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Breeding systems in the clam shrimp family Limnadiidae (Branchiopoda, Spinicaudata)

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Abstract. Crustaceans in the class Branchiopoda exhibit a wide range of breeding systems, including dioecy (gonochorism), androdioecy, parthenogenesis, cyclic parthenogenesis, and hermaphroditism. The largest subgroup of the Branchiopods, the Diplostraca, is reported to encompass all five of these breeding systems. However, many of these reports are based primarily on simple observations of sex ratios in natural populations. Herein we report the beginnings of a more rigorous approach to breeding system determination in the Diplostraca, starting with the family Limnadiidae. We combine measurements of sex ratio, offspring rearings, and behavior to identify three breeding systems within the Limnadiidae: dioecy, androdioecy, and selfing hermaphroditism. To date, no instances of parthenogenetic reproduction have been identified in this family. Comparisons of breeding system determination via simple population sex ratios with our more controlled studies show that simple sex ratios can be useful when these sex ratios are ~50% males (=dioecy) or 5–30% males (androdioecy). However, population sex ratios of 0–5% males or 35–45% males necessitate further investigation because estimates in these ranges cannot distinguish selfing hermaphroditism from androdioecy or androdioecy from dioecy, respectively. We conclude by noting that the genetic sex-determining system outlined for one of these limnadiid species, *Eulimnadia texana,* provides a parsimonious framework to describe the evolution of the three breeding systems observed within the Limnadiidae.

Additional key words: mating systems, androdioecy, selfing hermaphroditism

Branchiopods are a diverse assemblage of geographically widespread crustaceans that vary extensively in morphology (Olesen 2007) and the group is arguably the oldest crustacean class that contains living members (Martin & Davis 2001). Recent branchiopods include Anostraca (fairy shrimp), Notostraca (tadpole shrimp), ''Conchostraca'' (clam shrimp), and Cladocera (water fleas). The latter two taxa are often considered the ''Diplostraca'' (Olesen 1998, 2007), a classification that has been supported recently via DNA-based phylogenetic comparisons (Negrea et al. 1999; Braband et al. 2002).

A diverse array of breeding systems is found within the Diplostraca: dioecy (also called ''gonochorism''=separate males and females), androdioecy (males and hermaphrodites), selfing hermaphroditism, cyclic parthenogenesis (many bouts of parthenogenetic reproduction punctuated with single bouts of sexual reproduction), and parthenogenesis (Hebert & Finston 1993; Sassaman 1995). Most of the research on breeding systems has been on species in the order Cladocera (reviewed in Hebert 1987; Mort 1991a, b; Larsson & Weider 1995) with only limited coverage of breeding systems in the remaining Diplostraca (Sassaman 1995).

Sassaman (1995) surveyed the range of breeding systems described in the ''Conchostraca'': dioecy, androdioecy, parthenogenesis, and cyclic parthenogenesis. Almost all of the data covered in his review were simple observations of sex ratios in the various species: species with no males were assumed to be parthenogenetic, species with ''female''-biased sex ratios (herein, ''female'' is used to denote that the true sex [female or hermaphrodite] is not yet known) were assumed to be androdioecious, and species with 50:50 sex ratios were assumed to be dioecious. Inferring androdioecy from highly ''female''-biased (actually hermaphrodite-biased) sex ratios has been supported

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within the genus *Eulimnadia* (Weeks et al. 2006b). However, the validity of inferring dioecy and parthenogenesis from 50:50 sex ratios and lack of males, respectively, has not been empirically examined. In fact, species previously considered parthenogenetic on the basis of a lack of males (*Eulimnadia agassizii* Packard 1874 and *Limndadia lenticularis* Linné 1761) recently have proven to be selfing hermaphrodites (Scanabissi & Mondini 2002; Weeks et al. 2005). Thus, a more complete survey of the breeding systems of the ''Conchostraca'' is warranted to allow us to evaluate breeding system variability within the clam shrimp more accurately, as well as to test the notion that simple population sex ratios reflect underlying breeding systems in these crustaceans.

The family Limnadiidae is an appropriate place to start this survey. This family is the largest in the ''Conchostraca'' and purportedly includes three of the five breeding systems described in the Diplostraca: dioecy, selfing hermaphroditism, and androdioecy. However, except for a few studies (Sassaman & Weeks 1993; Weeks et al. 2005, 2006b), breeding system assignments for species in the Limnadiidae have been made only on the basis of sex ratios (Sassaman 1995). The remaining two diplostracan breeding systems (parthenogenesis and cyclic parthenogenesis) are primarily found in the Cladocera which, as stated above, have been well explored. Thus, the Limnadiidae appear to hold promise as a taxon that will substantially expand our understanding of the breeding systems within the Diplostraca.

Herein we report on a survey of sex ratios and breeding systems from 20 described species [*Eulimnadia africana* Brauer 1877, *Eulimnadia agassizii, Eulimnadia brasiliensis* Sars 1902, *Eulimnadia braueriana* Ishikawa 1895, *Eulimnadia colombiensis* Roessler 1989, *Eulimnadia cylindrova* Belk 1989, *Eulimnadia dahli* Sars 1896, *Eulimnadia diversa* Mattox 1937, *Eulimnadia feriensis* Dakin 1914, *Eulimnadia follisimilis* Pereira & Garcia 2001, *Eulimnadia inflecta* Mattox 1939, *Eulimnadia texana* Packard 1871, *Eulimnadia thompsoni* Mattox 1939, *Imnadia yeyetta* Hertzog 1935, *Limnadia lenticularis, Limnadia badia* Wolf 1911, *Limnadia sordida* King 1855, *Limnadia stanleyana* King 1855, *Limnadopsis parvispinus* Henry 1924, and *Limnadopsis tatei* Spencer & Hall 1896] and 12 populations of undescribed species (six *Eulimnadia,* three *Limnadia,* and three *Limnadopsis*). These species were collected from 86 populations that included samples from every continent except Antarctica (where no extant clam shrimp have been reported). Our intentions were to extend our understanding of breeding systems in the family Limnadiidae by expanding our descriptions of these systems beyond mere sex ratio information (Sassaman 1995).

To conduct such a broad scale examination of mating systems, we sought a relatively uncomplicated method of breeding system assessment in these clam shrimp that does not necessitate costly genetic assays (Sassaman & Weeks 1993) and/or transmission electron microscopic assays (Zucker et al. 1997; Scanabissi & Mondini 2002; Weeks et al. 2005). We settled on a combination of egg hatching, offspring rearing, and behavioral scoring that allowed us to assign 48 of the 86 limnadiid populations into three breeding system types: selfing hermaphrodites, androdioecy, and dioecy. We then compared population sex ratio data among these populations to gauge how well this simple metric reflects true breeding system assignments. These methods allow us to better document the variety of breeding systems in the family Limnadiidae and allow a wider view of the evolution of these systems in the Diplostraca more generally.

