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ANDRODIOECY INFERRED IN THE CLAM SHRIMP *EULIMNADIA AGASSIZII* (SPINICAUDATA: LIMNADIIDAE)

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ABSTRACT

Androdioecy (mixtures of males and hermaphrodites) is a rare mating system in both the plant and animal kingdoms. Androdioecy has been described in three branchiopod species, and is best known from the clam shrimp *Eulimnadia texana* Packard. Herein we describe sex ratio, genetic and histological evidence from the clam shrimp *Eulimnadia agassizii* Packard that suggest androdioecy is also found in this species. The *E. agassizii* population sampled had all-females, and when these females were isolated and allowed to produce eggs, those eggs yielded 100% female offspring in 15 out of 15 cases. Additionally, the originally isolated females proved to be completely homozygous at each of the six allozyme loci scored. The offspring from these isolated females also proved to be homozygous for the same alleles as their parent. Tissue sectioning of the gonad found that the "females" actually produced testicular tissue in the posterior portion of the gonad. Taken together, these data are entirely consistent with those of the androdioecious *E. texana*, and thus indicate that *E. agassizii* is also an androdioecious species, bringing the total number of branchiopod species with this form of reproduction to four.

Branchiopod crustaceans exhibit a broad array of reproductive mechanisms, ranging from complete apomixis in several cladoceran species (Hebert and Finston, 2001) to obligate outcrossing in most of the anostracan species (Clark and Bowen, 1976). The clam shrimp, originally classified as "conchostracans" but now split into the Laevicaudata, Spinicaudata, and Cyclestherida (Martin and Davis, 2001), encapsulate most of the overall reproductive variation found in the branchiopods as a whole (Sassaman, 1995). *Caenthestheriella gynecia* Mattox is described as strictly asexual (Mattox, 1950), *Cyclestheria hislopi* (Baird) exhibits cyclic parthenogenesis (Nair, 1968), *Limnadia lenticularis* (L.) is a self compatible hermaphrodite (Scanabissi and Mondini, 2002), *Eulimnadia texana* Packard is androdioecious (Sassaman and Weeks, 1993), and members of the genus *Lynceus* are obligately amphimictic (Sassaman, 1995).

Of these various mating systems, androdioecy is the least common (Pannell, 2002). Androdioecy is defined as populations comprised of males and hermaphrodites, but lacking true females. This mating system has been described in only a handful of animals, but has been found in three branchiopods: *Triops newberri* (Packard) (Sassaman, 1989b, 1991); *Eulimnadia antlei* Mackin (Sassaman, 1988); and *Eulimnadia texana* (Sassaman and Weeks, 1993). Of these three species, the specific mechanism of androdioecy has been most extensively examined in the latter species (Zucker *et al.*, 1997; Weeks *et al.*, 1999; Weeks and Zucker, 1999; Weeks *et al.*, 2000; 2001a; 2001b). In *E. texana*, maleness is determined by a recessive allele at a single sex-determining locus (Sassaman and Weeks, 1993). Hermaphrodites are comprised of two morphologically indistinguishable forms: the dominant homozygote, termed "monogenic," and the heterozygote, termed "amphigenic." Monogenic hermaphrodites can only produce hermaphrodites when self-fertilizing or mating with a male, while the amphigenic hermaphrodites produce 25% males when self-fertilizing and 50% males when mating with a male. Hermaphrodites lack the clasping appendages to allow the pairing necessary for mating, and thus hermaphrodites cannot fertilize one another (Sassaman and Weeks, 1993).

Although androdioecy has been found in two *Eulimnadia* species, most other species in this genus have not been examined for reproductive mode. The mating system of the uncommon *E. agassizii* has been only briefly mentioned in two publications. Asexuality has been inferred because of a lack of males in a population from Massachusetts (Zinn and Dexter, 1962), and Sassaman (1995) mentioned "unisexuality" in the rearing of offspring from eggs produced by isolated "females." However, neither author explored the mating system further.

