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Androdioecy and hermaphroditism in five species of clam shrimps (Crustacea: Branchiopoda: Spinicaudata) from India and Thailand

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Abstract. Crustaceans in the order Spinicaudata display a broad range of reproductive types, ranging from pure hermaphroditism to pure dioecy (separate males and females), and mixes in between. One particularly interesting genus of these "clam shrimps" is *Eulimnadia*. Based on offspring sex ratios, it has been suggested that all members of the genus are androdioecious: populations consist of mixtures of males and hermaphrodites. However, only two of the ~40 species in this genus have been examined histologically to confirm the presence of ovotestes in the purported hermaphrodites of this group. Here, we report both sex ratio and histological evidence that populations of five additional *Eulimnadia* species from India and Thailand are indeed mixes of males and hermaphrodites (four species) or hermaphrodite only (one species). Sex ratios of adults and offspring from isolated hermaphrodites are in accordance with those previously reported for 15 *Eulimnadia* species, and histological assays of four of the five species show the presence of both testicular and ovarian tissue in these hermaphrodites. As has been previously reported, the testicular tissue in members of these *Eulimnadia* spp. is located in a small section at the distal end of the gonad. In addition, the sperm produced in these hermaphrodites forms distinct plaques of compacted chromatin. Overall, these data are consistent with a single origin of hermaphroditism in the *Eulimnadia*, and support the notion that members of the entire genus are either androdioecious or all-hermaphroditic.

Additional key words: *Eulimnadia*, Conchostraca, breeding systems, ovotestes

The Branchiopoda (Arthropoda; Crustacea) represents one of the most ancient crustacean lineages that still includes extant representatives (Walossek 1993; Martin & Davis 2001; Olesen 2007). The class Branchiopoda is subdivided into the orders Anostraca, Notostraca, and Diplostraca (Martin & Davis 2001; Olesen 2007). These taxa have been extensively studied by evolutionary biologists interested in, among other things, the diversity of breeding systems that exist within and among branchiopod groups (Dumont & Negrea 2002). Specifically, members of the Diplostraca (which includes clam shrimps and water fleas) contain at least five different breeding systems: dioecy (true males and true females), hermaphroditism (individuals capable of self-fertilization due to testicular and ovarian tissue occurring concomitantly in the same reproductive tract), androdioecy (hermaphrodites and males), parthenogenesis (asexual reproduction), and cyclic parthenogenesis (multiple episodes of asexual reproduction followed by marked periods of dioecy) (Hebert & Finston 1993; Sassaman 1995; Martin & Davis 2001; Dumont & Negrea 2002; Olesen 2007; Weeks et al. 2008). Of these five breeding systems, four are found specifically in one suborder of the

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Diplostraca: the Spinicaudata. Such reproductive diversity makes these branchiopods ideal candidates for studies of the evolution of breeding systems.

The suborder Spinicaudata comprises three families (Cyzicidae, Leptestheriidae, and Limnadiidae) that are collectively known as "clam shrimps" (Martin & Davis 2001; Olesen 2007). Historically, sex ratio studies on the Spinicaudata have been the method used to identify breeding systems (Sassaman 1995). Sex ratio determination in clam shrimp populations is based solely on external morphological characters (i.e., claspers in males, and eggs in females/hermaphrodites). In many species of clam shrimps, sex determination occurs 5–8 d following hatching from desiccation-resistant eggs (Sassaman & Weeks 1993; Brendonck 1996; Weeks et al. 1997, 2002). Using these morphological indicators to identify the sex of individuals in populations of clam shrimps, four breeding systems have been identified in the suborder Spinicaudata: dioecy, androdioecy, hermaphroditism, and parthenogenesis.

Before evolutionary hypotheses regarding the evolution of breeding systems can be tested within the Spinicaudata, a more accurate assessment of the reproductive status of species is necessary. Such assessments require examinations that extend beyond mere sex ratio determinations. Aside from Wingstrand's (1978) descriptive account of branchiopod spermatology, which included examination of some representatives of Spinicaudata, few studies have looked at the reproductive biology of the Spinicaudata from a cellular and histological perspective, despite its importance in understanding phylogenetic relationships (Martin & Davis 2001). A few cellular and histological studies have examined clam shrimps in the family Leptestheridae, e.g., *Leptestheria dahalacensis* Rüppell 1837 and *Eoleptestheria ticinensis* Balsamo-Crivelli 1859 (Tommasini & Scanabissi Sabelli 1992; Scanabissi Sabelli & Tommasini 1994; Scanabissi & Mondini 2000). In addition, a few ultrastructural and histological studies have been conducted to assess reproductive diversity in the family Limnadiidae; these have focused on *Eulimnadia texana* Packard 1871 (Zucker et al. 1997; Scanabissi et al. 2006), *Eulimnadia agassizii* Packard 1874 (Weeks et al. 2005), and *Limnadia lenticularis* Linnaeus 1761 (Zaffagnini 1969; Tommasini & Scanabissi Sabelli 1992; Scanabissi & Mondini 2002a). Clearly, to better understand the evolution and diversity of breeding systems found within the Spinicaudata, a more extensive examination of the histological and cellular structure of clam shrimp reproductive systems must be undertaken.