Methods

Rearing from soil

For each of the populations that were reared from soil samples, we collected soil from the various field sites. Soil collection was carried out by sampling at many spots across the dried pools and then homogenizing the soil in plastic bags after collection (via breaking apart the soil into small particles). Approximately 500 mL of this field-collected soil was placed in the bottom of a 37-L aquarium and hydrated with deionized water. The aquarium was maintained under ''standard conditions'' (Weeks et al. 1997, 1999, 2001) of 25°–28°C, low aeration, constant light, and fed a mixture of baker's yeast and ground Tetramin™ flake fish food (2.5 g of each suspended in 500 mL of water).

Directly before sexual maturity, ''females'' were isolated in 500-mL plastic cups containing ~5 mL of soil from a source in New Mexico known not to contain branchiopod cysts (Weeks 2004) and filled with water from the above hatching tanks. There is a period of ~1 d before the shrimp become fully sexually mature, during which we can detect the developing eggs in the ovary/ovotestis for females/hermaphrodites and we can see the developing claspers for males. Thus, we can isolate individuals before they sexually mature and keep them from cross-fertilizing (Weeks et al. 2000). After the shrimp matured, total sex ratios (=population sex ratios) were calculated per hydration. Isolated ''females'' were observed for 7 d, and those that laid eggs were then frozen for later

projects. Eggs in the cups were dried, the cups were sealed with lids, and these "egg banks" were placed in the dark for >30 d for later hatching.

"Females" that did not produce eggs were then paired with a male in a separate, 500-mL isolation cup and allowed to produce eggs for another 7 d. At the end of this time, both male and "female" were frozen and the collected eggs were dried, the cups were sealed with lids, and egg banks were placed in the dark for >30 d for later hatching.

Field collections

In field-collected samples, adult shrimp were caught from natural pools using a small mesh dip net. Adults were sexed using a magnifying glass and a sub-sample was preserved in 100% ethanol for later morphological verification. In some cases, live individuals were transferred back to the laboratory to isolate in 500-mL plastic cups to collect egg banks.

Rearing from egg banks

After storing, the egg banks were hydrated in their 500-mL cups using deionized water and kept at 25°–28°C under constant light. The cups were checked daily for a period of 2 weeks for signs of hatching. Any hatching nauplii were transferred to 10-L rearing aquaria containing 100 mL of soil and ~10 L of deionized water. Aquaria were maintained under standard conditions. Upon sexual maturity, the clam shrimp were sexed.

Reproductive assignment

The methods by which we determined reproductive mode are based on those used to determine the breeding system for *Eulimnadia texana* (Sassaman & Weeks 1993) and for *Eulimnadia agassizii* (Weeks et al. 2005). To distinguish among the three breeding systems (selfing hermaphroditism, androdioecy, and dioecy), we used the following tiered assignment procedures. The most definitive assignment of breeding system occurred when offspring were successfully reared from isolations or matings done in the lab. To assign a population the breeding system of hermaphroditism or androdioecy, viable eggs would need to be successfully produced by "females" in isolation (Sassaman & Weeks 1993; Weeks et al. 2005). The resulting egg banks (denoted "Iso" egg banks) were dried and later hydrated. After hatching, the nauplii were reared to adulthood and sexed.

Populations were determined to be hermaphroditic if all of the isolated "females" produced 100% "female" offspring (Weeks et al. 2005). Populations were determined to be androdioecious if the isolated "females" were a mix of those that produced all "female" offspring (termed "monogenics") and those that produced ~20–25% males among their offspring (termed "amphigenics," Sassaman & Weeks 1993). Males are known to have higher mortality than hermaphrodites in androdioecious *Eulimnadia* (Zucker et al. 2001), and thus having offspring sex ratios <25% males at the age of sexual maturity is not unexpected. In the Zucker et al. (2001) study, offspring reared under similar laboratory conditions as in the current study showed that males survived at ~85% of the rate of hermaphrodites. Thus, we should expect that increased rates of male mortality should yield 20–25% male offspring at sexual maturity rather than the 25% males predicted at birth (Sassaman & Weeks 1993).

To assign a population the mating system dioecy, isolated "females" ("Iso" egg banks) had to produce few or no eggs during the 1-week period in an isolation cup without a male. These "females" were then paired with males and allowed to produce eggs for one additional week in a new cup ("Mate" egg banks). Both Iso and Mate egg banks were then dried for >30 d. These two categories of egg banks (Iso vs. Mate) from the same "female" were simultaneously hydrated to test whether the Iso egg banks had fewer hatches than the Mate egg banks. If eggs from either egg bank hatched, the resulting nauplii were reared to adulthood (when possible) and then sexed to note whether they produced ~50% males among their offspring. The combination of producing low to no offspring when in isolation (although no offspring should be produced without males, some dioecious females may have mated with early developing males before isolation, and these fertilized eggs then can be released into "isolation" cups; see results below) and producing ~50% males when eggs did hatch would place these populations into the dioecious category.

If egg banks (either those from "females" that produced eggs in isolation or from "females" that only produced eggs when paired with a male) did not produce offspring that survived to be sexed, then a second approach to breeding system assignment was attempted under the following conditions: if eggs were moved to the brood chamber only when "females" were paired with males and these eggs had a significantly higher hatching rate than eggs in the Iso egg banks, the "female" was determined to be dioecious.

In all other instances when offspring could not be reared to sexual maturity, and thus sexed, a breed-

Table 1. Numbers of male and hermaphrodite/female (H/F) offspring reared from isolated (Cup = I) or mated (Cup = M) hermaphrodites or females, respectively. In the androdioecious populations, columns labeled "Mono" or "Amph" denote the number of monogenic and amphigenic parents of the offspring represented in this table. In the hermaphroditic populations all individuals were labeled "Mono." In the dioecious populations, all individuals were labeled "Amph." "% Male" represents the percentage of offspring that were male and "SE%" is the standard error of that percentage. Entries of "N" in columns labeled "0%," "25%," and "50%" denote male percentages that do not significantly differ from 0%, 25%, and 50% males, respectively. Note that in populations with Cup = I and male percentages >0%, the sex ratios are for "amphigenic" hermaphrodites only. The column labeled "BrdSys" is for breeding system assignment: A = androdioecious, H = hermaphroditic, and D = dioecious (see text for determinations of breeding system). Three populations were assigned "A?" because they primarily conformed to expectations of androdioecy but had slight discrepancies (see text for outlines of these discrepancies). Four populations had offspring data that did not conform to expectations of any one breeding system and thus were assigned question marks to denote that breeding system could not be fully resolved.

