

Food Availability and Sex Reversal in *Mytella charruana*, an Introduced Bivalve in the Southeastern United States

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SUMMARY

We studied the reproductive biology of *Mytella charruana* to determine the potential reproductive success of this newly introduced bivalve species from Central/South America. We analyzed gonad morphology, gametogenesis, and the sex ratios of introduced populations throughout a 12 month period. In the non-native habitat *M. charruana* shows the same strategy of gametogenesis that had been observed in its native environment, which is an opportunistic type of gonadal cycle with gametes produced throughout the year. Instead, the spawning period of *M. charruana* along the southeastern US coast is extended compared to that found in the native environment. We determined the minimum size (shell length) of sexually reproductive mussels to be 1.25 cm. Interestingly, throughout the year the population samples were typically composed of a higher proportion of females. The female to male sex ratio varied within a wide range from 1:0 to 1:3.3. Upon this discovery we tested the effects of food availability on the gametogenesis of adult animals. The sex ratio of mussels collected from different locations and maintained in the laboratory with or without food changed toward a male-bias under starvation conditions within a month. This is the first study directly showing that food availability can trigger sex reversal in an adult bivalve. According to our data this mussel species will likely continue to spread along the east coast of the US. Moreover, *M. charruana* may prove to be a model organism in the study of alternative sexuality in bivalves.



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INTRODUCTION

Mytella charruana (subclass Pteriomorpha), synonymously known as *M. falcata*, *M. arciformis*, and *M. strigata*, is a new, introduced marine mussel along the southeastern US coastline (Boudreaux and Walters, 2006). *M. charruana* is native to Central and South America, extending from Southern Mexico to Ecuador in the Pacific (Oliveira et al., 2005) and from Trinidad through Brazil in the Atlantic (Paranagua, 1985; Kishore, 1995). In Florida, *M. charruana* was first discovered in Jacksonville waters in November 1986 (Lee, 1987). Eighteen years later, *M. charruana* was found in the Indian River Lagoon, approximately 160 km south of the first sighting (Boudreaux and Walters, 2006). Since 2004, the range of *M. charruana* rapidly extended

northward (Gillis et al., 2009), with individuals in 2008 found as far north as South Carolina (USGS, 2009; <http://nas.er.usgs.gov/queries/FactSheet.asp?speciesID=106>). The most likely source of *M. charruana* in US is from ballast water brought on ships from South America (Carlton, 1992; Gillis et al., 2009).

In this study, we investigated the reproductive biology of *M. charruana* to determine how this attribute may enable this bivalve to successfully invade new locations. *M. charruana* is a euryhaline species living in both marine and lagoon waters, and can tolerate salinity levels from 2 to 40 ppt (Yuan et al., 2009) and water temperatures between 6 and 31°C (Brodsky et al., 2009). Previous research on the reproductive patterns of *M. charruana* is very limited. Cardenas and Aranda (2000) determined that the spawning

period of *M. charruana* in two coastal lagoons along the Pacific Coast of Mexico occurred annually from July to October. Gross anatomy of *M. charruana* was described by Narchi and Galvao-Bueno (1983) and Villarroel and Stuardo (1995). They stated that the gonads are located in the mantle and the gonoducts open along the length of the renal duct.

Bivalve reproductive strategies vary greatly from species to species, starting with two main fertilization strategies (Kasyanov, 2001). Some species complete egg meiosis upon spawning and undergo external fertilization. Alternately, eggs complete meiosis after internal fertilization. In this strategy, the embryo usually develops for some time within the parental mussel (brooding). Observations of the fertilization strategy of *M. charruana* have not been reported.

Bivalves exhibit either ambisexuality (hermaphroditism) or unisexuality (gonochorism). Ambisexuality has been classified into four groups (Coe, 1943; Kasyanov, 2001): (1) Functional ambisexuality, where an animal concurrently develops both sperm and eggs (e.g., the ribbed scallop *Pecten irradians*). (2) Consecutive sexuality, once in the life of the bivalve, the animal undergoes a single sexual switch, usually from male to female (protandry, e.g., the quahog *Mercenaria mercenaria*). (3) Rhythmical consecutive sexuality, the animal experiences an equal number of sexual phases, changing from one sex to the other and maintaining a rhythmical pattern throughout its life (e.g., Olympia and European oysters, *Ostrea lurida* and *O. edulis*, respectively). (4) Alternative sexuality, whereby animals change sex depending on season or environmental triggers (e.g., the eastern and Japanese oysters, *Crassostrea virginica* and *C. gigas*, respectively).