One taxon for which a detailed analysis of reproductive systems is needed is the genus *Eulimnadia*. Members of *Eulimnadia* inhabit temporary playas, ditches, and many other ephemeral freshwater habitats throughout the world, from the tropics to the deserts (Dumont & Negrea 2002). Hermaphrodites produce desiccation-resistant cysts, which they bury within the top several millimeters of the soil. These cysts hatch rapidly following hydration under spring and summer conditions (at water temperatures above 18°C), releasing a nauplius larva. Larval and juvenile growth is extraordinarily rapid: most shrimp reach reproductive size in 4–7 d in the laboratory at 27–30°C (Sassaman & Weeks 1993; Weeks et al. 1997), and in as little as 4–6 d in the field (Vidrine et al. 1987). The hermaphrodites produce thousands of eggs in their lifetime, generating clutches ranging from 100 to 300 eggs, one to two times a day (Knoll 1995; Weeks et al. 1997). Sexual dimorphism is pronounced. The thoracic appendages of hermaphrodites are unmodified, but the first two pairs of thoracic appendages in males undergo differentiation into claw-like claspers, which are used to hold on to the margins of a hermaphrodite's carapace during mating. Natural populations of *Eulimnadia* are typically hermaphrodite-biased (Mattox 1954), with some populations completely lacking males (Zinn & Dexter 1962; Stern & Stern 1971). Weeks et al. (2008, 2009a) have inferred that the entire genus *Eulimnadia* comprises either androdioecious or all-hermaphroditic species. Androdioecious species typically are mixtures of all-hermaphrodite and mixed male/hermaphrodite populations (Sassaman & Weeks 1993; Weeks et al. 2008). Sex is determined in these clam shrimp by a Z-W chromosomal system (Weeks et al. 2010) in which there are three chromosomal types: ZZ males, ZW "amphigenic" hermaphrodites, and WW "monogenic" hermaphrodites; both hermaphroditic types can self-fertilize or mate with males (Sassaman & Weeks 1993). When selfing, the monogenic hermaphrodites "breed true", producing 100% monogenic hermaphroditic offspring, whereas the amphigenic hermaphrodites produce 25% male, 50% amphigenic hermaphrodite, and 25% monogenic hermaphrodite offspring when selfed (Sassaman & Weeks 1993). Weeks et al. (2008) used these sex ratio predictions to assess offspring rearings from isolated hermaphrodites and found 15 *Eulimnadia* species to be either androdioecious or all-hermaphroditic. Hermaphrodites from only two of these 15 species (*E. texana* and *E. agassizii*) have been examined histologically (Zucker et al. 1997; Weeks et al. 2005). Because the genus *Eulimnadia*

contains over 40 species (Brtek 1997), an assessment of reproduction in more members of the genus must be made before we can confidently assert that this genus contains only androdioecious and all-hermaphroditic species.

Toward that goal, this study combines microscopy with offspring rearings to assess reproductive mode in five species of *Eulimnadia* from India and Thailand: *Eulimnadia gibba* Sars 1900, *E. gunturensis* Radhakrishna & Durga Prasad 1976, *E. michaeli* Nayar & Nair 1968, *E. azisi* Subhash Babu & Bijoy Nandan 2011, and an undescribed *Eulimnadia* sp. We show that these five species are indeed a mixture of androdioecious and all-hermaphroditic reproductive modes, and note that the anatomical layout of the ovotestis in *E. gibba*, *E. gunturensis*, *E. michaeli*, and *E. azisi* has the same structural design as *E. texana* and *E. agassizii*. These findings are consistent with a single origin of hermaphroditism in this genus, as asserted previously (Weeks et al. 2009a).

Methods

Rearing of samples from soil

The cysts of clam shrimps were collected in soil samples from dry temporary pools (i.e., populations) as summarized in Table 1. Cysts of *Eulimnadia gibba* were collected from India (one population, PED1, from Pedakakani, Guntur district, Andhra Pradesh), *E. gunturensis* from India (one population, NN1 from Nagarjuna Nagar, Guntur district, Andhra Pradesh), *E. azisi* from India (two populations, Ghat 1 and Ghat 2, from the Western Ghats, Vettilapara area), *Eulimnadia* sp. from India (two populations: Veli and Karuvatta), and *E. michaeli* from Thailand (six populations, KK2A, KK4A, KK6A, KK7A, KK8A, and KK9A, from Khon Kaen). The soil samples were kept in a dry, dark cabinet for 6 months–2 years before hydration.

In cases where ample soil was available, approximately 500 mL of field site soil was added to a 27-L aquarium, and then hydrated using deionized (DI) water. In cases where only sparse amounts of soil were available, approximately 250 mL of field site soil was added to a 10-L aquarium, and then hydrated using DI water. "Standard conditions" were maintained for all aquaria (see Sassaman & Weeks 1993; Weeks et al. 1997, 2000, 2001, 2008, 2009a). These included a room temperature of 25–28°C, continuous light, minimal tank aeration (using air stones), and daily feeding of a mixture of ground baker's yeast and Tetramin™ fish food (Tetra Werke, Melle, Germany) (2.5 g of each in 500 mL of DI water). On day five after hydration, up to 100 immature clam shrimps were individually isolated in 500-mL plastic cups. Approximately, 6–10 d following the appearance of nauplii, the shrimps in both the isolated and larger aquaria were sexually matured. Sexual maturity was noted when males developed claspers and hermaphrodites produced eggs within "brood chambers" on their dorsal surface. Total population sex ratios were documented

Table 1. Population sex ratios of five species of *Eulimnadia* reared from soil samples. Herm., hermaphroditic; SE, standard error. Bolded numbers in the "% male" column indicate male percentages that were significantly different from zero (see Weeks et al. 2008). Breeding systems are hermaphroditic (Herm.) or androdioecious (Andro.); see text for determinations of breeding system.

Species	Location	Population	# Male	# Herm.	Total	% Male	SE (%)	Breeding System
E. azisi	Ghat, India	Ghat 1	0	112	112	0.0	0.0	
E. azisi	Ghat, India	Ghat 2	0	120	120	0.0	0.0	
		Total	0	232	232	0.0	0.0	Herm.
E. gunturensis	Nagarjuna Nagar, India	NN1	1	30	31	3.2	3.2	Herm.
E. michaeli	Khon Kaen, Thailand	KK2A	50	56	106	**47.2**	4.8	
E. michaeli	Khon Kaen, Thailand	KK4A	0	51	51	0.0	0.0	
E. michaeli	Khon Kaen, Thailand	KK6A	0	32	32	0.0	0.0	
E. michaeli	Khon Kaen, Thailand	KK7A	3	18	21	14.3	7.6	
E. michaeli	Khon Kaen, Thailand	KK8A	0	39	39	0.0	0.0	
E. michaeli	Khon Kaen, Thailand	KK9A	1	7	8	12.5	11.7	
		Total	54	203	257	**21.0**	2.5	Andro.
E. gibba	Pedakakani, India	PED1	11	40	51	**21.6**	5.8	Andro.
Eulimnadia sp.	Veli, India	Veli	27	159	186	**14.5**	2.6	
Eulimnadia sp.	Karuvatta, India	Karuvatta	0	41	41	0.0	0.0	
		Total	27	200	227	**11.9**	2.4	Andro.

for both isolated and aquarium-reared shrimp. Males from the isolated cups and all shrimp in the aquaria were frozen upon sexual maturity. Females/hermaphrodites in the isolated cups were allowed to produce eggs for egg banks (see below) for ~7 d, and then were frozen for potential future genetic analyses.