Herein we report the results of examinations of the mating system of *E. agassizii*, which is a rare clam shrimp found in the northeastern United States (Smith, 1992). Evidence from offspring sex ratios, allozyme data, and histological examination suggest that the mating system of *E. agassizii* is the same as that described for *E. texana* (Sassaman and Weeks, 1993; Zucker *et al.*, 1997), with the "all-female" populations described for *E. agassizii* actually being monogenic hermaphrodites that have lost all males and amphigenic hermaphrodites.

MATERIALS AND METHODS

Determining the mating system in clam shrimp is a multi-step process (Fig. 1) of raising and isolating "female" (herein, "female" is used to denote that we do not yet know whether the individuals are females or hermaphrodites) clam shrimp, raising their offspring, determining sex ratios, determining allozyme inheritance patterns (Sassaman and Weeks, 1993), and finally examining the gonad to confirm the presence of ovotestes (Zucker *et al.*, 1997). If the "females" produce viable offspring in isolation, then they are either parthenogenetic females or self-compatible hermaphrodites. To distinguish between these alternatives, one notes whether there

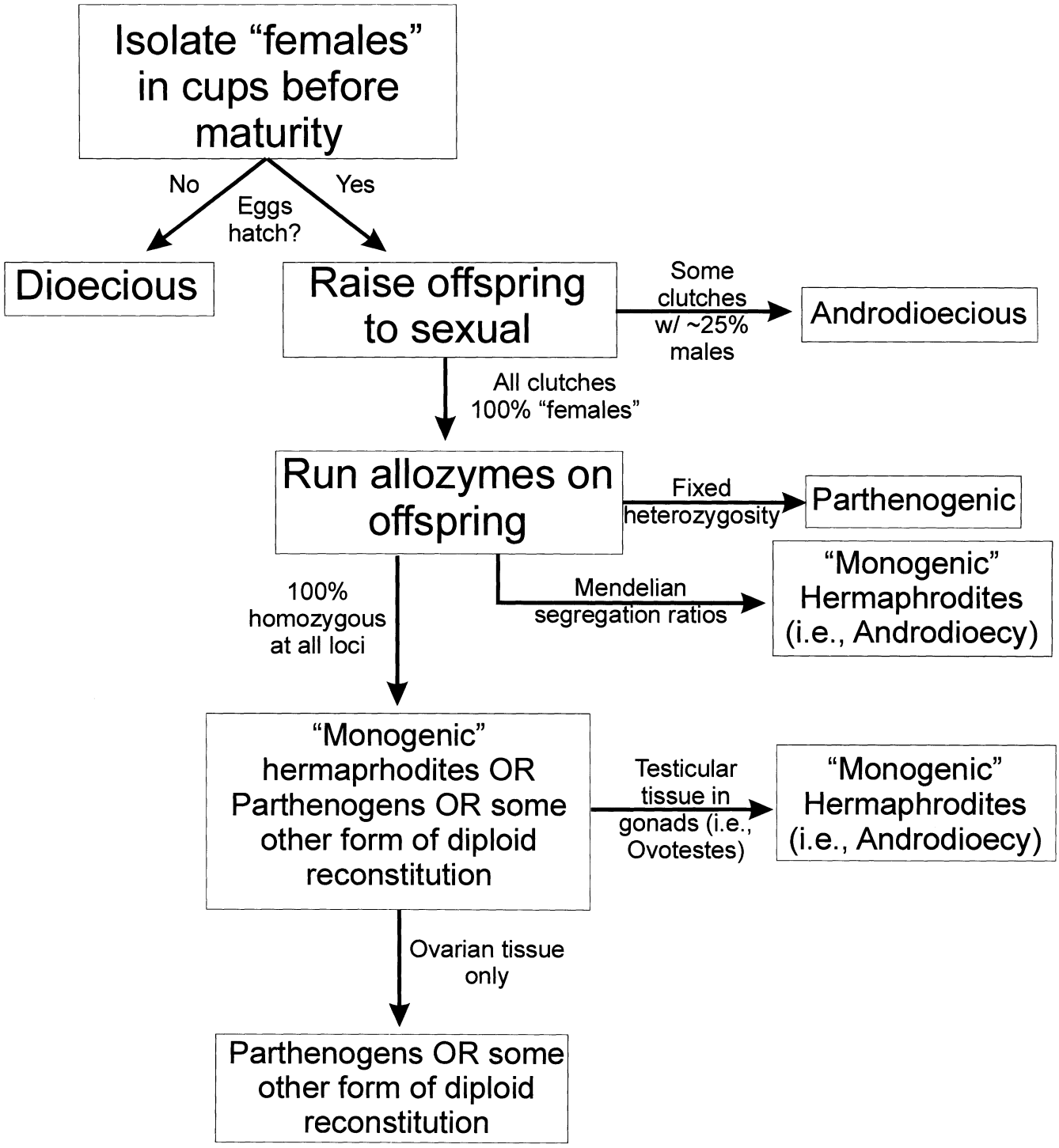

Fig. 1. Flow chart outlining procedures to detect type of mating system in clam shrimp.

are two classes of "females": ones that have no males among their unisexually produced offspring (monogenics) and ones that have ~25% males among their unisexually produced offspring (amphigenics; Sassaman and Weeks, 1993). The relative proportion of these two hermaphroditic types depends on the rate of outcrossing in the source population (Otto *et al.*, 1993). Because no other "female" organism has been described that produces 25% males when raised in isolation (Bull, 1983; Bell, 1985), finding any amphigenic hermaphrodites among the surveyed clam shrimp is most parsimoniously explained by assuming the species is androdioecious (Sassaman and Weeks, 1993). However, a lack of amphigenics can either be because the species is androdioecious but the population surveyed is comprised of only monogenic hermaphrodites, or because the species reproduces parthenogenetically (Fig. 1). Determining between these last two alternatives can be accomplished genetically or via sectioning of the gonad (Zucker *et al.