Genus	Species	Location	Pool	Cup	Mono	Amph	Male	H/F	% Male	SE %	0%	25%	50%	BrdSys
Eulimnadia	*africana*	Botswana	94-63	I	2	8	411	1332	23.6	1.0		N		A
Eulimnadia	*africana*	Botswana	Thomaga 13	I	1	8	146	270	35.1	2.3				A?
Eulimnadia	*agassizii*	Massachusetts		I	20	0	0	1772	0.0	0.0	N			H
Eulimnadia	*brasiliensis*	Brazil	1	I	4	3	89	460	16.2	1.6				A?
Eulimnadia	*brasiliensis*	Brazil	2	I	10	7	67	293	18.6	2.1				A
Eulimnadia	*brasiliensis*	Brazil	3	I	10	12	161	739	17.9	1.3				A
Eulimnadia	*braueriana*	Japan	Hojo	I	7	13	249	791	23.9	1.3		N		A
Eulimnadia	*braueriana*	Japan	Otsu	I	20	26	425	1838	18.8	0.8				A
Eulimnadia	*braueriana*	Taiwan		I	29	3	126	366	25.6	2.0		N		A
Eulimnadia	*colombiensis*	Venezuela	Margarita	I	5	11	118	449	20.8	1.7				A
Eulimnadia	*cylindrova*	Galapagos		I	6	12	85	380	18.3	1.8				A
Eulimnadia	*cylindrova*	Mexico	Baja, CA	I	7	6	102	236	30.2	2.5		N		A
Eulimnadia	*dahli*	Australia	Bunjil	I	9	4	25	37	40.3	6.2			N	?
Eulimnadia	*dahli*	Australia	The Humps	I	11	10	241	679	26.2	1.4		N		A
Eulimnadia	*diversa*	Arizona	Alb	I	12	1	25	75	25.0	4.3		N		A
Eulimnadia	*diversa*	Arizona	Bap	I	14	10	105	455	18.8	1.6				A
Eulimnadia	*diversa*	Arizona	Gaz	I	2	0	0	176	0.0	0.0	N			H
Eulimnadia	*diversa*	Arizona	Tri	I	4	2	37	105	26.1	3.7		N		A
Eulimnadia	*diversa*	Florida	Gain4	I	4	0	0	172	0.0	0.0	N			H
Eulimnadia	*diversa*	Indiana		I	6	12	55	214	20.4	2.5		N		A
Eulimnadia	*diversa*	Mississippi	#8	I	2	4	120	441	21.4	1.7		N		A
Eulimnadia	*diversa*	Nebraska	1	I	6	9	292	1099	21.0	1.1				A
Eulimnadia	*feriensis*	Australia	Cairns Rock	I	17	21	237	895	20.9	1.2				A
Eulimnadia	*feriensis*	Australia	Outcrop	I	23	1	1	47	2.1	2.1	N			?
Eulimnadia	*feriensis*	Australia	TAM2	I	10	0	0	240	0.0	0.0	N			H
Eulimnadia	*feriensis*	Australia	WAC2	I	0	2	15	59	20.3	4.7		N		A
Eulimnadia	*feriensis*	Australia	WAR9	I	2	2	9	102	8.1	2.6				A?
Eulimnadia	*feriensis*	Australia	Wave Rock	I	14	21	106	267	28.4	2.3		N		A
Eulimnadia	*feriensis*	Australia	YOR1	I	2	5	65	271	19.3	2.2				A
Eulimnadia	*feriensis*	Australia	YOR2	I	22	0	0	1939	0.0	0.0	N			H
Eulimnadia	*feriensis*	Australia	YOR10	I	4	10	43	121	26.2	3.4		N		A

Table 1. Continued.

Genus	Species	Location	Pool	Cup	Mono	Amph	Male	H/F	% Male	SE %	0%	25%	50%	BrdSys
Eulimnadia	*follisimilis*	New Mexico		I	15	15	120	461	20.7	1.7				A
Eulimnadia	*inflecta*	Louisiana	CD	I	13	0	0	1626	0.0	0.0	N			H
Eulimnadia	*texana*	New Mexico	JD1	I	5	33	500	1515	24.8	1.0		N		A
Eulimnadia	*texana*	New Mexico	JT4	I	23	37	262	949	21.6	1.2				A
Eulimnadia	*texana*	New Mexico	SWP5	I	17	42	546	1799	23	0.9		N		A
Eulimnadia	*texana*	Arizona	WAL	I	18	30	240	1102	17.9	1.0				A
Eulimnadia	*thompsoni*	Illinois	URB3	I	6	9	246	1120	18.0	1.0				A
Eulimnadia	n sp.	Georgia	Stone Mtn	I	4	7	18	81	18.2	3.9		N		A
Eulimnadia	sp.	Australia	DevMarb1	I	0	8	30	88	25.4	4.0		N		A
Eulimnadia	sp.	Australia	Jim Jim	I	3	2	6	39	13.3	5.1				A
Imnadia	*yeyetta*	Austria		M		26	439	482	47.7	1.6			N	D
Limnadia	*lenticularis*	Florida	Pond 19	I	4	0	0	47	0.0	0.0	N			H
Limnadia	*lenticularis*	Florida	Pond 21	I	6	1	3	6	33.3	15.7		N	N	?
Limnadia	*sordida*	Australia	WAC2	M		10	27	34	44.3	6.4			N	D
Limnadia	*stanleyana*	Australia	Kanangra3	M	2	2	3	10	23.1	11.7		N		?
Limnadopsis	*parvispinus?*	Australia	KAD	M		3	55	68	44.7	4.5			N	D

ing system was not assigned. Additionally, if the offspring rearings produced conflicting or incomplete results, the breeding system was assigned "?" to denote the inconsistent rearing results (as opposed to a blank assignment, which denoted a lack of offspring rearing data). There were two levels of questionable assignments in the offspring rearing experiments: (1) populations in which data was primarily, but not completely, consistent with one breeding system were denoted by adding the question mark behind the breeding system assignment (e.g., "A?") or (2) populations in which offspring data were not consistent with any one breeding system were simply denoted as "?".

Statistical comparisons

Deviations of sex ratios from expectations were examined by constructing 95% confidence intervals. Expected sex ratios for dioecious species were 50% males for both "population" and "offspring" sex ratio comparisons. Expected sex ratios for hermaphroditic populations were 0% males for both "population" and "offspring" comparisons (Weeks et al. 2006b). Expected sex ratios for androdioecious populations were that some "females" produced 0% males (monogenics) while others produced 25% males (amphigenics) among their offspring (Sassaman & Weeks 1993). Because the ratio of monogenics to amphigenics in natural populations determines overall ("population") sex ratios (Otto et al. 1993), no specific sex ratio is expected in androdioecious populations. Chi-squared analyses were performed on (1) comparing hatching success of egg banks collected from "females" that were isolated from males (Iso), versus these same "females" that were then mated to males (Mate), and (2) comparing the number of the three breeding system types that were categorized as being significantly different from 0% or 50% males in the population sex ratio data.

