerning groups as 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. 6.5A) is of an order of magnitude higher than that seen in the monomorphic E. texana dataset (Fig. 6.4A) and is very similar to distance measures in other dimorphic taxa studied. 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 that seen in the
dimorphic data. These patterns either reflect the presence of multiple species in a collection labeled as a single species, or are caused by severe taphonomic interference in these specimens. Thus, for these taxa, no sexual system could be inferred.

For the remaining samples, morphotypes were assigned when cluster analysis of the informative eigenshape scores produced two distinct groups (an example is outlined in Figure 6.54B). Observations of the informative eigenshapes allowed interpretation of the mode of shape change between either grouping. Sexes may be assumed for the 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% male/70% "female": androdioecious). This has been recorded in extant, wild populations of dioecious species such as *Cyzicus tetracerus* (Popović and Gottstein-Matočec, 2006), *Leptestheria gigas* (Karande and Inamdar, 1959) and the androdioecious species *Eulimnadia 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.
Figure 6.4. Cluster analyses of a monomorphic sample of *Eulimnadia texana* (A) and a dimorphic sample (B) based on scores of individuals along the first four eigenshape axes.

Figure 6.5. Morphotype identification in the fossil species *Estheria middendorffii* based on a cluster analyses of the first two most informative eigenshape axes.
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 as it is close to the 30/70 ratio that is observed in most extant androdioecious species (male/female respectively) but leaves some room for sampling error. Examples of morphotypes in fossil species can be seen in Figure 6.6.

Eight of the 29 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 (Figure 6.7): 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.

Polytomies in the tree presented in Figure 6.7 originate from uncertain intra-familial relationships inferred by (Zhang et al., 1976). However, by time-calibrating the tree using the 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 (Figure 6.8). 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.
### Figure 6.6

A sample of the fossil taxa studied, their diagnosed sexual systems and overlaid mean-shapes of the detected morphotypes (M1+M2).

<table>
<thead>
<tr>
<th>Taxon Name</th>
<th>Geologic Information</th>
<th>Diagnosed Sexual System</th>
<th>Diagram</th>
</tr>
</thead>
</table>
| *Martinestheria codoensis* (Antronestheriidae) | Lower Cretaceous, Argentina | N=15  
M1=46.5%  
M2=53.5% | Dioecy |
| *Wolfestheria smekali* (Fushunograptidae) | Upper Jurassic, Argentina | N=33  
M1=43%  
M2=57% | Dioecy |
| *Carapacesteria dispararis* (Eoestertheriidae) | Jurassic, Antarctica | N=34  
M1=50%  
M2=50% | Dioecy |
| *Leasia gondwanella* (Lealidae) | Mid-Upper Permian, Antarctica | N=16  
M1=37.5%  
M2=62.5% | Dioecy |
| *Chaliaolmadiopsida mendozaensis* (‘Pemphillmadiopsida’ Eoesthe-
ridae) | Triassic, Argentina | N=14  
M1=50%  
M2=50% | Dioecy |
| *Cyzicus (Euestheria) crustapatulus* (Eoestertheriidae) | Lower Jurassic, Argentina | N=33  
M1=30%  
M2=70% | Androdioecy |
| *Estheria forbesi* (Eoestertheriidae) | Triassic, Argentina | N=116  
M1=54%  
M2=46% | Dioecy |
| *Menucoestheria wichmanni* (Eoestertheriidae) | Upper Triassic, Argentina | N=23  
M1=40%  
M2=60% | Dioecy |
Figure 6.7. Distribution of predicted sexual systems in fossil taxa analyzed in this study. NA = taxa in which sexual system prediction was impossible (see Figure 6.8).

Figure 6.8. A time calibrated phylogeny with polytomies largely resolved by stratigraphic occurrence and branch lengths approximate to generic range. X axis = millions of years.
The durations of the branches (Figure 6.8) are reflective of the first and last known occurrences of specie within that genus in the fossil record. In order to test the assumption that androdioecy is an unstable transitional sexual system that should not persist over long periods of time (Nunney, 1989), a simple Student's t-test was performed comparing the duration of genera suggested by this analysis to exhibit either dioecious or androdioecious sexual systems (Figure 6.9). The result of the test indicates that there is no significant difference in the duration of lineages that exhibit either sexual system (p=0.11; Fig. 6.9).

