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EVIDENCE OF SELFING HERMAPHRODITISM IN THE CLAM SHRIMP *CYZICUS GYNECIA* (BRANCHIOPODA: SPINICAUDATA)

Justin S. Brantner [1], Donald W. Ott [1], R. Joel Duff [1], Jonelle I. Orridge [2], John R. Waldman [2], and Stephen C. Weeks [1,*]

[1] Integrated Bioscience Program, Department of Biology, University of Akron, Akron, OH 44325-3908, USA
[2] Biology Department, City University of New York Graduate Center and Queens College, 65-30 Kissena Boulevard, Flushing, NY 11367, USA

ABSTRACT

The branchiopods display a broad range of reproductive modes, including dioecy, hermaphroditism and parthenogenesis. An order within Branchiopoda, Spinicaudata or the "clam shrimp" are also reported to have all three of these breeding systems; yet parthenogenesis has only been inferred on the basis of a lack of males in several clam shrimp species. Herein we report a detailed analysis of the breeding system of one of these supposed parthenogenetic clam shrimp: *Cyzicus gynecia* (Mattox, 1950). A RAPD genetic analysis across a three state geographic range (New Jersey, Massachusetts, and New York) showed high levels of genetic differentiation among populations indicative of reproduction without males. Additionally, functional male gametes were found to be produced in a small region of the gonad located just posterior to the head. Thus, we posit that the purported parthenogenetic females of *C. gynecia* are instead functioning hermaphrodites that produce a small amount of sperm anteriorly in an ovotestis that they then use to fertilize their own eggs. These findings suggest that there are, in fact, no parthenogenetic species within Spinicaudata, but rather all "female" species are most likely self-compatible hermaphrodites.

KEY WORDS: conchostracans, evolution of breeding systems, gonad structure, parthenogenesis, self-fertilizing hermaphrodites, Spinicaudata

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INTRODUCTION

Within Crustacea, class Branchiopoda is interesting both because of its phylogenetic importance (Martin and Davis, 2001), but also increasingly because of its diversity of reproductive forms (Sassaman, 1995; Dumont and Negrea, 2002; Weeks et al., 2009). One particularly diverse group of branchiopods, Diplostraca (clam shrimp and water fleas) has five modes of reproduction: dioecy (males + females = gonochorism), hermaphroditism (organism possesses a functional ovotestis and is thus capable of self-fertilization), parthenogenesis (reproduction via asexual means), cyclic parthenogenesis (many bouts of parthenogenesis with periodic sexual reproduction) and androdioecy (males + hermaphrodites; Dumont and Negrea, 2002). Among the diplostracans, one group, the spinicaudatans (clam shrimp), is reported to contain four of these five breeding systems, only lacking cyclic parthenogenesis (Sassaman, 1995).

Within Spinicaudata, breeding systems have often been inferred from sex ratios: "When males are abundant, the species is considered to be sexual (gonochoric); when males are absent, the species is considered to be 'parthenogenetic'; and when males are present but very rare, a mixed-mating system of sexual and 'parthenogenetic' reproduction is usually deduced" (Sassaman, 1995: 47). However, simply relying on sex ratios to deduce breeding system has been misleading (but see: Weeks et al., 2008), especially when parthenogenesis has been deduced from the absence or rarity of males. Sassaman and Weeks (1993) noted that *Eulimnadia texana* Packard, 1871, which had been classified as a mixture of sexual and parthenogenetic lineages, was actually androdioecious on the basis of genetic and offspring rearing studies. An anatomical study of the gonad of this species confirmed the presence of sperm and eggs occurring concomitantly in the ovotestes of the hermaphrodites (Zucker et al., 1997). Similarly, *Limnadia lenticularis* Linnaeus, 1761 was also purported to be parthenogenetic (Zaffagnini, 1969) until Scanabissi and Mondini (2002b) determined that sperm within the supposedly "rudimentary" ovotestis was, in fact, functional rather than rudimentary. Thus, to date, we have no solid evidence that any Spinicaudata are truly parthenogenetic.