Rearing from egg banks

Egg banks generated above were used to assess the sex ratios of offspring produced by isolated individuals. Eggs and DI water were added to 500-mL cups along with 2 mL of baker's yeast/Tetramin™ food. Cups were maintained under continuous light at temperatures of 25–28°C. In addition, 10-L rearing tanks were prepared with water from "conditioning" tanks that had 27 L DI water and 500 mL of soil collected from either Arizona or Indiana (United States). This water was screened through a 63-μm sieve to ensure no cysts or nauplii were transferred to the rearing tanks. These rearing tanks allowed growth of the nauplii once they hatched. The 500-mL cups were periodically checked for nauplii over a period of 2 days. Once observed, nauplii were transferred from the 500-mL cups to the 10-L rearing aquaria, which were then kept under the standard conditions noted above. Isolated sex ratios were determined after sexual maturity, as noted above.

Testing sex ratios

To assess whether the five species of *Eulimnadia* studied here are mixtures of all-hermaphrodite and androdioecious populations, as noted in other *Eulimnadia* species (Weeks et al. 2008), we compared both population and isolated sex ratios as determined above to expectations derived from previously studied species. Expected population sex ratios for all-hermaphroditic populations were 0% males: 100% hermaphrodite (Weeks et al. 2006b), whereas there were no specific population sex ratio expectations for androdioecious populations because the ratio of monogenics to amphigenics in natural populations determines overall ("population") sex ratios (Otto et al. 1993). Deviations of population sex ratios from expectations of all-hermaphrodite species were examined by constructing 95% confidence intervals around the sex ratios measured, and noting whether the CI included 0% males (see Weeks et al. 2008).

Sex ratios from isolated females/hermaphrodites were used to test both whether the isolated individuals were female (no viable offspring expected) versus asexual/hermaphroditic (viable offspring produced in isolation; see Sassaman & Weeks 1993). If isolated individuals produced viable offspring, sex ratios of their offspring can be used to distinguish hermaphroditic type (Sassaman & Weeks 1993). For all-hermaphroditic populations, all isolated hermaphrodites were expected to produce 100% hermaphroditic offspring, termed "monogenic" hermaphrodites (Sassaman & Weeks 1993). For androdioecious populations, expectations were that some isolates would produce 0% males (monogenics) while others would produce ~25% males (amphigenics) among their offspring (Sassaman & Weeks 1993). To test for a 3:1 ratio of hermaphrodites to males in amphigenic clutches, a Chi-squared analysis was used (Sassaman & Weeks 1993).

Microscopy

Sexually mature clam shrimp hermaphrodites from populations of four clam shrimp species (*E. gibba*, *E. gunturensis*, *E. michaeli*, and *E. azisi*) were removed from rearing tanks and prepared for fixation. A minimum of three hermaphrodites were surveyed from each of these four species. All fixation and embedding procedures were carried out at room temperature unless otherwise noted. Samples were placed into small glass vials and pre-fixed in a 2% glutaraldehyde solution buffered with 0.1 mol L^{-1} sodium cacodylate (pH of 7.2) for 2 h. At this point, the carapace of each individual was removed. The samples were then placed into small glass vials that contained fresh 2% glutaraldehyde buffered with 0.1 mol L^{-1} sodium cacodylate (pH of 7.2) for 1 h. The samples were then washed in three changes of 0.1 mol L^{-1} sodium cacodylate buffer for 1 h. Next, the samples were post-fixed in 2% osmium tetroxide (OsO_4) with 0.1 mol L^{-1} sodium cacodylate buffer (pH of 7.2) for 1.5 h, following which samples were washed with three changes of deionized (DI) water for 30 min. Samples were *en bloc* stained for 30 min using an aqueous 2% uranyl acetate solution, then washed with three changes of DI water at 10-min intervals. After washing the samples thoroughly, the specimens were dehydrated overnight (approximately 13 h) in an acetone desiccator (Ott & Brown 1974). To ensure complete dehydration, 100% acetone was added and then removed seven times at 10-min intervals the following day. Next, samples were covered in a 90% acetone-10% plastic (Embed-812; Electron Microscopy Science, Hatfield, PA, USA) solution, covered with aluminum foil with several holes punched in the foil, and

placed under a fume hood overnight (~13 h) to allow slow evaporation of the acetone and total infiltration of the plastic. To ensure complete infiltration, 100% plastic was added, and then partially removed three times from the samples at 30-min intervals the following day. Samples were placed in a mold, covered in 100% plastic, and placed in a 60°C oven for 48 h. The embedded specimens were removed from the oven and allowed to cool. The specimens were then sectioned with a Reichert OMU-3 ultramicrotome using a diamond knife. Thick sections (1.5 μm) were gently placed on a slide, heat-fixed, and stained with a 1% toluidine blue-1% sodium borate solution for light microscopy. Thick sections were examined using an Olympus BX60 digital light microscope (Olympus America Inc) equipped with an Olympus DP71 digital camera.

Results

Sex ratios

The population samples of *Eulimnadia azisi* produced all hermaphrodites (i.e., 0% males), suggesting the species has an "all-hermaphroditic" breeding system (Table 1). One other species, *E. gunturensis*, had a low enough male sex ratio (3.2%) to not be significantly different than 0% males (Table 1), which is consistent with an all-hermaphroditic breeding system (but see below). The remaining three species had average male percentages ranging from 12 to 22% in the population samples, which were all significantly different from 0% male (Table 1). These male percentages are consistent with an androdioecious breeding system. Two of these three species (*E. michaeli* and *Eulimnadia* sp.) had one or more populations that had no males (Table 1), which is also consistent with most androdioecious species (Weeks et al. 2008).

Although population sex ratios are generally indicative of breeding system type in clam shrimps (Weeks et al. 2008), sex ratios from egg banks collected from isolated females/hermaphrodites (Table 2) are better indicators of breeding systems because these sex ratios can be tested against specific predictions (Sassaman & Weeks 1993). All four species tested produced viable offspring from eggs collected from females/hermaphrodites reared in isolation, indicating that they were either self-compatible hermaphrodites or asexual females (Weeks et al. 2008). Isolated females/hermaphrodites produced broods without males, or broods that contained 13–30% males. Although the 0% male isolates could have been produced by asexual females, the most parsimonious inference is that these isolates comprised monogenic and amphigenic hermaphrodites, as has been found in all other species of *Eulimnadia* examined to date (Sassaman & Weeks 1993; Weeks et al. 2008).