Results

Table 1 shows the results of offspring hatching for egg banks collected from "females" in this study. For example, 10 total isolated (I) "females" from Botswana (pool 94-63) produced viable offspring. Two of these 10 sets of offspring were 100% "females" and thus these two isolated parents were inferred to be monogenics (see Sassaman & Weeks 1993). The remaining eight egg banks produced males and "females," and among these offspring, a total of 411 males and 1332 "females" were produced

(23.6 ± 1.0% males). This male sex ratio was significantly different from both 0% and 50% male but not different from 25% male. These sex ratios are predicted by the genetic sex determining model for androdioecy in the genus *Eulimnadia* (see Weeks et al. 2006b), and thus the "females" in this population were inferred to be mixtures of monogenic and amphigenic hermaphrodites and the population was assigned the breeding system of androdioecy (see A in Table 1: Sassaman & Weeks 1993). Successful hatching of collected egg banks occurred in 47 of the 86 populations (Table 1).

There are three separate expected offspring sex ratios for the three breeding systems present in this family. Selfing hermaphroditic populations should have individuals that successfully produce offspring in isolation and all of the resulting offspring should be "female." Seven of the 47 populations had only individuals that produced 0% males among their offspring: one population of *Limnadia lenticularis* and six of *Eulimnadia* (Table 1). A combined total of 5972 offspring were reared from these seven populations. The "females" from these populations were therefore considered hermaphrodites on the basis of producing offspring in isolation and having no males among these offspring. There was one remaining population (Outcrop) that had low male offspring production: 23 "females" produced no males out of a total of 687 offspring, while one "female" produced one male out of 48 offspring (2.1% male). Because the one "female" that produced males did not produce nearly enough males to be considered "amphigenic" (i.e., to be amphigenic, ~12 males should have been produced out of 48 total offspring, see Sassaman & Weeks 1993), we did not consider the population androdioecious. Because this "female" could not be considered either monogenic or amphigenic, we assigned "?" to denote conflicting data for this population.

Androdioecious limnadiid populations should have individuals that successfully produce offspring when reared in isolation, and these isolated individuals should be mixtures of "monogenic" and "amphigenic" hermaphrodites—the former producing no males while the latter producing ~20–25% males when reared in isolation (see Sassaman & Weeks 1993; Weeks et al. 2006b). "Amphigenic" hermaphrodites are unique to branchiopods (see Weeks et al. 2006a), and thus comparing observed sex ratios to the expectation of 20–25% males can determine androdioecy in these clam shrimp (see Weeks et al. 2006b). In Table 1, therefore, the number of "females" that produced no males among their offspring when reared in isolation (monogenics) were separated from those that produced males when reared in isolation (amphigenics). Thirty-seven of the 47 populations had mixtures of monogenics and amphigenics, indicative of androdioecious populations. All but two of these 37 were *Eulimnadia;* the two exceptions were in the genus *Limnadia:* one population of *L. lenticularis* (Pond 21) and one of *L. stanleyana* (Kanangra 3).

We tested whether the offspring produced by amphigenics significantly deviated from the expected 20–25% males. We found that 32 of the 47 populations had offspring sex ratios that did not differ from 20–25% males; again, all but two of these 32 were in the genus *Eulimnadia* (Table 1). The same two populations noted above (Pond 21 and Kanangra 3) had small total sample sizes (9 and 13 offspring, respectively). For Pond 21, offspring sex ratio was not significantly different from either 25% or 50% males (Table 1). In this population, one "female" had three males out of a total of nine offspring, while six other "females" had no males out of a total of 134 offspring. In the Kanangra 3 population, two "females" had 50% males each and two "females" had 0% males each. Because of the small sample sizes and conflicting data for these two populations, breeding system assignments were denoted as "?" in these comparisons. The remaining 30 populations (having mixtures of monogenics and amphigenics plus having sex ratios among their offspring in amphigenic clutches that did not differ from 20–25% males) were all classified as androdioecious.

An additional three populations (Thomaga 13, Brazil 1, and WAR9) had mixtures of monogenics and amphigenics but had sex ratios that differed significantly from 25% males in the amphigenic clutches (Table 1). All three populations have offspring sex ratios that do not conform to any of the three breeding systems (i.e., they are significantly different from 0%, 25%, and 50% males). Because they substantially conformed to the predictions of an androdioecious population (i.e., all had mixtures of monogenics and amphigenics and produced a substantial number of male—combined total of 244 males), we tentatively assigned them into the androdioecious category (i.e., designated them "A?").

One population of *Eulimnadia dahli* (the Bunjil population) had four amphigenics, only one of which deviated significantly from 25% male (61.9 ± 10.6% male for 21 total offspring). The remaining three amphigenics from this population produced 29.3 ± 7.1% males (41 total offspring). Because the total male production of amphigenics did not differ from 50% males (a dioecious trait) even though the population contained both monogenics and

Table 2. Hatching success in egg banks from ''females'' isolated from (Iso) and subsequently mated to (Mate) males. ''Hatch'' and ''No Hatch'' refer to the number of egg banks that had some level of hatching or no hatching, respectively. Chi-square and p-values are for deviation from expected equal hatching rates in isolated versus mated cups.

Genus	Species	Location	Pool	Cup	Hatch	No Hatch	Chi-square	p
Imnadia	*yeyetta*	Austria		Iso	11	67	31.63	<0.0001
Imnadia				Mate	27	15		
Limnadia	sp.	Australia	TAR	Iso	0	6	9.42	0.0022
Limnadia				Mate	4	1		
Limnadia	*stanleyana*	Australia	Kanangra2	Iso	0	3	5.06	0.0245
Limnadia				Mate	3	1		
Limnadia	*stanleyana*	Australia	Kanangra 3	Iso	0	28	5.85	0.0155
Limnadia				Mate	4	24		
Limnadopsis	*parvispinus?*	Australia	KAD	Iso	2	22	10.91	0.0010
Limnadopsis				Mate	12	12		
Limnadopsis	sp.	Australia	Pabellup	Iso	0	9	6.70	0.0096
Limnadopsis				Mate	4	5		

amphigenics (an androdioecious trait) we considered these data to be conflicting and assigned the Bunjil population an undetermined breeding system (''?'').