*, 1997).

Rearing and Isolating Methods

Approximately 50 ml, by volume, of field-collected soil containing clam shrimp cysts was placed in the bottom of three-liter aquarium and hydrated with deionized water. The aquarium was maintained under "standard conditions" (Weeks *et al.*, 1997; 1999; 2001a) of 25°C, low aeration, constant light, and fed a mixture of baker's yeast and ground Tetramin™ flake fish food.

Table 1. Sex ratios among offspring from 15 isolated "female" *E. agassizii*. AAM = age at maturity; F = "females"; M = males. Note: sexing of offspring was discontinued after the first 100 randomly chosen offspring were sexed, if more than 100 offspring were available.

		Sex ratio	
ID	AAM	F	M
A4	9	100	0
A5	10	42	0
A12	9	27	0
A25	7	18	0
A26	13	100	0
A28	12	100	0
A30	10	80	0
A33	12	100	0
A36	12	100	0
A37	11	100	0
A39	13	80	0
A40	15	100	0
A52	11	100	0
A56	7	98	0
A63	11	100	0

Directly before sexual maturity, 73 "females" were isolated in 500-mL plastic cups containing approximately 5 mL of soil and filled with water from the above hatching tanks. Isolated "females" were allowed to lay eggs for seven days, and were then frozen for later electrophoretic typing. Eggs in the cups were then dried; the cups were sealed with lids, and then placed in the dark for more than 30 days.

Rearing of Laboratory Egg Banks

After storing, the 22 egg banks were hydrated using the methods above. The cups were checked daily for a period of two weeks for signs of hatching. Twenty of the 22 egg banks hatched, and the resulting nauplii were transferred to 10-L rearing aquaria containing 100 mL of soil and deionized water. Aquaria were maintained under standard conditions. Upon sexual maturity, the clam shrimp were sexed and frozen for electrophoretic typing.