What is needed is further in depth studies of the reproductive biology and genetics of Branchiopoda that are assumed to be parthenogenetic. Although Wingstrand (1978) has previously reported detailed spermatozoa ultrastructure for some members of the order Spinicaudata, few reports exist in the literature detailing the complete reproductive anatomy for the different clam shrimp families. Of the few studies that have examined the reproductive anatomy of the clam shrimp, both the testes and ovaries of the males and females, respectively, have been described as bi-lobed organs, lying within the majority of the hemocoel located adjacent to and running parallel with the intestinal tract (Tommasini and

* Corresponding author; e-mail: scw@uakron.edu

Scanabissi Sabelli, 1992; Scanabissi Sabelli and Tommasini, 1994; Zucker et al., 1997). Using such anatomical evidence, females from certain clam shrimp representatives (*E. texana*, *L. lenticularis*, and *E. agassizii* Packard, 1874) previously reported to be parthenogenetic were in fact identified as self-compatible hermaphrodites (Zucker et al., 1997; Scanabissi and Mondini, 2002b; Weeks et al., 2005). This demonstrates that the use of anatomical data is critical in discerning true sexuality in members of Spinicaudata and such evidence proves useful in supporting or refuting the claim that all-female clam shrimp species are either capable of parthenogenesis or are rather self-compatible hermaphrodites.

In Cyzicidae, only one study to date has been carried out using cellular ultrastructure to examine reproductive biology (Wingstrand, 1978). While there are a number of representatives in Cyzicidae, the one species that is perhaps most intriguing is *Cyzicus gynecia* (Mattox, 1950). According to Sassaman (1995), although all other species in the cyzicids are dioecious, *C. gynecia* is assumed to be parthenogenetic due to a lack of males in surveyed populations. Further, Weeks et al. (2009) suggested a need to study *C. gynecia* from a cellular/histological perspective because: "...it would be constructive to assess [...] the presence/absence of testicular tissue to determine the true mode of reproduction." Therefore, a solid examination of representatives in the cyzicid family, e.g., *C. gynecia*, using cellular and histological techniques would not only provide an understanding of the reproductive biology of Cyzicidae in general, but questions regarding mating system evolution could also be addressed.

The current study examines *C. gynecia* using both microscopy and genetic techniques to assess the assumed parthenogenetic breeding system of this species. We report that, in fact, *C. gynecia* is a self-fertilizing hermaphrodite, as has been found in both the *Eulimnadia* and in *L. lenticularis*.

Methods and Materials

A brief technical note must be made regarding the systematic placement of *C. gynecia*. Mattox (1950) reported the first record of populations of *C. gynecia* in small, ephemeral pools near Oxford, Ohio. Since the initial discovery, populations of *C. gynecia* have been reported not only in Ohio (Mattox, 1950; Mattox and Velardo, 1950; Emberton, 1980; Weeks and Marcus, 1997), but also in Massachusetts (Smith and Gola, 2001), New York, New Jersey (Schmidt and Kiviat, 2007), and Pennsylvania (Schwentner et al., 2009; Weeks et al., 2009). Initially, *C. gynecia* was described as a member of the genus *Caenestheriella* by Mattox (1950) and has most often been referred to in previous literature as *Caenestheriella gynecia* (Mattox and Velardo, 1950; Emberton, 1980). However, more recent studies have questioned not only the criterion initially used to distinguish the two genera *Caenestheriella* and *Cyzicus* (Smith and Gola, 2001), but also in addition the placement of this species within *Caenestheriella* itself (Sassaman, 1995). Most current literature has begun to refer to this species as *Cyzicus gynecia* (Schwentner et al., 2009; Weeks et al., 2009). In keeping with this trend to place this species within the genus *Cyzicus*, the present study will refer to this particular species as *Cyzicus gynecia*.

Cyzicus gynecia samples were collected from seventeen locations throughout the Northeastern United States (Table 1) including sites in Pennsylvania (PA), New York (NY), Massachusetts (MA), and New Jersey (NJ). The Pymatuning site was used for the histological study, in which dried soil collected from Pymatuning was hydrated in the Akron laboratory under "standard" clam shrimp rearing conditions (see Weeks et al., 1997 for a complete outline of these rearing procedures). The other remaining sites were used for the genetic analyses.

Table 1. GPS coordinates for collection locations.

Location	Puddle	Latitude	Longitude
Bergen Co., NJ	1	N 40.82747	W 74.04084
Bergen Co., NJ	2	N 40.82715	W 74.04072
Bergen Co., NJ	3	N 40.82679	W 74.04062
Bergen Co., NJ	4	N 40.82656	W 74.04049
Bergen Co., NJ	5	N 40.82586	W 74.04036
Bergen Co., NJ	6	N 40.82526	W 74.04020
Bergen Co., NJ	7	N 40.82496	W 74.04009
Bergen Co., NJ	8	N 40.82473	W 74.03997
Bergen Co., NJ	9	N 40.82449	W 74.03988
Bergen Co., NJ	10	N 40.82422	W 74.03985
Lenox, MA	1	N 42.21731	W 73.14731
Pittsfield, MA	1	N 42.28637	W 73.13438
Pittsfield, MA	2	N 42.28637	W 73.13438
Pittsfield, MA	3	N 42.29046	W 73.13578
Saugerties, NY	1	N 42.10917	W 73.93278
Hyde Park, NY	1	N 41.78333	W 73.9000
Pymatuning, PA	1	N 41.61955	W 80.45439