Dioecious species should have females that cannot produce viable offspring in the absence of males, and when offspring are produced, males should be produced in equal numbers to females. Six populations had ''females'' that produced few or no viable eggs in isolation but significantly more viable eggs when paired with males (Table 2). These populations were members of three genera: *Imnadia* (one species), *Limnadia* (two species), and *Limnadopsis* (two species). ''Females'' from these six populations plus an additional six populations (Dingo, Paperbark, WAC2, Waterlily, Pingaring, and 7J Creek) were never seen with eggs in their brood chambers when these ''females'' were held in isolation (Table 3, column ''BC'') but did move eggs into their brood chambers when males were added to their cups. Unfortunately, no eggs hatched from five of these additional six populations (all but WAC2) and thus a test of hatching proportions in isolated relative to mated cups could not be performed. WAC2 differed from the remaining populations in that ''females'' were isolated from the field and brought back into the lab. Initially, these ''females'' were mistakenly identified as *Eulimnadia,* and thus were set up in isolation and no males were collected for an Iso vs. Mate comparison. Once it was noted that these ''females'' did not produce additional clutches of eggs once they were isolated in the lab, they were examined more closely and found to be *Limnadia*. Their resulting eggs were later hydrated to examine offspring sex ratios.

Of the six populations that did have hatching eggs (Table 2), only three had offspring that survived until adulthood (Austria, Kanangra 3, and KAD; Table 1). In two of these three populations (Austria and KAD), offspring sex ratios were not significantly different from 50% males (Table 1). The third population (Kanangra 3) had two ''females'' that produced only female offspring (two total) and two ''females'' that produced 37.5% males (eight total). Additionally, the offspring raised from the single clutches produced by the field-collected WAC2 also did not significantly differ from 50% males (Table 1).

Considering all three sets of data together (relative hatching, propensity to move eggs to the brood chamber, and offspring sex ratios), two populations (Austria and KAD) had consistent patterns of dioecy in that they produced few to no viable eggs without males, did not move eggs to their brood chambers in the absence of males, and after mating with males had $\sim$50% males among their resulting offspring. Both of these populations had some hatching in egg banks produced by isolated females (11 in Austria and 2 in KAD; Table 2), which should not happen if they are dioecious. Nonetheless, other evidence strongly suggests dioecy for these two populations. We therefore suggest that in both of these populations, one or more males developed early in the rearing tanks and were able to fertilize these few females before they were isolated. Evidence that these few hatching egg banks contained contaminant, fertilized eggs comes from sex ratio information: if these hatchlings were the products of self-fertilization by isolated ''females,'' we would expect that there would be either 0% or 25% males among the resulting hatchlings (Sassaman & Weeks 1993). In both populations

Table 3. Population sex ratios (% Male) for field caught (Source = F) or lab reared (Source = L) limnadiid clam shrimp. SE% = standard error of percent male. "#" = sample size. Entries of "N" in columns labeled "0%" and "50%" denote male percentages that do not significantly differ from 0% and 50% males, respectively. An entry of "N" in the column labeled "BC" denotes that female clam shrimp were never observed moving eggs into their brood chambers when kept isolated from a male. The column labeled "BrdSys" is for breeding system assignment (from Table 1): A = androdioecious, H = hermaphroditic, and D = dioecious (see text for determinations of breeding system). "?" denotes contradictory information disallowing breeding system assignment.

Genus	Species	Location	Pool	Source	%Male	SE%	#	0%	50%	BC	BrdSys
Eulimnadia	*africana*	Botswana	94-63	L	30.4	9.6	23				A
Eulimnadia	*africana*	Botswana	Thomaga 13	L	23.5	11.8	13				A?
Eulimnadia	*agassizii*	Massachusetts		L	0.0	0.0	176	N			H
Eulimnadia	*brasiliensis*	Brazil	1	L	22.9	5.0	70				A?
Eulimnadia	*brasiliensis*	Brazil	2	L	12.0	3.4	92				A
Eulimnadia	*brasiliensis*	Brazil	3	L	14.0	3.5	100				A
Eulimnadia	*braueriana*	Japan	Hojo	L	19.4	2.7	216				A
Eulimnadia	*braueriana*	Japan	Otsu	L	17.5	2.6	211				A
Eulimnadia	*braueriana*	Taiwan		L	5.5	1.9	145				A
Eulimnadia	*colombiensis*	Venezuela	Margarita	L	0.8	0.8	118	N			A
Eulimnadia	*cylindrova*	Galapagos		L	16.3	2.9	166				A
Eulimnadia	*cylindrova*	Martinique	26	L	0.0	0.0	35	N			
Eulimnadia	*cylindrova*	Mexico	Baja California	L	27.0	7.3	37				A
Eulimnadia	*dahli*	Australia	Bunjil	L	25.0	4.2	108				?
Eulimnadia	*dahli*	Australia	Green Rock 1	F	47.6	7.7	42		N		
Eulimnadia	*dahli*	Australia	Green Rock 2	F	50.0	14.4	12		N		
Eulimnadia	*dahli*	Australia	Green Rock 4	F	22.2	5.7	54				
Eulimnadia	*dahli*	Australia	Green Rock 7	F	0.0	0.0	11	N			
Eulimnadia	*dahli*	Australia	Green Rock 8	F	37.5	12.1	16		N		
Eulimnadia	*dahli*	Australia	The Humps	L	27.7	3.7	148				A
Eulimnadia	*diversa*	Arizona	Alb	L	1.5	1.5	66	N			A
Eulimnadia	*diversa*	Arizona	Bap	L	8.6	1.7	278				A
Eulimnadia	*diversa*	Arizona	Gaz	L	0.7	0.7	138	N			H
Eulimnadia	*diversa*	Arizona	TDtch	L	4.0	3.9	25	N			
Eulimnadia	*diversa*	Arizona	Tri	L	0.0	0.0	379	N			A
Eulimnadia	*diversa*	Florida	Gain4	L	1.5	1.1	133	N			H
Eulimnadia	*diversa*	Indiana		L	20.6	3.4	141				A
Eulimnadia	*diversa*	Mississippi	#8	L	0.0	0.0	6	N			A
Eulimnadia	*diversa*	Nebraska	1	L	17.6	6.5	34				A
Eulimnadia	*feriensis*	Australia	Albany Hwy Rail	L	9.8	4.2	51				
Eulimnadia	*feriensis*	Australia	Cairns Rock	L	15.6	2.2	282				A
Eulimnadia	*feriensis*	Australia	Outcrop	L	0.0	0.0	128	N			?
Eulimnadia	*feriensis*	Australia	TAM2	L	0.0	0.0	64	N			H
Eulimnadia	*feriensis*	Australia	TAM9	L	0.0	0.0	108	N			
Eulimnadia	*feriensis*	Australia	WAC2	L	13.6	7.3	22	N			A
Eulimnadia	*feriensis*	Australia	WAR3	F	10.0	9.5	10	N			

Table 3. Continued.