Allozymes

To distinguish parthenogenetic females from monogenic hermaphrodites, allozyme patterns were scored on six enzyme loci: *Fum* (fumarate hydratase, EC 4.2.1.2), *Idh-1, Idh-2* (isocitrate dehydrogenase, EC 1.1.1.42), *Mpi* (mannose-phosphate isomerase, EC 5.3.1.8), *Pgm,* (phosphoglucomutase, EC 5.4.2.2), and *Pgi* (Glucosephosphate isomerase, EC 5.1.3.09). These six loci were scored in 44 adults and offspring from six egg banks. Electrophoretic assays were conducted using cellulose acetate electrophoresis (Richardson *et al.*, 1986). All gels were run using "Buffer C" from Richardson *et al.* (1986). Allele designations were labeled by increasing anodal mobility. They are reported relative to allele mobility in *E. texana* (Weeks and Duff, 2002).

Fixing and Embedding for Transmission Electron Microscopy (TEM)

The specimens were fixed in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2) for two hours at 4°C. The specimens were further washed in 0.1 M buffer and were then postfixed in 1% OsO_4 in the same buffer for one hour at 4°C. Samples were processed in a graded acetone series using propylene oxide and then embedded in an Epon-Araldite mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and observed through a Philips EM 410 electron microscope. Semi-thin sections, 0.5–1.0 μm thick, were stained with toluidine blue for light microscope observations.

Results

From the initial soil sample from Connecticut, 361 "females" were reared, and no males were found. Seventy-three of these "females" were isolated for seven days to produce eggs, and 22 of these were hydrated to determine whether the "females" produced viable offspring in the absence of males. Twenty of these 22 egg banks (91%) hatched, of which 15 (75%) sets of offspring successfully grew to adulthood. From these 15 egg banks, a total of 1772 offspring were reared (Table 1). Not a single male was found in any of these 1772 offspring.

Table 2. Genotypic scores from six allozyme loci from 44 shrimp. No heterozygotes were found for any shrimp. Anodal scores are reported relative to those reported in Weeks and Duff, 2002.

Genotype	*Pgm*	*Idh-1*	*Idh-2*	*Fum*	*Mpi*	*Pgi*
aa	20	1		43		
bb		42				
cc			44			
dd					40	44

Allozyme patterns were scored from 44 of the original 361 clam shrimp. We found no heterozygotes for any of the six allozyme loci scored (Table 2). In fact, five of the six allozyme loci were fixed for a single allele, and the sixth locus (*Idh-1*) had one single individual that was found to be homozygous for one additional allele (Table 2).

In six of the above 44 clam shrimp, we found ambiguous banding patterns that could have been interpreted as being either homozygous or heterozygous at one or more loci. We then proceeded to rear offspring from those "females" to note the inheritance of the alleles among their offspring. For each of the original six parents, all offspring within clutches were found to be completely homozygous for the ambiguous score of their "female" parent (Table 3). Thus, none of the original allozyme scores were actually heterozygous, and the offspring from a homozygous parent were identically homozygous for any particular locus examined.

Tissue sectioning through the posterior region of the *E. agassizi* gonad showed a tubular organ which lies between the midgut and the body wall. Two different organizations and distributions of the germ cells were found (Fig. 2). The first was identical to those observed in an ovary of other described Limnadiidae species, presenting a wall around the lumen acting as an eggshell secretory organ and intermixed female germ cells that develop towards the hemocoel.

The second organization differed from the first in that it clearly was organized as an ovotestis, with an unusual

Table 3. Inheritance patterns in clutches of offspring from an isolated "female" which had ambiguous scores at one or more allozyme loci (ambiguity noted by "?").