Where some degree of unisexuality (self-fertilizing hermaphroditism) occurs in a fossil Spinicaudatan family, resulting in androdioecious taxa (in these cases), there is no evidence of them being less speciose than their fully dioecious counterpart; in fact the trend is for more species in families with androdioecious species relative to those without (Figure 6.10), although this trend is clearly not significant (p=0.54). An example of this is the dioecious Eosestheriidae (seven species) and the Euestheriidae which contains eight total species, four dioecious and four androdioecious.
Figure 6.9. Box plot of lineage duration between predicted dioecious lineages (~50/50) and androdioecious lineages (~30/70).
Figure 6.10. Plot of the number of species within a fossil family (of those used in this study) against the % of lineages within that family that exhibit some degree of hermaphroditism (androdioecy in these cases). *: The families Antronestheriidae, Diestheriidae and Loxomegaglyptidae all represented by one dioecious species. Red line = linear fit. Difference in specific diversity between dioecious lineages and those that contain some degree of inferred hermaphroditism p=0.54
Discussion

The results of this study provide, for the first time, empirical evidence that allows us to address two important, canonical hypotheses of reproductive evolutionary theory: (1) that unisexual species should be short lived and less speciose than their outcrossing counterparts (Nunney 1989) and (2) that androdioecy is an unstable, transitional system that should not persist over long periods of time (Lloyd 1975; Charlesworth 1984; Pannell 1997; Wolf and Takebayashi 2004).

The complete absence of monomorphism in the fossil taxa studied here supports the evolutionary theory that unisexuality (in the case of the Spinicaudata, species that are exclusively self-fertilizing hermaphrodites) should be short lived and prone to extinction (Fisher, 1930; Muller, 1964; Muller, 1932; Weismann, 1889). If purely hermaphroditic taxa occurred in the Spinicaudatan clade in the geologic past, they are not represented in the fossil record (covering the past 370 million years) so far examined.

A likely cause of this absence is the previously hypothesized short duration of unisexual lineages (Nunney, 1989). In the Spinicaudata, a drift to unisexuality (i.e., female-biased hermaphrodites) would occur if hermaphroditic females outcompeted males via differential reproductive success. If conditions arise where hermaphroditism is favored (e.g., "reproductive assurance"; Pannell 1997, 2002), other selective forces must promote the maintenance of males (e.g., increase in genotypic diversity or avoidance of inbreeding depression) for the population to remain androdioecious and avoid the
The latter is predicted due to the accumulation of new deleterious alleles (see ‘Muller’s ratchet’ (Muller, 1964) and ‘mutational meltdown’ (Lynch et al., 1993)) or the severe loss of heterozygosity that accrues as gene conversion exposes existing, recessive deleterious alleles (seen in the closely related Cladocera (Tucker et al., 2013)). The results of the current study provide the first supportive evidence for this hypothesized phenomena occurring on a geologic time scale.

It is likely that the ancestor of the Spinicaudata was dioecious and that some form of ‘sexual lability’ (similar to that suggested for Notostracan tadpole shrimp (Mathers et al., 2013)) leads to the repeated evolution of sperm production in females. However once hermaphrodites evolve, a subsequent exclusion of males from populations (resulting from the increase in progeny produced via self-fertilization over that of outcrossing) (Weeks et al., 2013) can result in monomorphic lineages developing that can be subsequently prone to extinction. These micro-evolutionary phenomena likely explain the absence of completely hermaphroditic lineages in the fossil record as purely selfing lineages (unisexual lineages) are much more likely to face extinction in the face of variation in selective pressures that their outcrossing (dioecious/androdioecious) counterparts.

The idea of an underlying genetic lability influencing the repeated occurrence of sperm production in females (Sassaman, 1995; Weeks et al., 2006b) fits well with Spinicaudatan life histories in that they are known to inhabit (presently and throughout the past) freshwater systems that experience regular or periodic environmental
fluctuations, usually through evaporation and ultimately desiccation (Dumont and Negrea, 2002). The dispersal of Spinicaudatan eggs by birds (Schwentner et al., 2012), and in the Mesozoic, likely by dinosauria and other extinct macrofauna frequenting such environs, may have led to small founder populations being established in new pioneer habitats. The evolution of minimal sperm production in the reproductive tract of females would bestow a huge advantage to reproductive assurance in such situations. Indeed, this is likely the strategy employed by living branchiopods in similar environments (Brantner et al., 2013; Weeks et al., 2013). However, as is evidenced from the fossil data in this study, lineages that lose males to reproduce exclusively via hermaphroditism appear to be evolutionarily short lived.