Genus	Species	Location	Pool	Source	%Male	SE%	#	0%	50%	BC	BrdSys
Eulimnadia	*feriensis*	Australia	WAR4	F	50.0	13.4	14		N		
Eulimnadia	*feriensis*	Australia	WAR8	F	0.0	0.0	12	N			
Eulimnadia	*feriensis*	Australia	WAR9	L	11.8	7.8	17	N			A?
Eulimnadia	*feriensis*	Australia	Wave Rock	L	17.0	3.5	112				A
Eulimnadia	*feriensis*	Australia	YOR1	L	26.7	8.1	30				A
Eulimnadia	*feriensis*	Australia	YOR2	L	0.0	0.0	27	N			H
Eulimnadia	*feriensis*	Australia	YOR5	L	7.0	2.6	100				
Eulimnadia	*feriensis*	Australia	YOR9	L	1.8	1.3	111	N			
Eulimnadia	*feriensis*	Australia	YOR10	L	14.6	3.7	89				A
Eulimnadia	*follisimilis*	New Mexico		L	24.6	5.3	65				A
Eulimnadia	*follisimilis*	Venezuela	Caracas	F	0.0	0.0	300	N			
Eulimnadia	*inflecta*	Louisiana	CD	L	0.0	0.0	27	N			H
Eulimnadia	*texana*	Arizona	WAL	L	18.9	0.9	1709				A
Eulimnadia	*texana*	New Mexico	AMT1	L	19.1	3.4	131				
Eulimnadia	*texana*	New Mexico	JD1	L	15.9	3.0	145				A
Eulimnadia	*texana*	New Mexico	JT3	L	9.1	8.7	11	N			
Eulimnadia	*texana*	New Mexico	JT4	L	22.5	0.9	1964				A
Eulimnadia	*texana*	New Mexico	JT5	L	29.2	4.3	113				
Eulimnadia	*texana*	New Mexico	LTER	L	18.9	3.3	143				
Eulimnadia	*texana*	New Mexico	SWP3	L	17.5	4.8	63				
Eulimnadia	*texana*	New Mexico	SWP4	L	32.5	3.3	203				
Eulimnadia	*texana*	New Mexico	SWP5	L	22.6	1.7	637				A
Eulimnadia	*thompsoni*	Illinois	URB3	L	20.5	3.7	122				A
Eulimnadia	sp.	Georgia	Stone Mtn	L	40.1	3.8	167				A
Eulimnadia	sp.	Australia	7J Creek	L	5.3	5.1	19	N			
Eulimnadia	sp.	Australia	DevMarb1	L	0.0	0.0	107	N			A
Eulimnadia	sp.	Australia	DevMarb2	L	15.7	5.1	51				
Eulimnadia	sp.	Desirade	3	L	0.0	0.0	46	N			
Eulimnadia	sp.	Florida	JP	L	0.0	0.0	33	N			
Eulimnadia	sp.	Australia	Jim Jim	L	0.0	0.0	19	N			A
Imnadia	*yeyetta*	Austria		L	66.7	4.7	99		N	N	D
Limnadia	*badia*	Australia	Dingo	L	46.3	4.1	147		N	N	
Limnadia	*badia*	Australia	Dunn Rock	L	29.8	4.3	114				
Limnadia	*badia*	Australia	WAR5	F	60.9	10.2	23		N		
Limnadia	*lenticularis*	Austria		L	0.0	0.0	60	N			
Limnadia	*lenticularis*	Florida	Pond 19	L	0.0	0.0	283	N			H
Limnadia	*lenticularis*	Florida	Pond 21	L	0.0	0.0	129	N			?
Limnadia	*sordida*	Australia	Paperbark	L	56.0	9.9	25		N	N	
Limnadia	*sordida*	Australia	WAC2	F	34.5	11.6	58		N	N	D
Limnadia	*sordida?*	Australia	Waterlily	L	37.3	6.8	51		N	N	
Limnadia	*stanleyana*	Australia	Kanangra1	L	0.0	0.0	11	N			

Table 3. Continued.

Genus	Species	Location	Pool	Source	%Male	SE%	#	0%	50%	BC	BrdSys
Limnadia	*stanleyana*	Australia	Kanangra1	F	27.3	13.4	11		N		
Limnadia	*stanleyana*	Australia	Kanangra2	L	47.6	10.9	21		N	N	D
Limnadia	*stanleyana*	Australia	Kanangra2	F	21.7	8.6	23				D
Limnadia	*stanleyana*	Australia	Kanangra3	L	55.5	3.2	238		N	N	D
Limnadia	sp.	Australia	TAR	L	50.0	6.2	20		N	N	D
Limnadia	sp.	Australia	Pingaring	L	59.4	4.8	106		N	N	
Limnadia	sp.	Australia	Witjira	L	33.3	7.9	36				
Limnadopsis	*parvispinus?*	Australia	KAD	L	49.6	4.4	127		N	N	D
Limnadopsis	*tatei*	Australia	7J Creek	L	39.0	5.6	77		N	N	
Limnadopsis	*tatei*	Australia	Lasseter Hwy	F	50.0	9.4	28		N		
Limnadopsis	sp.	Australia	Melaleaca	L	40.9	10.5	22		N		
Limnadopsis	sp.	Australia	Pabellup	L	40.4	5.1	94		N	N	D
Limnadopsis	sp.	Australia	Tjulum	F	31.6	10.7	19	N	N		

there were ~50% males among offspring reared to adulthood (Table 1), again suggesting that they were produced by cryptic outcrossing between males and females that occurred before we isolated the females rather than by self-fertilization or parthenogenesis. Thus, in view of the combined evidence, these two populations were considered to be dioecious.

WAC2 had no information on Iso vs. Mate hatching, but did not move eggs to their brood chambers in the absence of males and had ~50% males among their hatched offspring. We thus also considered this population to be dioecious. Although Kanangra 3 had inconsistent offspring data (Table 1), the fact that "females" from this population did not produce viable offspring in the absence of males and were never seen to move eggs to their brood chambers without males led us to infer that this population was also dioecious.

The three additional populations whose hatchlings we were unable to grow to adulthood (TAR, Kanangra 2, and Pabellup) also had patterns that indicated they were dioecious: none of the "females" held in isolation produced viable offspring in the absence of males, plus they were never observed moving eggs into their brood chambers when males were not present. We therefore considered these three populations to also be dioecious. Thus, a total of seven populations and six species were inferred to be dioecious using these combined data: one species of *Imnadia,* three species of *Limnadia,* and two species of *Limnadopsis* (Table 3).