RAPD Analysis

Sixty-six individuals were dissected and digestive tracts were removed. Genomic DNA was extracted from the remaining animal sections by placing them in 250 μl aliquots of proteinase K extraction buffer (2 M Tris. HCl, pH 8.0; 0.5 M EDTA, pH 8; 2.5 M NaCl; 10% SDS; 10 μl proteinase K; stored at 4°C) and were incubated at 55°C overnight. DNA was extracted with 500 μl of phenol/chloroform/isoamyl alcohol (25:24:1), shaken for 10 min and followed with a 5-min, high-speed microcentrifuge (10 000 rpm) spin at room temperature. This step was repeated two more times and then ethanol precipitation of DNA was carried out by adding equal volume of sodium acetate to supernatant and 800 μl of 100% ethanol, followed with 10-min high speed centrifuge. The DNA pellets were washed with 400 μl of 70% ethanol, dried for 20 min in a SpeedVac Concentrator (Savant Instruments, Inc.; Farmingdale, New York, U.S.A.), resuspended in 100 μl sterile water, incubated for 30 min at 37°C and stored at 4°C.

RAPD reactions were performed in a final volume of 25 μl, containing 25 pmol RAPD primer, 50-100 ng template DNA and a standard quantity of Ready To Go RAPD Analysis mixture (Amersham Pharmacia Biotech, Inc. #27-9502-01). The mixture was denatured for 5 minutes at 95°C followed by 45 cycles of 1 minute denaturation at 95°C, 1 minute annealing at 36°C, and 2 minutes extension at 72°C. The amplified product was resolved by electrophoresis on 1.5% agarose gel in 1x TAE buffer for 1 hour at 100 volts. The gel was stained with ethidium bromide and immediately photographed under UV light. Sizes were inferred by comparison with a 100 bp ladder. Six primers (P1-P6; Table 2), obtained from the Ready to Go RAPD analysis kit, were tested for amplification and appropriate levels of variation.

RAPD bands produced were scored and entered into a binary data matrix as present/absent (1/0). An all-inclusive approach was taken when scoring bands as absent and present as faint bands were considered to be the result of low amounts of DNA extracted from individuals. Each individual was genetically characterized by the presence or absence of every visible amplification product. Selection of primers (Table 2) was based on reproducibility of the RAPD profiles and its consistency of producing

Table 2. RAPD analysis primers.

RAPD analysis primers	Primer sequence
Primer 1	5′-d[GGTGCGGGAA]-3′
Primer 2	5′-d[GTTTCGCTCC]-3′
Primer 3	5′-d[GTAGACCCGT]-3′
Primer 4	5′-d[AAGAGCCCGT]-3′
Primer 5	5′-d[AACGCGCAAC]-3′
Primer 6	5′-d[CCCGTCAGCA]-3′

polymorphic bands per standardized DNA concentration (1-5 ng μl^{-1}; Williams et al., 1990).

Intra- and inter-population analyses of gene diversity were performed using ARLEQUIN v. 3.5 (Kautenberger, 2006; Martinez et al., 2006). Puddles/pools were grouped by location for analysis among populations. RAPD results of individuals from Pittsfield and Lenox, MA were combined due to low sample size from each location. Individual pools were analyzed for differences within a given population. RAPD primers 2 and 3 were combined to create a master data set. The number of clones in each puddle varied depending on which RAPD primer was used. Primer 3 produced more (if not the same) clones per puddle than Primer 2 88% of the time. This questions the reliability of which primer could accurately determine the true number of clones in a puddle. For this reason, results will focus on the combined RAPD results as the combination of more than one molecular marker increases the chance of detecting differences among clone mates (Meirmans and Van Tienderen, 2004).