Isolated sex ratios averaged by population are shown in Table 2A. Populations Ghat 1, Karuvatta, and KK4A comprised only monogenic hermaphrodites, while the remaining populations had 23 to 52% (mean 34.1%) monogenic hermaphrodites (Table 2A). As was found for the population sex

Table 2. Sex ratios of offspring produced by isolated hermaphrodites of *Eulimnadia*. **A.** Overall sex ratios combined across hermaphroditic parents (each parent produced one clutch). % Monogenic indicates the percentage of the isolated hermaphroditic parents that were monogenic (i.e., had only hermaphrodite offspring). **B.** Sex ratios of offspring from isolated amphigenic hermaphrodites only. Chi-square is the calculated deviation from a 3:1 sex ratio expected for hermaphrodites:males among the offspring of a selfing amphigenic hermaphrodite. Other columns are as in Table 1.

A. Species	Population	*N* parents	# Male	# Herm.	Total	% Male	SE (%)	% Monogenic
E. azisi	Ghat1	14	0	861	861	0.0	0.0	100
E. gibba	PED1	6	36	274	310	**11.6**	1.8	33.3
Eulimnadia sp.	Veli	44	150	984	1134	**13.2**	1.0	22.7
Eulimnadia sp.	Karuvatta	3	0	82	82	0.0	0.0	100
E. michaeli	KK2A	20	232	2116	2348	**9.9**	0.6	40.0
E. michaeli	KK4A	22	0	1416	1416	0.0	0.0	100
E. michaeli	KK8A	21	83	1023	1106	**7.5**	0.8	52.4

B. Species	Population	# Male	# Herm.	Total	% Male	SE (%)	Chi-square	p-value
E. gibba	PED1	38	129	167	22.8%	3.2%	0.45	0.5023
Eulimnadia sp.	Veli	150	588	738	20.3%	1.5%	8.6	0.0033
E. michaeli	KK2A	232	1130	1362	17.0%	1.0%	46.1	<0.0001
E. michaeli	KK8A	81	537	618	13.1%	1.4%	46.6	<0.0001

ratios, Ghat1, Karuvatta, and KK4A all had average isolate sex ratios not significantly different from 0% males, while PED1, Veli, and KK2A were all significantly different from 0% males. KK8A was significantly different from 0% males in the isolated rearings (Table 2A), which differed from the 0% males seen in the population rearings (Table 1).

Average sex ratios for isolates producing males (i.e., amphigenics) ranged from 13 to 23% males (Table 2B), with only one of the four populations (PED1) having a sex ratio not significantly different from the 3:1 hermaphrodite to male ratio predicted by the ZW chromosomal system of other species of *Eulimnadia*.

Histology

To determine whether the conclusions of hermaphroditism and androdioecy inferred from the sex ratio data were correct, we longitudinally sectioned the gonads of hermaphrodites of four of the purported hermaphroditic/androdioecious species (*E. gibba, E. gunturensis*, *E. michaeli,* and *E. azisi*) to test for the presence of ovotestes. Positioned adjacent to the digestive tract within the hemocoel was a bi-lobed, tubular gonad. Beginning at the anterior tip and constituting the majority of the gonad was a distinct female wall. This thick female wall (which surrounded a central luminal space) consisted mostly of closely compacted epithelial cells, sporadically interrupted by developing oocytes. The female wall continued down the length of the gonad until it was interrupted by a thin-walled region (Fig. 1). This thin wall represented the male wall, and the amoeboid cells strewn throughout the wall (as well as located in the lumen of the gonad) were clearly defined male gametes (Figs. 1, 2). Often, male gametes toward the center of the lumen