Population sex ratios for the 86 populations surveyed in this study ranged 0–67% males (Table 3). Of the 90 samples measured for population sex ratio (75 from the laboratory and 15 from the field; note there are four more samples than populations because WAC2 and 7J Creek had two species, and Kanangra 1 and Kanangra 2 had both lab and field estimates), 32 had male percentages that did not significantly differ from zero. Fifteen of these 32 populations were among the 47 populations identified for breeding system in Table 1. The distribution of these 15 samples was non-random with respect to breeding system ($\chi^2_{(2)} = 22.311$; $p < 0.0001$): seven of these 15 samples not differing from 0% males were from populations determined to be hermaphroditic from offspring rearings while the remaining 8 were from populations determined to be androdioecious from offspring rearings (Tables 1 and 3). None of these 15 samples were from dioecious populations.

Considering only the 40 populations determined to be either androdioecious or hermaphroditic, none of the hermaphroditic populations differed from 0% males while 76% of the androdioecious populations

did (Table 3), a difference that was significant ($\chi^2_{(1)} = 16.370$; $p < 0.0001$). Nonetheless, eight of the 33 androdioecious populations did not significantly differ from 0% males (Table 3).

Twenty-one of these 90 samples had population sex ratios that did not differ from a 1:1 sex ratio (Table 3). Again, these populations were not randomly distributed across the populations positively identified for breeding system (Table 3). Seven of the 21 were from populations positively identified in Table 3, and all seven of these were from dioecious populations. All androdioecious and hermaphroditic populations noted in Table 1 had population sex ratios that significantly differed from 50% males (Table 3). This difference among breeding systems was significant both when considering populations of all three breeding systems ($\chi^2_{(2)} = 39.561$; $p < 0.0001$) or when only comparing androdioecious and dioecious populations ($\chi^2_{(1)} = 37.098$; $p < 0.0001$).

Discussion

The goal of this study was to deepen our understanding of the taxonomic distribution of the three known breeding systems in the family Limnadiidae: selfing hermaphroditism (Zucker et al. 1997; Scanabissi & Mondini 2002; Weeks et al. 2005), androdioecy (Sassaman & Weeks 1993; Weeks et al. 2006b), and dioecy (Sassaman 1995). To accomplish this goal, we sampled 86 populations (32 total species) from across the world. Because of the scale of the comparisons, we sought a set of criteria that would allow us to assign species/populations to these three breeding systems without the need to perform costly and time consuming genetic and transmission electron microscopic studies (Zucker et al. 1997; Scanabissi & Mondini 2002; Weeks et al. 2005; Scanabissi et al. 2006). We settled on procedures modified from Weeks et al (2005) in which offspring are reared from egg banks that are collected from isolated or mated "females." Data, in the form of hatching success, propensity to move eggs into the brood chamber in the absence of males, and offspring sex ratios, allowed us to make the breeding system assignments. These methods should be considered as supplemental to the traditional methods of genetic and anatomical assessment of breeding systems rather than as general replacements for these more traditional methods in these crustaceans. On the basis of these criteria, 47 total populations were assigned a breeding system in this study: 33 androdioecious (three of these populations were questionable), 7 hermaphroditic, and 7 dioecious (Table 3). Two additional populations had conflicting offspring data, and thus could not be assigned a breeding system.

Because we also have information on population sex ratios (Table 3), we can compare the traditional methods of identifying breeding system in clam shrimp to those we have developed herein. Traditionally, sex ratios near unity were defined as dioecious (= gonochoric), those with no males were termed parthenogenetic, and those with female-biased sex ratios were thought to be either mixtures of parthenogenetic and dioecious individuals or mixtures of males and hermaphrodites (i.e., androdioecious, Sassaman 1995). Only using the 47 populations positively identified as to breeding system in Table 3, comparisons of simple sex ratio data suggest that population sex ratios can be useful to identify breeding systems in some instances. For example, all seven dioecious populations had sex ratios that did not significantly differ from 50% males, while all 40 remaining populations (seven hermaphroditic and 33 androdioecious) significantly differed from 50% males. Although it would be convenient to assume that this suggests dioecy can be definitively noted on the basis of population sex ratio alone, two issues should be noted. First, we only sampled seven populations of dioecious limnadiids, and a broader survey of dioecious populations may indeed reveal that the distribution of sex ratios of dioecious populations may include some that significantly deviate from 50% males. Second, although none of the population sex ratios from dioecious populations significantly deviated from 50% males, their distribution did overlap that of the androdioecious populations.

Plotting the population sex ratios (Table 3) from these 47 populations, but restricting comparisons with populations with ≥ 20 individuals (including the three "A?" populations but eliminating Thomaga 13, Mississippi 8, WAR9, and Jim Jim for sample sizes < 20), there was some overlap in sex ratios in the 35–45% male range from populations exhibiting androdioecious and dioecious breeding systems (Fig. 1). This overlap suggests that populations in that sex ratio range could be mistaken for the incorrect breeding system by using population sex ratio data alone. Thus, the traditional method of inferring that species with 50:50 sex ratios are dioecious and female-biased sex ratios are androdioecious appears useful if sample sizes are large and sex ratios are not in the 35–45% male range. For populations in the 35–45% male range, combining sex ratio data with isolations and egg bank rearings are minimally required to infer breeding system.

Distinguishing androdioecy from selfing hermaphroditism is more problematic. Although the

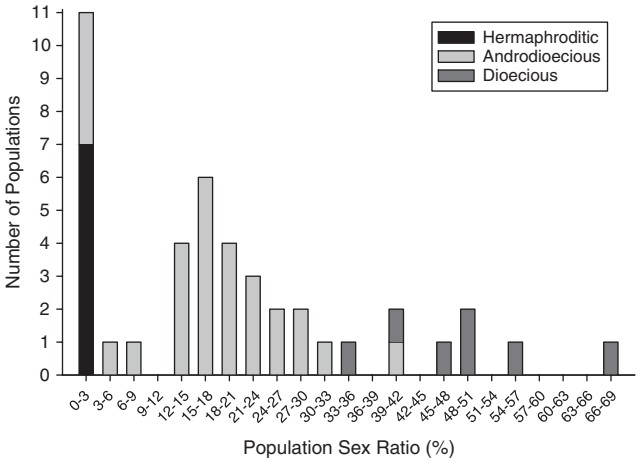

Fig. 1. Distribution of male population sex ratios for hermaphroditic (n = 7), androdioecious (n = 29), and dioecious (n = 7) populations of field-collected adults or adults raised from field-collected soil for samples of $\geq$20 individuals per population (see Table 3 for specific populations).

androdioecious species had significantly more samples with population sex ratios that differed from 0% males, there were still a number of androdioecious populations with low-to-no males in the population samples (Fig. 1). This makes simple sex ratio comparisons ineffective at distinguishing these two mating systems when males are rare or absent.