Fixation, Light Microscopy, and Transmission Electron Microscopy (TEM)

Fixation and light microscopy methods were as previously reported (see Brantner et al., in press). Briefly, four adult specimens of *C. gynecia* (adult state noted by the presence of eggs along the lateral side of the soft body underneath the carapace) were initially fixed in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH = 7.2) for two hours. Shrimp were removed, placed in fresh 2% glutaraldehyde/0.1 M sodium cacodylate (pH = 7.2) solution and carapaces were removed. The soft bodies were then placed for an additional one hour in fresh 2% glutaraldehyde/0.1 M sodium cacodylate (pH = 7.2) solution. Samples were washed in corresponding buffer and then post-fixed in 2% osmium tetroxide (OsO_4) buffered with 0.1 M sodium cacodylate (pH = 7.2) for 1.5 hours. The specimens were then washed with deionized water (DI water) and then en bloc stained in a 2% uranyl acetate solution for 0.5 hours. Samples were then washed in DI water and placed in an acetone desiccator overnight (Ott and Brown, 1974). Dehydrated samples were placed in a 90% acetone-10% plastic (Embed-812; Electron Microscopy Science, Hatfield, PA) solution and placed under a laminar air flow fume hood overnight. The following day, the specimens (in 100% plastic due to acetone evaporation) were placed in molds to which fresh 100% plastic was added. The molds were placed in a 60°C oven for 48 hours to cure the plastic. The plastic blocks were sectioned using a diamond knife on a Reichert OMU-3 ultramicrotome. Thick sections (1.5 μm) were placed on glass slides, heat fixed, stained with a solution consisting of 1% toluidine blue-1% sodium borate, and viewed with an Olympus BX60 digital light microscope (Olympus America Inc, PA) using an Olympus DP71 digital camera (Olympus America Inc, PA). Thin sections were mounted onto 300 mesh copper grids, pre-stained for eight minutes with 1% methanolic uranyl acetate, and further post-stained for 15 minutes in Reynolds' lead citrate (Reynolds, 1963). Thin sections were examined using a Hitachi H-7500 transmission electron microscope (Hitachi High-Technologies, Hitachinaka, Japan) at the Molecular and Cellular Imaging Center (MCIC) [part of the Ohio Agricultural Research and Development Center (OARDC)].

RESULTS

RAPD Analysis

Sufficient variation in RAPDs was found among populations (Fig. 1) to allow estimates of genetic divergence using two of the six tested primers (Primers 2 and 3; Table 2). Bands produced by primers 1, 4, 5, and 6 generated the same bands in individuals tested within puddles, among puddles, and among populations. Among the 66 individuals that were sequenced for both primer 2 and 3, the combination of the two revealed the presence of 18 haplotypes within and among populations of NJ, NY and MA (Table 3). Haplotype A was found in all populations in both 2007 and 2008. Haplotype B was present in NJ 2 and NJ 6 in both 2007 and 2008. In 2007, haplotype C was unique to NJ 4. NJ 6 contained three unique haplotypes – D, E, and F. NJ 8 contained four unique haplotypes – G, H, I and J. In 2008, haplotypes K and L appeared in NJ 2, haplotypes M, N, O appeared in NJ 6, haplotypes P and Q appeared in NY and haplotype R appeared in the MA population (Tables 3). Frequencies of each haplotype are found in Table 3. The majority of the variation was found between populations (Tables 3 and 4), which resulted in high estimates of F_{ST} (Table 4).

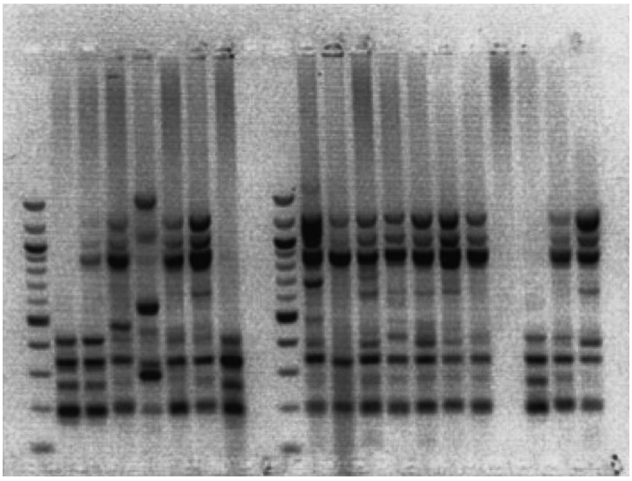

Fig. 1. RAPD profiles for 18 individuals obtained using Primer 3. Lanes 2-10: Puddle 6 (2007) individuals. Lanes 13-21: Puddle 8 (2007) individuals. All specimens were collected from the Meadowlands, NJ.