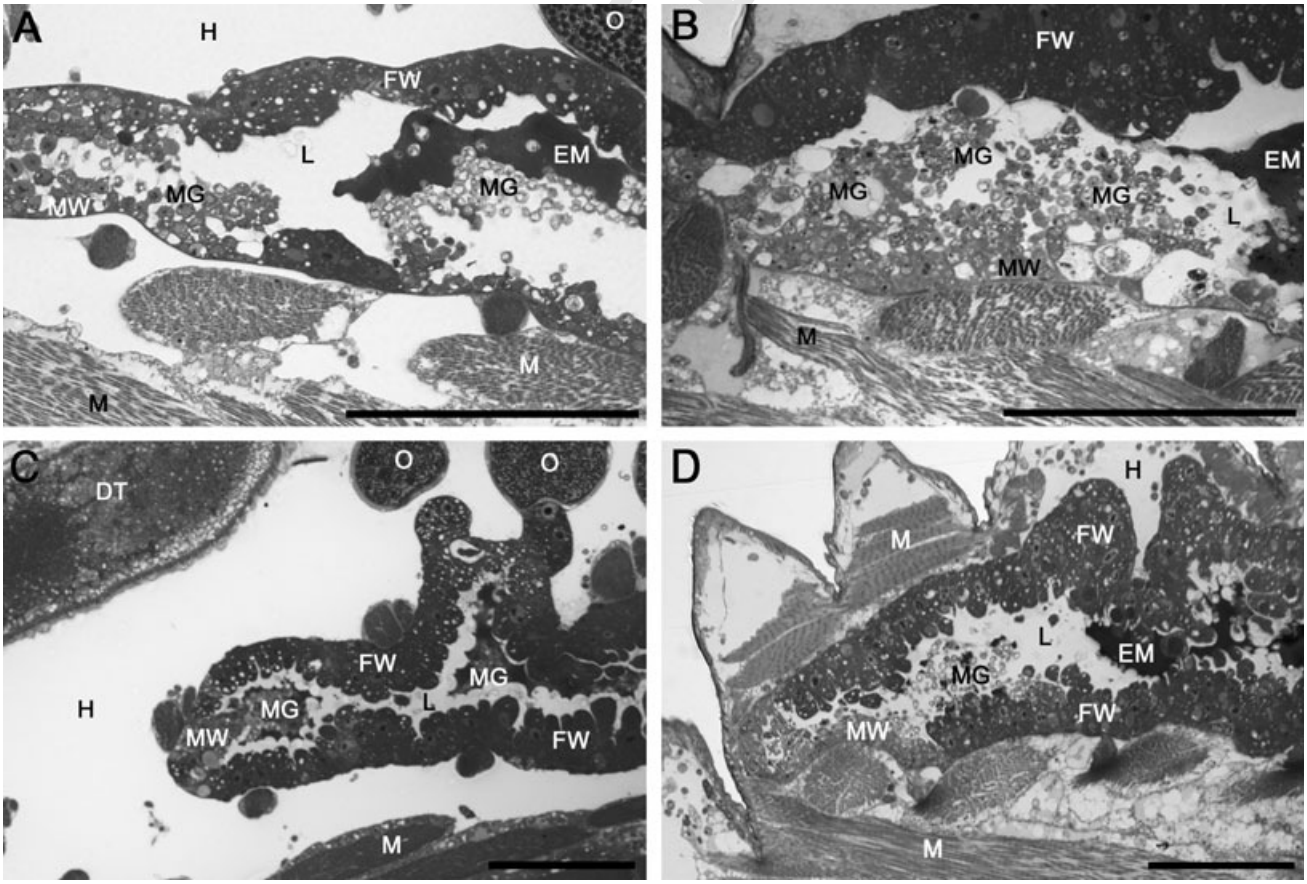

Fig. 1. Longitudinal thick sections of the posterior region of the hermaphroditic gonads of *Eulimnadia* spp. **A**. *Eulimnadia gibba*. **B**. *Eulimnadia gunturensis*. **C**. *Eulimnadia michaeli*. **D**. *Eulimnadia azisi*. In each of these species, the gonad is surrounded by muscle (M). Internally, the gonad includes a female wall (FW) that is interrupted at the posterior-most tip by a male wall (MW). From the male wall, male gametes (MG) are released into the gonad lumen (L) and are often found surrounded by eggshell secretory matter (EM). DT, digestive tract; H, hemocoel; O, oocyte. Scale bars=200 μm.

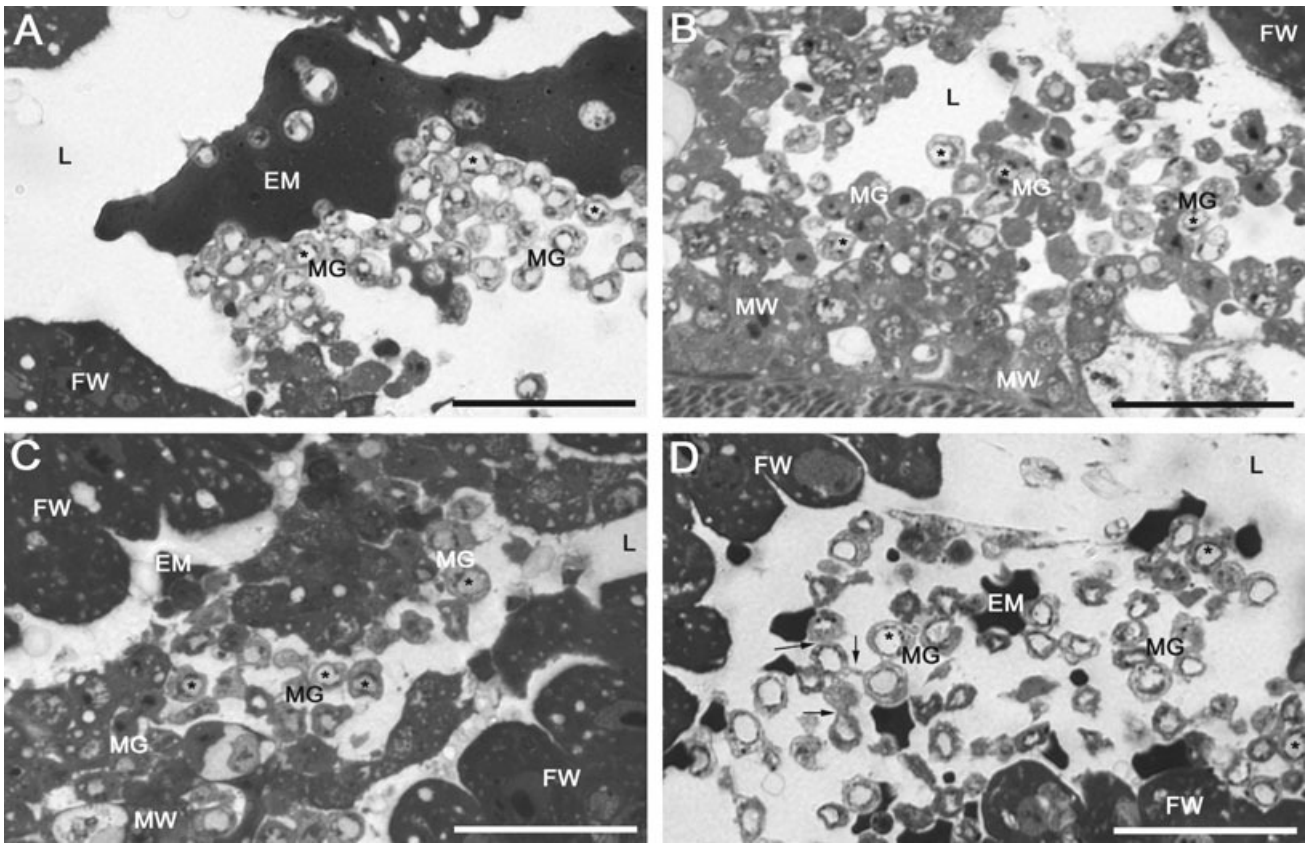

Fig. 2. Male gametes (MG) released from the male wall (MW) into the lumen (L) of the hermaphroditic gonads of *Eulimnadia* spp. **A**. *Eulimnadia gibba*. **B**. *Eulimnadia gunturensis*. **C**. *Eulimnadia michaeli*. **D**. *Eulimnadia azisi*. Many of the luminal male gametes possess sporadically arranged chromatin blocks (*) against the nuclear membrane. Intercellular bridges (arrows) are evident between the male gametes in *E. azisi* (D). EM, eggshell secretory matter; FW, female wall. Scale bars=50 μm.

could be seen in clusters associated with the eggshell matter secreted by cells of the female wall of the gonad (Figs. 1, 2).