			Offspring Score			
Parent	Locus	Adult Score	aa	bb	cc	dd
A-1	*Fum*	aa?	8			
A-4	*Fum*	aa?	10			
	Mpi	dd?				10
A-5	*Fum*	aa?	10			
	Mpi	dd?				10
A-7	*Fum*	aa?	8			
A-39	*Fum*	aa?	10			
	Idh-1	aa?	6			
	Mpi	dd?				7
	Pgi	dd?				10
A-56	*Fum*	aa?	10			
	Idh-1	aa?	10			
	Pgi	dd?				10

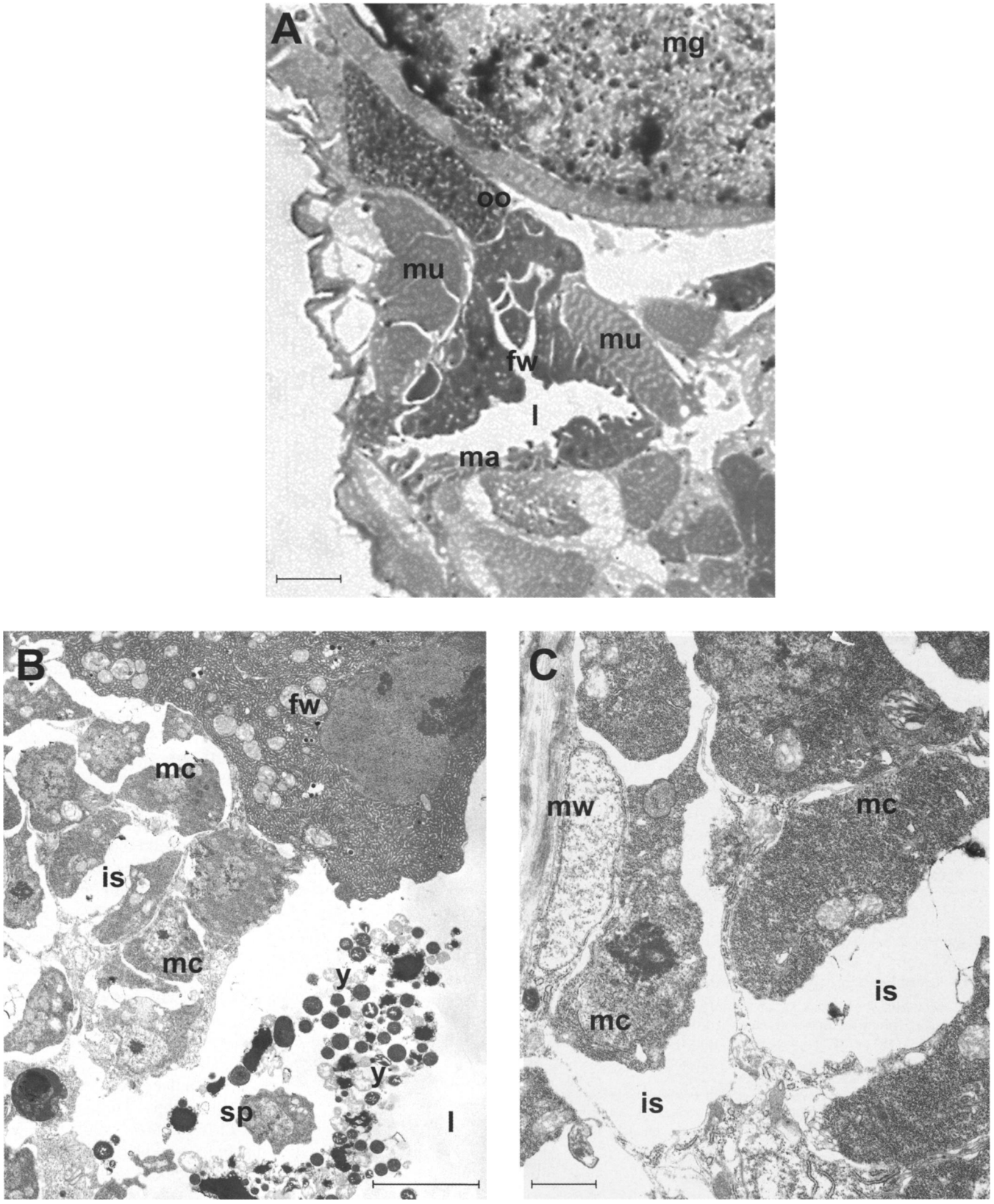

Fig. 2. A. Semithin section of *E. agassizii* posterior quarter. At the center the ovotestis, squeezed by the muscles (mu), two regions are found around the lumen (l): the female region fills approximately three quarters of the gonad (female wall, fw) and faces the midgut (mg); the thin male area (ma) is located towards the body wall. One mature oocyte (oo) is inside the hemocoel. Scale bar, 33 μm. B. Ultrastructural view of the ovotestis. Upper, female wall (fw); lower, male area, composed by male germ cell (mc) inside the intracellular spaces (is) of the squamous epithelium. In the gonad's lumen (l) a sperm (sp) and yolk remnants (y) are visible. Scale bar, 5 μm. C. Male zone. Epithelial cells act as male wall (mw). Their large intracellular spaces (is) contain the male germ cells (mc); Scale bar, 1 μm.

distribution of the male region. The latter is a well-defined area occupying a quarter of the gonad. The male area is made up of a multilayered epithelium (thickness approximately 6 μm) acting as a wall, and by male germ cells. The epithelial cells are stretched and present a thin cytoplasm extended in several bridles (showing many septate desmosomes) thus forming large intracellular spaces, typical of the "maturation in vacuoles" model described by Wingstrand (1978). The epithelial cells do not show any glandular activity, as found in the female wall cells that are linked to eggshell construction and are more distinctly protruding towards the lumen. Every male germ cell originates and matures in the intracellular spaces of a single somatic cell, which envelops them until they pour into the gonad's lumen. The sperm are typical, ameboid cells, of ~2.5 μm diameter. The earlier stages are not well distinguished from the mature stage, having not found any sign of meiosis. The cytoplasm appears very electrondense due to the ribosomes and mitochondria, strongly contrasting with the wall cell appearance. No signs of sperm degeneration have been found.