Histology and Cell Ultrastructure

Adult *C. gynecia* specimens were longitudinally sectioned and examined. Situated in the hemocoel and running parallel to the digestive tract down the length of the organism was a tubular, twin lobed gonad. Beginning at the posterior-most region and continuing towards the anterior region, the gonad was composed of a thick 'female' wall that surrounded a central luminal cavity and consisted of two primary cell types: epithelial cells and female germ cells (Fig. 2A). The epithelial cells comprised the majority of the 'female' wall. Throughout the central luminal cavity, a dense, amorphous substance was found. This substance (eggshell secretory matter) directly resembles the material found in gonads of other female Spinicaudata and owes its origination to the epithelial cells of the 'female' wall (Tommasini and Scanabissi Sabelli, 1992; Zucker et al., 1997; Scanabissi and Mondini, 2000, 2002b; Weeks et al., 2005). Commonly found branching from the tubular gonad were dilations that consisted of developing, four-celled ovarian follicles (Figs. 2A, B). In order to accommodate the enlarging follicles, dilations in the gonad wall containing the maturing ovarian follicles protruded into available hemocoel space. Ultimately, one of the four cells from the ovarian follicle increased in cellular volume, a result of undergoing vitellogenesis, whereby the cytoplasm of the cell becomes richly filled with vitelline droplets and the true oöcyte becomes apparent. The remaining cells from the ovarian follicle did not appear to increase in cellular volume but rather remained in close association at the basal portion of the developing oöcyte (Figs. 2A, B).

In the anterior-most region of the gonad, the 'female' wall was partially interrupted by a clearly defined, thin 'male' wall region from which large nucleated cells originated prior

Table 3. Relative frequencies of RAPD clones in each puddle and population.

Haplotype	2007 (n)				2008 (n)			
	NJ 2 (4)	NJ 4 (10)	NJ 6 (8)	NJ 8 (9)	NJ 2 (7)	NJ 6 (10)	NY (13)	MA (5)
A	0.75	0.9	0.25	0.444	0.143	0.2	0.077	0.8
B	0.25		0.375		0.571	0.4		
C		0.1						
D			0.125					
E			0.125					
F			0.125					
G				0.111				
H				0.111				
I				0.222				
J				0.111				
K					0.143			
L					0.143			
M						0.1		
N						0.2		
O						0.1		
P							0.615	
Q							0.308	
R								0.2

Table 4. Results of the AMOVA testing for significant differences among sub-populations of *C. gynecia* using RAPD results from primers 2, 3 and a combination of both ($P < 0.0001$).

	Primer 2	Primer 3	Combined
Among population	62.93	51.86	44.78
Within population	37.07	48.14	55.22
F_{ST} index	0.629	0.519	0.448

to movement into the gonad lumen (Figs. 2C, D). These cells represent male gametes at various developmental stages. Delineating male gamete development stages in Spinicaudata is particularly challenging since diameter size between spermatids and mature sperm can be as small as $\sim$1.3 μm (Scanabissi et al., 2006). Thus, although maturation appeared to continue in the lumen, morphological changes in male gametes were not readily evident, making it difficult to determine the exact stage of development following liberation into the gonad lumen (Figs. 2C, D).

In thin section, male gametes consisted of a regularly dense cytoplasm with mitochondria of varying sizes strewn throughout (Figs. 3A, B). Intercellular bridges were evident connecting many gametes throughout the luminal cavity. Nuclei of male gametes contained widely spread chromatin with no evident gradient or localization against the inner nuclear membrane. Spherical nucleoli were visible in some nuclei, often found in close proximity to the inner nuclear membrane (Figs. 3A, B). Unique to some male gametes within the lumen was a small invagination that began to form from within the nucleus. Nuclear and cellular membranes appeared fully intact, indicating that these openings were not artifacts of fixative preservation (Fig. 3B). Although these invaginations appeared to resemble early stages of cellular degeneration, thorough examination of male gametes provided no evidence to suggest a complete or widespread gametic cellular degenerative process.