Nuclear arrangement appeared to differ depending on the location of the male gametes. Male gametes that were closely associated with the male wall typically had well-defined nuclei with less compacted nuclear material. As male gametes were liberated from the male wall and began to mature, nuclei were still readily identifiable, although many began to show pronounced nuclear condensation. These condensed chromatin blocks formed along the nuclear membrane were not prevalent in male gametes still attached to the male wall, and appeared to increase in frequency within male gametes toward the center of the gonad lumen (Fig. 2). Interestingly, many of the luminal male gametes of *E. azisi* were connected to each other via pronounced intercellular bridges (Fig. 2D).

Discussion

Phylogenetic evidence suggests that the common ancestor of the genus *Eulimnadia* was androdioecious (Weeks et al. 2006b, 2009a). To date, all species of *Eulimnadia* that have been surveyed have either been androdioecious or all-hermaphroditic (Weeks et al. 2008). Herein, we have evidence of an additional five species of *Eulimnadia* that are also a mix of androdioecious and all-hermaphroditic populations.

Weeks et al. (2006b, 2008, 2009a) used sex ratio data combined with offspring-rearing studies and genetic assays to determine hermaphroditism in 15 species of *Eulimnadia* from all over the world. In this study, population sex ratios ranged from 0% to 47% males (Table 1). These ratios fall within the range previously reported for *Eulimnadia* species: 0–50% males with a mode of 15–18% males (Weeks et al. 2008). These ratios suggested that *E. azisi* is

all-hermaphroditic (sex ratios not significantly different from 0% males) and that *E. gibba, E. michaeli,* and *Eulimnadia* sp. are androdioecious (sex ratios significantly different than 0% males, and a mix of androdioecious and all-hermaphroditic populations for the latter two species). The one population of *E. gunturensis* provided ambiguous results: even though one male was found, the population's sex ratio was not significantly different from 0% males (Table 1). The smaller sample size (31 total shrimp) and single population studied makes the assignment of all-hermaphroditism to this species quite tentative.

A second method for assessing androdioecy and hermaphroditism is to assess sex ratios from isolated hermaphrodites that are forced to self-fertilize (Sassaman & Weeks 1993; Weeks et al. 2006b, 2008). We were able to do this in four of the five species studied (Table 2), indicating that these isolates were capable of producing viable offspring without being fertilized by males, which is consistent with the self-fertilizing hermaphroditism noted in all other species of *Eulimnadia* studied to date (Weeks et al. 2008). When these offspring were reared to sexual maturity, overall offspring sex ratios ranged from 0% to 13% males (Table 2A). These male percentages were low compared with those of other species of *Eulimnadia*, which ranged from 0% to 43% males (Weeks et al. 2008). Offspring sex ratios from isolated hermaphrodites can include all-hermaphrodite clutches (i.e., from isolated monogenic hermaphrodites) and those that have ~25% males (i.e., from isolated amphigenic hermaphrodites). When considering sex ratios from isolates from the populations with males, the current range was 7.5–13.2% males (mean 10.5%). This is half of that found in previous studies of *Eulimnadia* (21.8% male; Weeks et al. 2008). Previous studies have shown that male mortality is partly due to expression of deleterious recessive alleles on the Z sex chromosome (Weeks et al. 2011). It is possible that the Z chromosomes in the current species have a greater number of deleterious alleles, which could then cause lower male frequency due to higher mortality rates because of expression of these alleles (Weeks et al. 2010). To test this, very specific crosses would need to be made to assess the possibility of the expression of these deleterious recessive alleles (Weeks et al. 2010).

In populations that had no males (i.e., Ghat 1, Ghat 2, KK4A, KK6A, KK8A, and Karuvatta), we expected all monogenic hermaphrodites, and thus overall offspring sex ratios should have been 0% male among egg banks reared from isolated hermaphrodites. Offspring were successfully reared from isolated hermaphrodites in four of these six populations (Table 2A), and three of these four populations (Ghat 1, KK4A, and Karuvatta) indeed had no males among a total of 2359 offspring reared, suggesting that these populations were quite likely all-monogenic. In the one remaining population (KK8A), 83 males were found of a total of 1106 offspring, a sex ratio of 7.5% males, with 11 of the 21 isolates producing no males (i.e., 52% of the isolates were monogenic). Thus, although the population sex ratio suggested all-hermaphroditism for this population, it instead turned out to be androdioecious, with ~50% of the isolated hermaphrodites being amphigenic (see below).

Of the four populations containing males (PED1, Veli, KK2A, and KK8A), the percentage of monogenics ranged from 23% to 52% (mean 37.1%). This is very similar to the average percentage of monogenics in other androdioecious *Eulimnadia* species (mean 41.1%; Weeks et al. 2008), indicating that these populations were also likely androdioecious.

To further test for androdioecy consistent with that previously found in other *Eulimnadia* species, sex ratios of clutches produced by isolated amphigenic hermaphrodites were compared with the 3:1 hermaphrodite:male sex ratio predicted by the ZW sex determining system of related clam shrimp species (Sassaman & Weeks 1993; Weeks et al. 2010). Sex ratios for amphigenics only (Table 2B) ranged from 13% to 23% males (mean 18.3%), which is somewhat lower than reported for *E. texana* (mean 23.1%; Sassaman & Weeks 1993). Only one population, PED1, had sex ratios that strictly conformed to the 3:1 prediction. The remaining three populations had male sex ratios significantly lower than the predicted 25% (Table 2B). Finding fewer males than the predicted 25% is common in androdioecious *Eulimnadia* species (Sassaman & Weeks 1993; Weeks et al. 2006b, 2008). Males have poorer survival than hermaphrodites (Strenth 1977; Zucker et al. 2001) due to both expression of deleterious alleles on the Z-chromosome (as noted above), as well as increased energy expenditures of males relative to hermaphrodites (Weeks et al. 2011). The lower percentage of males was mainly in the two Thai populations, which may suggest that males of *E. michaeli* have a lower relative survival than other *Eulimnadia* males (as suggested above).

Considering population and isolate sex ratios together, *E. azisi* appears to be all-hermaphroditic, *E. michaeli*, *E. gibba*, and *Eulimnadia* sp. appear to be androdioecious, and we await further evidence

for *E. gunturensis*, which we had difficulty rearing in the laboratory.