Weeks et al. (2006b) concluded that clam shrimp in the genus *Eulimnadia* all have the same breeding system first outlined for *Eulimnadia texana* by Sassaman & Weeks (1993): (1) mixtures of males, monogenic hermaphrodites, and amphigenic hermaphrodites, or (2) only monogenic hermaphrodites. In our current categorization scheme, the former would be classified as androdioecious while the latter would be classified as selfing hermaphroditism. The population sex ratio distribution for the populations of *Eulimnadia* underscores this dichotomy: ~30% of the populations have sex ratios at or near 0% males, while the remaining ~70% of the populations have a broad distribution of sex ratios that have a mode in the 15–20% male range (Table 3, Fig. 1). This dichotomy and sex ratio distribution is similar to that reported in other studies of *Eulimnadia* (Sassaman 1989; Weeks & Zucker 1999; Weeks et al. 2006b). Because androdioecious populations can include population sex ratios with no males and some hermaphroditic populations had low proportions of males (Fig. 1), we suggest that, similar to distinguishing dioecy from androdioecy, for samples with population sex ratios in the range of 0–5% males, only offspring rearing can distinguish selfing-hermaphroditism from androdioecy.

Additionally, because androdioecious populations contain monogenic hermaphrodites that only produce hermaphroditic offspring (Sassaman & Weeks 1993), ''definitively'' determining that a population is composed of selfing hermaphrodites does not necessarily determine whether the species as a whole comprises only selfing hermaphrodites; other populations of the species may include males and hermaphrodites. In fact, in this study, *Eulimnadia diversa* and *Eulimnadia feriensis* both have hermaphroditic and androdioecious populations (Table 3), and population sex ratio data suggests that *Eulimnadia colombiensis, Eulimnadia follisimilis,* and *Eulimnadia texana* are similarly composed of both all-hermaphrodite and androdioecious populations (Sassaman 1989; Weeks et al. 2006b). When sampling populations in these five species, an all-hermaphrodite population may just be one that was initially colonized by monogenic hermaphrodites and will eventually become androdioecious once males migrate to the pool. Thus, to determine whether a species of *Eulimnadia* is truly selfing hermaphroditic, many replicate populations need to be sampled (Weeks et al. 2006b).

To summarize, using simple sex ratios to infer breeding system can be valid if sex ratios are 1:1 or strongly female biased. Populations with 1:1 sex ratios were reliably scored as dioecious while highly ''female''-biased populations were reliably scored as androdioecious. Sex ratios in the 35–45% male range could be either androdioecious or dioecious, and sex ratios in the 0–5% male range could be either selfing hermaphroditic or androdioecious. For both of these ranges, more detailed information would need to be collected to make any sort of inference about breeding system. In no instances has parthenogenesis been documented in the Limnadiidae, and thus inferring parthenogenesis from the simple absence of males in a population is certainly not warranted.

One of the species examined herein deserves special attention. Members of *Limnadia lenticularis* have been repeatedly described as being ''all-female'' and assumed to be parthenogenetic (Zaffagnini 1969; Sassaman 1995). A few males have been found (including Pond 21 in this study; Table 1), but males are so rare that finding them in this species warranted a separate paper (Eder et al. 2000). Although Zaffagnini (1969) suggested that hermaphroditism in *L. lenticularis* was ''rudimentary'' and that this species was actually parthenogenetic, it is likely that he mistook a self-fertilization event for his described ''refusion of a polar body.'' Scanabissi & Mondini (2002) determined that ''females'' of *L. lenticularis*

were actually functional hermaphrodites, and when combined with genetic evidence of self fertilization (Tinti & Scanabissi 1996), we can conclude that these clam shrimp are actually self-fertilizing hermaphrodites. In the current study, we found 10 of 11 individuals to be self-compatible hermaphrodites, but the 11th produced ~30% males among its nine offspring. This latter individual produced males at the rate expected for amphigenic hermaphrodites. The low numbers of males produced in this and other studies (Eder et al. 2000) is intriguing. If some populations of *L. lenticularis* have low numbers of viable males that can outcross with hermaphrodites, and the underlying sex determining mechanism is the same as described in *Eulimnadia* (Sassaman & Weeks 1993; Weeks et al. 2006b), we should find low numbers of amphigenic hermaphrodites in these populations. More detailed examinations of isolated hermaphrodites from the Florida and other populations of *L. lenticularis* are needed to detect whether low levels of amphigenics do exist in these populations, which would indicate that these populations are monogenic-biased androdioecious populations.

Regardless of whether *L. lenticularis* is a species comprising mixtures of monogenic-only and androdioecious populations, the current evidence is that hermaphroditism is found in two genera in the family Limnadiidae: *Eulimnadia* and *Limnadia*. The obvious question is whether these represent a single evolutionary event or two separate events. Gonad development may shed light on this question: the ovotestes in *Eulimnadia* have testicular tissue concentrated into a small region at the distal end of the gonad (Zucker et al. 1997; Weeks et al. 2005), whereas testicular tissue is interspersed with ovarian tissue throughout the ovotestes in *L. lenticularis* (Scanabissi & Mondini 2002). This suggests two separate derivations of hermaphroditism in these lineages, which is consistent with phylogenetic analyses of the relationship of these genera (Hoeh et al. 2006). We still do not know whether the genetic system that underlies androdioecy in *Eulimnadia* (a Z/W chromosomal system, Sassaman & Weeks 1993; Weeks et al. 2006b) may also underlie the selfing hermaphroditism found in *L. lenticularis*.

Together, these data suggest that the clam shrimp species so far studied in the Australian genera *Limnadia* and *Limnadopsis,* and the European genus *Imnadia,* are all dioecious, whereas the broadly distributed members of *L. lenticularis* are likely primarily selfing hermaphroditic (although there are interesting signs that this species may also include some androdioecious populations). The largest genus, *Eulimnadia,* comprises species that contain a mixture of androdioecious and selfing hermaphroditic populations. This latter observation may be parsimoniously explained by assuming that all members of *Eulimnadia* have the basic genetic sex-determining mechanism outlined for *E. texana* (Sassaman & Weeks 1993), which is predicted to result in populations that are either mixtures of males and hermaphrodites or hermaphrodite-only (Otto et al. 1993).

In fact, all three breeding systems noted in the Limnadiidae could be explained given different selective pressures on the Z/W sex-determining system found in the *Eulimnadia:* selection for complete outcrossing and allocation away from male gamete production in hermaphrodites would lead to dioecy (ZZ+ZW); selection for complete self fertilization would eliminate the Z chromosome resulting in all selfing hermaphrodites (WW); and selection for mixed levels of selfing and outcrossing would maintain all three chromosomal combinations (ZZ, ZW, and WW) in an androdioecious breeding system (Sassaman & Weeks 1993). We hope that the current data can be combined with additional studies to test whether these larger-scale breeding system patterns in the Limnadiidae all stem from a similar underlying genetic system, as suggested by Sassaman (1995).

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