Discussion

The genetic data is consistent with our hypothesis that *C. gynecia* reproduces via unisexual reproduction, e.g., selfing hermaphroditism. The RAPD analyses found that among-population genetic divergence was high in these clam shrimp populations. Unisexually reproducing species typically have very high among-population genetic divergence (De Meester et al., 2002; Nybom, 2004). De Meester et al. (2002) point out that the small pool environment typical for these clam shrimp tend to generate high among-pool genetic differentiation, likely caused by the early colonizing nature of the pool inhabitants (their "monopolization hypothesis"). Indeed, in a comparison of small pool species (anostracans, copepods, clacoderans, and notostracans), Boileau et al. (1992) found significant levels of F_{ST} ranging from 0.02 to 0.37 (ave = 0.12). Our estimates of among-population divergence (F_{ST}) using RAPD markers showed higher divergence rates than those reported by Boileau et al. (1992), with a combined F_{ST} value of 0.45 (Table 4). This value of F_{ST} is very similar to those reported for unisexually reproducing plants (average $F_{ST} = 0.42$; Nybom, 2004) and cladocerans ($F_{ST} = 0.38$; De Meester et al., 2002) and much higher than those reported for outcrossing (average $F_{ST} = 0.22$) or mixed mating (average $F_{ST} = 0.26$) plants (Nybom, 2004). Thus, these genetic data are suggestive of a unisexual mode of reproduction for *C. gynecia*, which is consistent with self-fertilizing hermaphroditism.

In addition to the genetic evidence for self-fertilization, we found direct anatomical evidence of an ovotestis in these shrimp (Figs. 2A-D and 3A, B). Developing in the walls of the anterior region of the gonad tract and liberated into the luminal cavity of the gonad of *C. gynecia* gonads were cells similar in appearance to sperm from the other Spini-