Discussion

Androdioecy in the branchiopods was first elucidated in the clam shrimp *Eulimnadia texana* (see Sassaman and Weeks, 1993). By performing a series of progeny tests of offspring produced by either "females" reared in isolation or reared with a male, and by noting Mendelian inheritance of alleles among offspring of heterozygous "females," Sassaman and Weeks determined that *E. texana* populations were actually mixtures of two types of hermaphrodites (monogenics and amphigenics) and males. Since then, androdioecy has been inferred in two other branchiopods: *Triops newberri* and *Eulimnadia antlei* (see Sassaman, 1995), but has not been described in any other crustacean.

All of the data reported herein are completely consistent with the notion that this population of *E. agassizii* is androdioecious and that all "females" are actually monogenic hermaphrodites. Monogenic hermaphrodites of *E. texana* are homozygous for the sex determining allele, and thus when selfed, produce only monogenic hermaphroditic offspring (Sassaman and Weeks, 1993).

In populations that are founded by either one or a few monogenic propagules, or in which selfing has been the favored form of reproduction, heterozygosity across all loci will quickly be lost due to inbreeding. In fact, in 10 generations of selfing, over 99.9% of the initial heterozygosity of the population is lost due to inbreeding (Wright, 1958). Additionally, overall allelic diversity in such populations is usually low due to genetic drift and the potentially small number of founders for such populations (Kimura, 1983). Thus, a population of all hermaphrodites that cannot cross with one another (as in *E. texana*) should be expected to be highly homozygous and have low levels of allelic diversity. This pattern contrasts sharply with the expectations from a parthenogenetic species (Fig. 1), in which heterozygosity is fixed in parents and offspring, and in fact should accumulate over time, as alleles at a given locus mutate and diverge over generations (Normark *et al.*, 2003).