Although parsimonious interpretation of sex ratios to infer androdioecy and all-hermaphroditism is reasonable, a stronger conclusion for these two breeding systems would be to find ovotestes in the purported hermaphrodites of these species. Our anatomical examinations demonstrate that *E. gibba, E. gunturensis, E. michaeli,* and *E. azisi* definitively contain hermaphrodites (Fig. 1). As in the previous species of *Eulimnadia* assayed, *E. gibba*, *E. gunturensis*, *E. michaeli*, and *E. azisi* possess only a small section of the ovotestis devoted to sperm production, and this section is located at the posterior end of the gonad. Such a bias toward egg production in hermaphrodites is expected in species that can only self-fertilize, because hermaphrodites only need to produce a small number of sperm to fertilize their own eggs; any extra sperm production would be wasted energy (Charnov 1982). Thus, to date, all six species examined for the presence of testicular tissue in the hermaphroditic gonad (*E. texana, E. agassizii*, *E. gibba, E. gunturensis*, *E. michaeli*, and *E. azisi*) have exhibited the same pattern: a small portion of the gonad at the distal end of the ovotestis devoted to sperm production (Zucker et al. 1997; Weeks et al. 2005).

Among the *Eulimnadia* analyzed histologically in this study, male gametes liberated into the lumen of the ovotestis appear to contain distinct plaques formed against the nuclear membrane (Fig. 2). These plaques represent highly compacted chromatin (chromatin blocks), and are similar to those found in male gametes reported in other male branchiopods (Scanabissi & Mondini 2002b; Scanabissi et al. 2005, 2006). Although these plaques have been previously associated with degenerating sperm in *E. texana* (Scanabissi et al. 2006), the hermaphrodites surveyed here (*E. gibba, E. gunturensis, E. michaeli,* and *E. asizi*) did not display any appreciable signs of male gamete degeneration, such as the widespread cytoplasmic voiding found in males of *E. texana*. However, to completely rule out all features suggestive of complete male gamete degeneration in the hermaphrodites surveyed, future studies using transmission electron microscopy would be helpful. In addition, it would be beneficial in future studies to examine male specimens from the present androdioecious populations to determine if, in fact, there are any signs of male gamete degeneration, as have been reported in various other branchiopods (Wingstrand 1978; Scanabissi & Mondini 2002b; Scanabissi et al. 2006; Weeks et al. 2009b).

Combining the sex ratio with the anatomical evidence, we propose that three (*E. gibba, E. michaeli*, and *Eulimnadia* sp.) of the five species studied herein are androdioecious. *Eulimnadia gunturensis* is definitively hermaphroditic, but sex ratio data are not sufficient to determine whether it is all-hermaphroditic or androdioecious. The fifth species, *E. azisi*, appears to be solely hermaphroditic, as noted in *E. agassizii*. However, it is possible that *E. azisi* is also androdioecious, but that we have only sampled two all-hermaphroditic populations. As noted here and elsewhere (Sassaman 1989; Weeks et al. 2008), androdioecious species of *Eulimnadia* are a mix of all-hermaphroditic and androdioecious populations. The all-hermaphrodite populations are likely the products of colonization by monogenic hermaphrodites, which cannot produce males (Pannell 2008). Thus, it would be helpful to study more populations of both *E. gunturensis* and *E. azisi* to determine whether they are fully hermaphroditic or androdioecious.

All of the findings reported above are consistent with the prediction that the genus *Eulimnadia* comprises either androdioecious or all-hermaphroditic species (Weeks et al. 2006b, 2009a). These data strengthen the hypothesis that androdioecy is the ancestral breeding system for these shrimps. As a result, we can be confident that this breeding system has been in place for at least 25 million and possibly up to 180 million years (Weeks et al. 2006b). Hence, this crustacean taxon represents the longest lived androdioecious clade in the animal or plant kingdoms (Pannell 2002; Weeks et al. 2006a).

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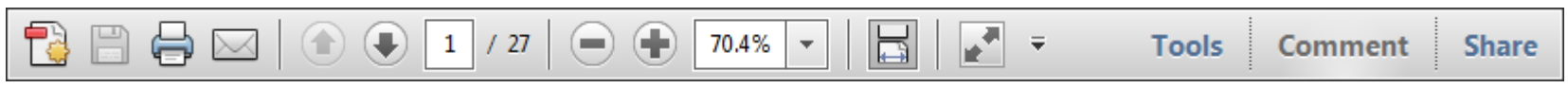

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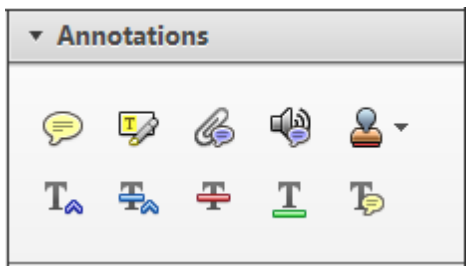

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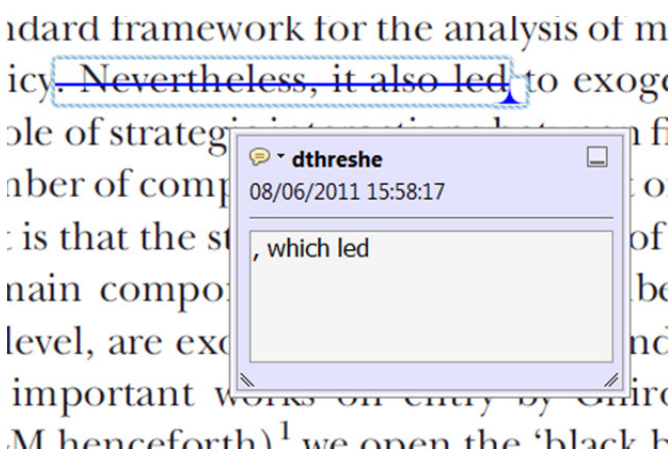

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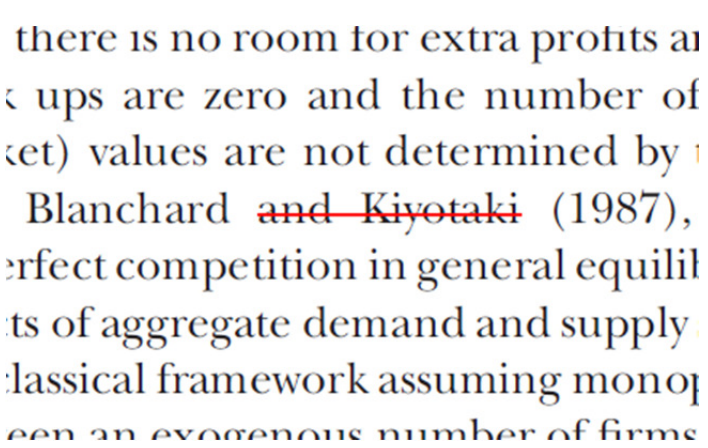