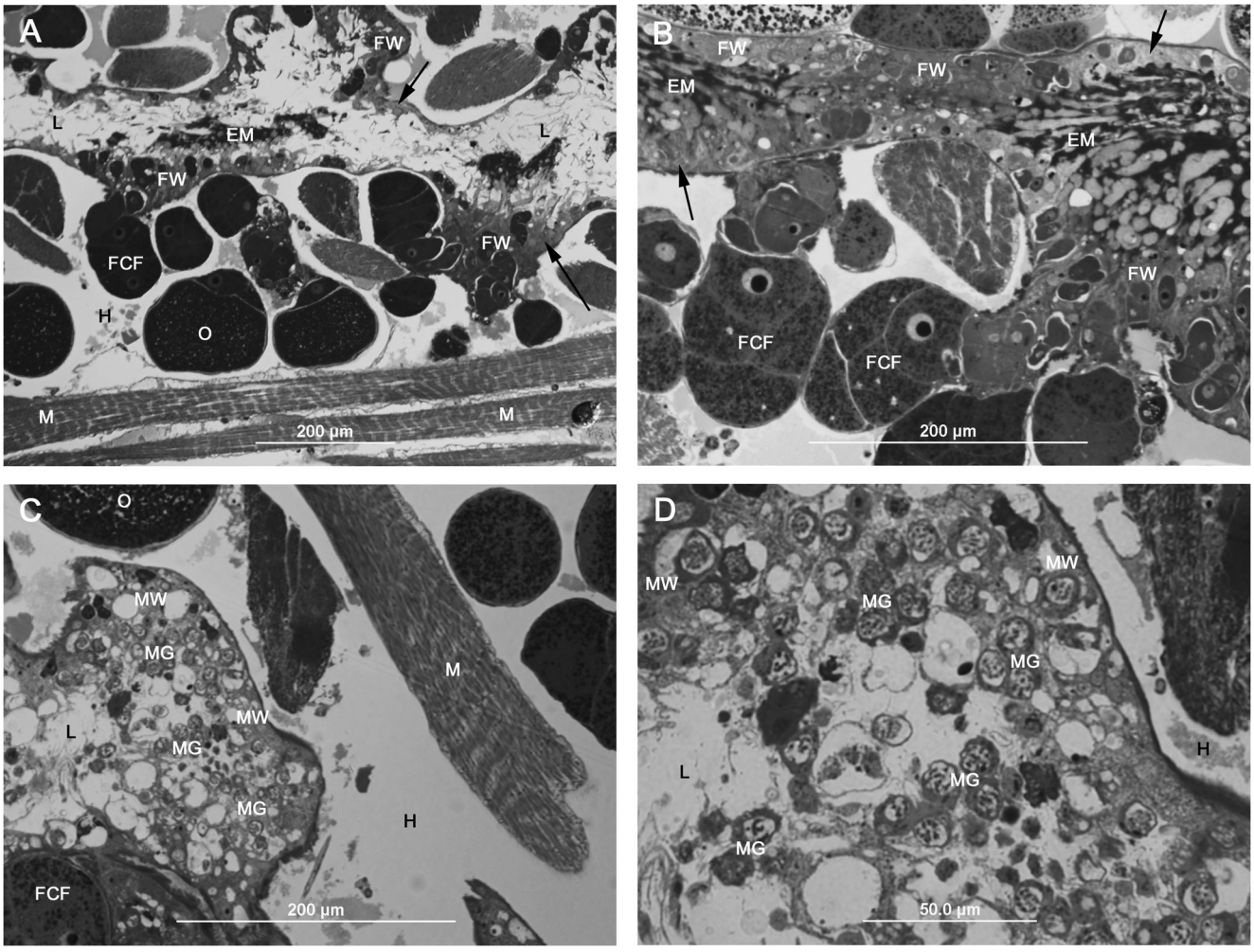

Fig. 2. Low (A) and high (B) magnifications of longitudinal thick sections of one lobe of the mid to posterior region of the gonad of *Cyzicus gynecia* surrounded by muscle (M). The 'female' wall (FW) of the gonad consists of epithelial tissue (arrows), multiple four-celled ovarian follicles (FCF) and mature oöcytes (O) that protrude into the hemocoel (H). Internally, the gonad consists of a large central lumen (L) that contains regions of eggshell secretory matter (EM). Low (C) and high (D) magnification of longitudinal thick sections of the 'male' wall (MW) area of the anterior region of the gonad. Detached from the 'male' wall into the lumen are large nucleated male gametes (MG) that possess significant amounts of loosely compacted nuclear material.

caudata, *Leptestheria* and *Eulimnadia* (Scanabissi Sabelli and Tommasini, 1994; Zucker et al., 1997; Weeks et al., 2005; Scanabissi et al., 2006). Using TEM, cellular ultrastructure of *C. gynecia* male gametes also appeared similar to previously reported Spinicaudata male gametes in that numerous mitochondria (situated in an electron dense cytoplasm) surrounded a large, singular nucleus (Wingstrand, 1978). Specimens of *C. gynecia* were observed to possess some cells close to the 'male' wall region of the gonad that contained small invaginations forming from within the nucleus. Despite the fact that widespread cellular degeneration has been previously observed in some male gametes from certain branchiopod species (Wingstrand, 1978; Scanabissi and Mondini, 2002a; Scanabissi et al., 2006), the observation that only a few cells possessed these openings, in addition to the lack of evidence for a complete cellular degeneration process (no cytoplasmic voiding observed), it is reasonable to infer that most male gametes are fully functional inside the ovotestis of *C. gynecia*.

Although thoroughly examined, we found no evidence that the populations of *C. gynecia* contained any males, nor have males been reported in previous studies of this clam shrimp species (Mattox, 1950; Mattox and Velardo, 1950; Weeks and Marcus, 1997; Smith and Gola, 2001). These observations, combined with the genetic signature of self-fertilization and the anatomical evidence for clearly defined male and female regions occurring concomitantly within the gonad, lead us to infer that the mating system for *Cyzicus gynecia* is self-fertilizing hermaphroditism.

The current evidence for hermaphroditism in *C. gynecia* refutes previous inferences that this species is parthenogenetic (Sassaman, 1995). Sassaman inferred that *C. gynecia* would be composed of heterogametic, parthenogenetic females that likely evolved a modified meiosis in which polar bodies re-fused to form diploid gametes with alternate sex chromosomes (S/s in Sassaman, 1995). Such a scenario would predict high levels of genetic heterozygosity and no male gamete production, neither of which is verified herein. In fact, to date there is no hard evidence of any partheno-