Low levels of heterozygosity and low allelic diversity typify populations of *E. texana* (Sassaman, 1989a; Weeks and Zucker, 1999; Weeks and Duff, 2002). In fact, level of heterozygosity is directly proportional to the frequency of males in the population, with the lowest levels of heterozygosity being reported from populations with no males (Sassaman, 1989a; Weeks and Zucker, 1999). Hermaphrodites isolated from these populations have been found to be entirely monogenic (Weeks, unpubl. data).

The *E. agassizii* population studied herein revealed sex ratio and genetic patterns entirely consistent with these previous reports on *E. texana*. No males were found out of 361 clam shrimp, and 15 out of 15 egg banks produced 100% "female" individuals when reared to maturity. Additionally, all 44 adults surveyed were completely homozygous at all six loci examined, and offspring surveyed from six of these parents were also homozygous for the same loci as their parents. All of these data are consistent with the patterns exhibited by all-monogenic populations of *E. texana*, as noted above.

Anatomical evidence from *E. texana* also confirms the genetic results of Sassaman and Weeks (1993). Zucker *et al.* (1997) reported finding a small area of the posterior portion of the ovotestis that was devoted to sperm production. This result confirmed the previous genetic surveys, and thus verified that *E. texana* "females" were in fact truly hermaphroditic.

The results of surveys of *E. agassizii* "females" mirrored those found in *E. texana*. The testicular portion of the ovotestis was also found to be in a small posterior portion of the ovotestis, similar to that found in *E. texana*, as noted in Zucker *et al.* (1997). Interestingly, only one of the two hermaphrodites surveyed displayed this tissue, suggesting that some "hermaphrodites" may in fact be truly females or that hermaphroditism may be sequential in this species. Further examinations of these clam shrimp must be undertaken to distinguish these two possibilities.

Taken together, the offspring sex ratio, genetic, and anatomical evidence all suggest that *E. agassizii* has the same form of reproduction as *E. texana*, and thus suggest that the "all-female" populations of *E. agassizii* (see Zinn and Dexter, 1962) are in fact monogenic hermaphrodites. Interestingly another species in the family Limnadiidae, *Limnadia lenticularis*, also has genetic and anatomical patterns consistent with androdioecy, although it has yet to be formally described as androdioecious. Populations of *L. lenticularis* from Europe have exceptionally low levels of heterozygosity (Tinti and Scanabissi, 1996), have very low levels of males (Eder *et al.*, 2000), and have testicular tissue in their gonad (Scanabissi and Mondini, 2002). However, previous authors have assumed that this testicular tissue was rudimentary and nonfunctional, and assumed that unisexual reproduction was the product of polar body re-fusion in "females" (Zaffagnini, 1969). Since then, Tinti and Scanabissi (1996) have shown that *L. lenticularis* produce viable sperm, and that they are, thus, functional hermaphrodites.

Because *Eulimnadia texana*, *Eulimnadia antlei*, *Limnadia lenticularis*, and now *Eulimnadia agassizii* all are showing consistent patterns among their reproductive systems, it is probable that all four have the same sex determining mechanism outlined by Sassaman and Weeks (1993) for *E. texana*, namely a single locus (or a group of tightly linked loci that are inherited as virtually a single locus

(Weeks *et al.*, 2001a), with the dominant locus coding for hermaphrodites and males being recessive homozygotes. If *Limnadia* and *Eulimnadia* form a monophyletic clade, then it is likely that this reproductive mode is representative of the common ancestor to both genera. Future examinations of species in the genus *Limnadia* that show equal sex ratios (Sassaman, 1995) need to be undertaken to note the reproductive mechanisms in these species, from which we may paint a more complete picture of the mode of evolution of reproductive systems in these interesting crustaceans.

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