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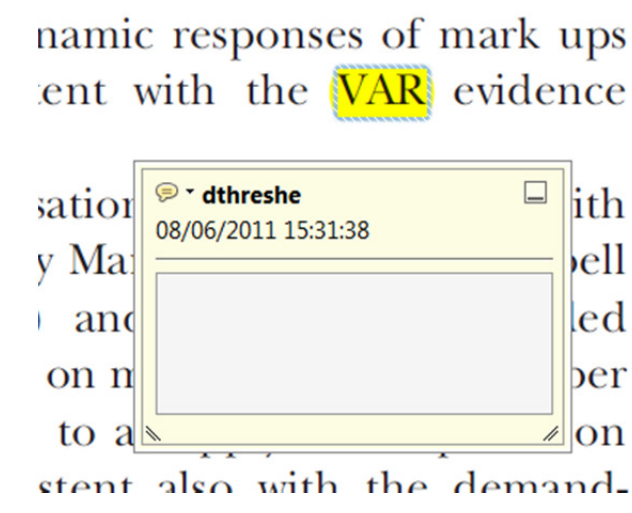

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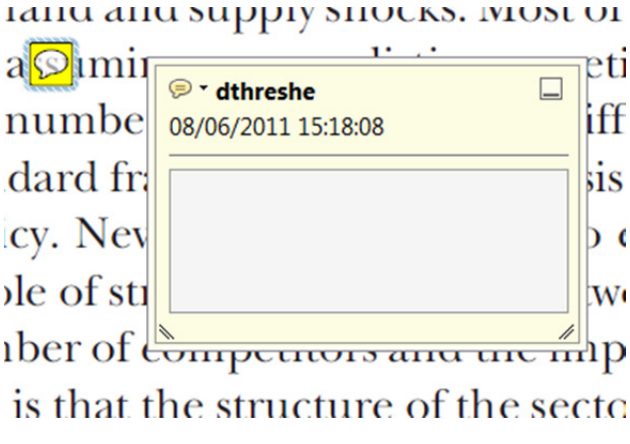

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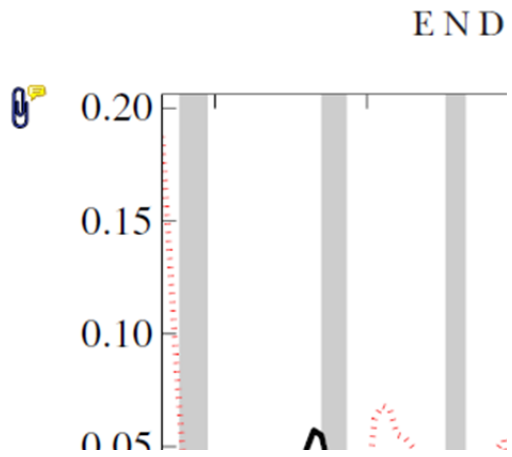

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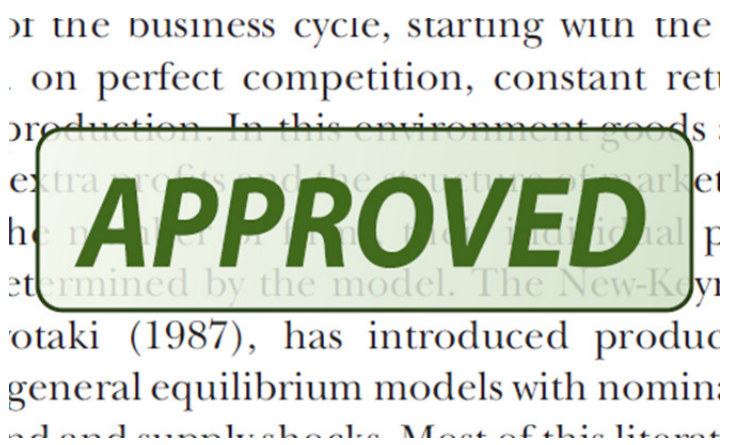

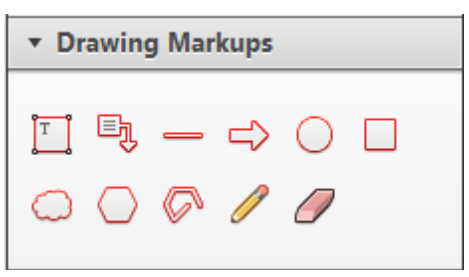

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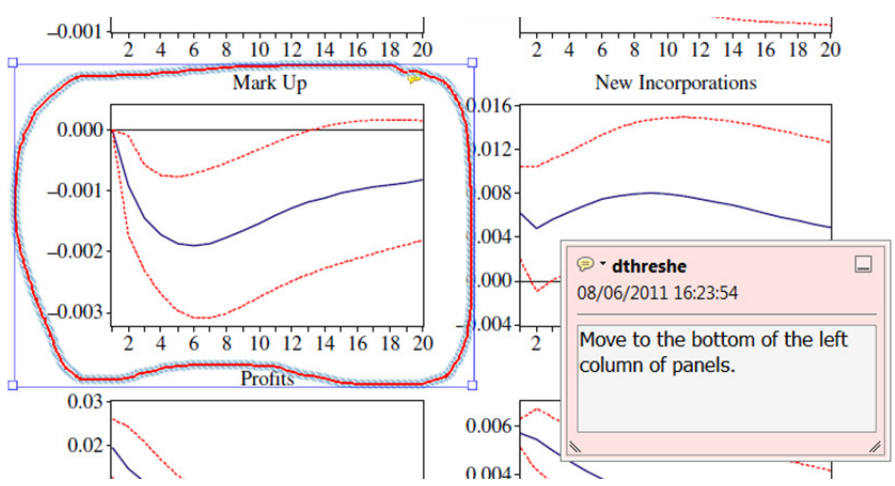

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