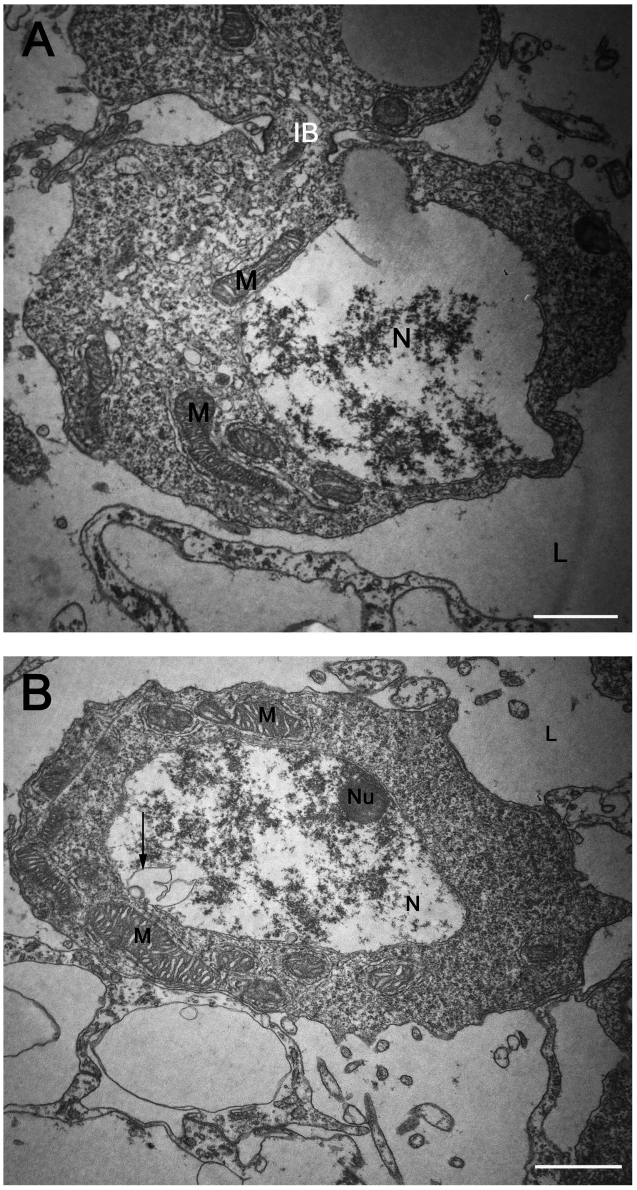

Fig. 3. A, TEM micrograph of two male gametes connected via an intercellular bridge (IB) recently released from the 'male' wall of the gonad of *Cyzicus gynecia* into the gonad lumen (L). Mitochondria (M) are dispersed throughout a regularly dense cytoplasm. The nucleus (N) of one of the male gametes consists of loosely compacted nuclear material. Scale bar = 1 μm. B, forming from within the nucleus of a male gamete (which contains both loosely compacted nuclear material and a well defined nucleolus (Nu) closely associated with the inner nuclear membrane) is a small invagination (arrow) against the inner nuclear membrane. Scale bar = 1 μm.

genetic reproduction within Spinicaudata. The inference of parthenogenesis has been refuted within *Eulimnadia* (Sassaman and Weeks, 1993; Zucker et al., 1997), *Limnadia* (Scanabissi and Mondini, 2002b), and now *Cyzicus*. The one last potential parthenogenetic species is an unidentified *Leptestheria* from Columbia (Roessler, 1995) that has also been inferred to be parthenogenetic only on the basis of a lack of males (Sassaman, 1995). Our prediction is that further study will prove that this *Leptestheria* sp. is also an all-hermaphroditic species, as has been found for all other in-depth examinations of purported parthenogenetic clam shrimp species.

The current finding of a unique derivation of selfing hermaphroditism within the otherwise dioecious Cyzicidae, combined with the previous reports of two separate derivations of selfing hermaphroditism from dioecy within Limnadiidae (Weeks et al., 2006, 2009), suggest a total of three separate derivations of selfing hermaphroditism within Spinicaudata. This supposition is supported by the physical location of the testicular tissue in these three lineages. It has previously been shown that testicular tissue in *E. texana* and *E. agassizii* is restricted to the posterior region of the hermaphroditic gonad (Zucker et al., 1997; Weeks et al., 2005). In hermaphrodites of *Limnadia lenticularis*, however, testicular tissue appears haphazardly scattered throughout the gonad tract (Zaffagnini, 1969; Scanabissi and Mondini, 2002b). In *C. gynecia*, the testicular tissue was found toward the anterior region of the gonad tract (directly behind the head region), suggestive of a third, independent derivation of hermaphroditism in this species. Additionally, within Notostraca, selfing hermaphroditism has also evolved at least once, if not many times (Sassaman, 1991; Sassaman et al., 1997; Zierold et al., 2007; Murugan et al., 2009). Thus, it appears that something about the ecology of these branchiopod crustaceans selects for unisexual reproduction, probably the selection for "reproductive assurance" in these early colonizing species (Pannell, 1997, 2002).

The repeated derivation of selfing hermaphroditism from dioecy suggests that the evolution of testicular tissue in formerly female branchiopods to form hermaphrodites (Weeks et al., 2009) must be more likely than the modification of meiosis to form parthenogenetic females (Sassaman, 1995). In some species/populations, the spread of such a hermaphroditic mutant has replaced the females to form androdioecious species (Sassaman et al., 1997; Weeks et al., 2009). In others, the hermaphrodites appear to have outcompeted both males and females to form all-hermaphroditic populations/species (Weeks et al., 2005, 2008). Future studies of these crustaceans that compare the life history and ecology of the dioecious to the androdioecious/hermaphroditic species may elucidate the conditions that select for hermaphroditism in the Branchiopoda.

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