Contrary to the second hypothesis stated in the introduction (i.e., androdioecy is unstable and should not persist over evolutionary time), strong evidence exists for both its occurrence in the geologic past and its persistence over geologic time. The standard paradigm would predict that fossil lineages that exhibit dioecy should persist in the geologic record substantially longer and have more species than those that exhibit androdioecy. Results show that neither of these predictions are true: there is no significant difference in the duration of lineages that exhibit either sexual system (p=0.11; Fig. 6.9) nor do families with higher proportions of androdioecious species have fewer species than more dioecious families (Fig. 6.10). In fact, lineages with sex ratios indicating androdioecy often last slightly longer and have more species (though not significantly) than dioecious lineages in the taxa studied (Figs. 6.9 & 6.10).
Eight of the fossil taxa examined show sex ratios indicative of androdioecy. These independent occurrences are not likely to be chance observations of a short lived phenomenon. It is far more likely that androdioecy can be stable, given the right conditions. This inference is clearly supported when considering the extent of androdioecy found in the extant, specious Limnadiid clam shrimp genus *Eulimnadia*, which consists of 20+ androdioecious and hermaphroditic species. The frequency that androdioecy occurs in the fossil record of Spinicaudatan clam shrimp (32%) versus fossil species that exhibit dioecy (68%) is similar to that seen in known extant Spinicaudata clam shrimp where 26% are androdioecious and 58% are dioecious (the remaining 16% of extant Spinicaudata display unisexual hermaphroditism only). These results verify the inference of long-lived androdioecy within the Limnadiidae suggested by Weeks et al. (2006), and coupled with the current data set, suggest that androdioecy can persist for long enough to leave a palaeontological signal.

Weeks et al. (2009) note that the evolution of androdioecy from dioecy is more flexible than that of all-hermaphroditism from dioecy, because both the Z and W chromosomes are retained in androdioecy allowing either all hermaphroditism (WW) or dioecy (ZW and ZZ) to evolve from an androdioecious ancestor. When a lineage evolves all (monogenic) hermaphroditism, the male-specific genes have been lost (through the fixation of the W chromosome in WW hermaphrodites), and thus reestablishment of fully male individuals should be exceptionally improbable (Weeks et al. 2009). Where androdioecy occurs, two phenotypically identical but genetically different self-fertilizing hermaphrodites can exist. Amphigenic hermaphrodites are heterozygous for sex
determining chromosomes (ZW) while monogenic hermaphrodites are homozygous (WW) (Weeks et al., 2001a). In this situation, self-fertilizing hermaphrodites can reproduce on their own or by outcrossing with males. Amphigenic individuals may produce males via selfing, allowing populations to be seeded by unisexual individuals that subsequently give rise to populations that become androdioecious and benefit from outcrossing. Monogenic hermaphrodites do not have this advantage and will produce monogenic hermaphrodites only. Monogenic hermaphrodites are less fit than their amphigenic counterparts, likely due to increased genetic load (Weeks et al., 2001b), and subsequently suffer from smaller population sizes and higher rates of mortality (Weeks 2004).

The current data suggest that Spinicaudata reproduced via androdioecy in the geologic past, and that androdioecy is more stable than previously assumed. Given these observations, and the widespread occurrence of androdioecy and hermaphroditism in extant Spinicaudata, there is likely some type of underlying genetic predisposition in Spinicaudata (and possibly Branchiopoda more generally) that allows the repeated evolution of sperm production in females. 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 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 maintaining males. This would quickly eliminate outcrossing from a lineage, ultimately leading to extinction. Essentially, we can explain the macro-evolutionary patterns we are seeing in these fossil data if we extrapolate the micro-evolutionary processes generated by the genetic dynamics of sexual systems observed in the extant Spinicaudata.

The exact pattern of the emergence and maintenance of androdioecy and dioecy in the Spinicaudata is only beginning to be explored (Hoeh et al., 2006; Weeks et al., 2009; 2013). However, the current exploration of the Spinicaudatan fossil data begins to shed light on the duration, emergence and disappearance of lineages that exhibit different sexual systems. Figure 6.11 represents two possible patterns of sexual system dynamics 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 Phyllopodan 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 is also supported
by recent molecular analyses and attempts at ancestral state reconstruction (Mathers et
al., 2013; Weeks et al., 2013). The fossil data taxa in this study carry a clear
palaeontological signal that suggests the hypothesized sexual lability inferred from
studies of living Spinicaudata is ancient, occurring multiple times over the past 430
million years. This unique crustacean group has successfully used this lability throughout
the geologic past to claim both the benefits of unisexual and sexual reproduction while
limiting the long term effects of engaging in prolonged periods of selfing and the
increased costs of obligate outcrossing.

Conclusions

This research has uncovered the first empirical evidence for multiple sexual
systems occurring in the fossil record 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, evidence for a hypothesized microevolutionary phenomenon occurring at a
macroevolutionary level over geologic time has been uncovered. The complete 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 uncovered
evidence that did not support the idea that androdioecy is an unstable, transitional
sexual system, occurring in fossil taxa as old as 430 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 my hope that the integrated, multi-pronged approach to investigating the evolution of sexual systems in living and fossil Spinicaudata be utilized for similar investigations of biological interactions in other fossil taxa.
Figure 6.11. 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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