Sex ratios in bivalve populations are usually close to 1:1, but there are examples of sex ratios biased toward either females or males (Coe, 1943; Morton, 1991). Generally, there seems to be a correlation between sex ratio and habitat (Morton, 1991). Freshwater species are either hermaphroditic or gonochoristic, and the latter shows a female-biased sex ratio (Morton, 1991). Mangrove and brackish water species are gonochoristic with either a slight overall female or male bias (Morton, 1991). Marine species are usually gonochoristic with a 1:1 sex ratio (Morton, 1991). Furthermore, for some freshwater and brackish species, the sex ratio varies with age, with the juvenile sex bias being species dependent (Morton, 1991). Adult *M. charruana* in one native environment (coastal lagoons in the Pacific Coast of Mexico) had an annual sex ratio close to 1:1 (Cardenas and Aranda, 2000). This is similar to other closely related marine species, including *Mytella guyanensis* (Cruz and Villalobos, 1993) and *Perna viridis* (Lee, 1988; Morton, 1991; Gillis et al., 2009). It is not known whether the sex ratio of *M. charruana* varies with age. Additionally, at the population level, it has been hypothesized that bivalve sex ratios vary in response to environmental factors such as food availability, injury and water temperature (Egami, 1953; Sastry, 1968; Davis and Hillman, 1971). In natural populations of hermaphroditic bay scallops, a correlation was observed between a higher proportion of males and a combination of cooler temperatures and low food supply (Sastry, 1968).

Furthermore, gonadal development in bivalves is variable and two primary strategies have been described (reviewed in Kasyanov, 2001). Gametogenesis can either occur under favorable food availability at any time of the year (opportunistic strategy) or only during specific seasons (conservative strategy). Both *M. charruana* and *M. guyanensis* appear to utilize an opportunistic type of gonadal cycle in their native waters (Cruz and Villalobos, 1993; Cardenas and Aranda, 2000).

There are several reproductive mechanisms that could contribute to the success of an introduced species in a new location, but ultimately these strategies must be conducive to rapid expansion upon founding with few individuals. In this study we characterized the reproductive biology of *M. charruana* to determine if this species exhibits a reproductive strategy that favors rapid establishment and subsequent spreading. First, we characterized gonadal morphology in females and males in introduced populations along the southeastern US. Second, we determined the sex ratio and reproductive status monthly for 12 months. We also determined the minimum size for gamete production, and hence reproduction, for *M. charruana*. Third, we conducted experiments to determine if food availability altered gametogenesis. This study brings new insights into the reproductive strategy of *M. charruana* and adds to the general knowledge of bivalve reproductive biology. Our work is also vital to the understanding of the invasive potential of this species and adds information on the general capabilities of mussels to spread beyond their native range.

RESULTS

Characterization of Gonads and Gametes

Both fresh tissue and histological sections of female gonads showed that the eggs of *M. charruana* are contained in follicles within the mantle of the mussel (Fig. 1A,B). The egg-containing follicles were interconnected and organized into larger sacs, forming acinous structures. Eggs isolated from fresh tissue were approximately 50 µm in diameter (Fig. 1B). Eggs from all collections contained a large germinal vesicle, which indicated they were immature (Fig. 1B). In males, sperm was found within sacs in the mantle, similar to the structures observed in the females (Fig. 1D). The head length of the sperm was approximately 3 µm (Fig. 1D).

Adult mussels spawned gametes spontaneously in laboratory aquaria in June 2008. Unfertilized eggs were observed within the first 24 hr after spawning, and did not show a germinal vesicle (Fig. 1C), which indicated that they matured before spawning or within the first 24 hr post-spawning. After spawning, eggs were approximately 20 µm in diameter (Fig. 1C).

Minimum Size for Reproduction and Temporal Analysis of Sex Ratio

Among 826 mussels measured, the minimum shell length of sexually reproductive mussels was 1.25 cm (Table 1). The smallest mussel producing gametes was a female collected in St. Marys, GA in August 2007. The smallest reproductive

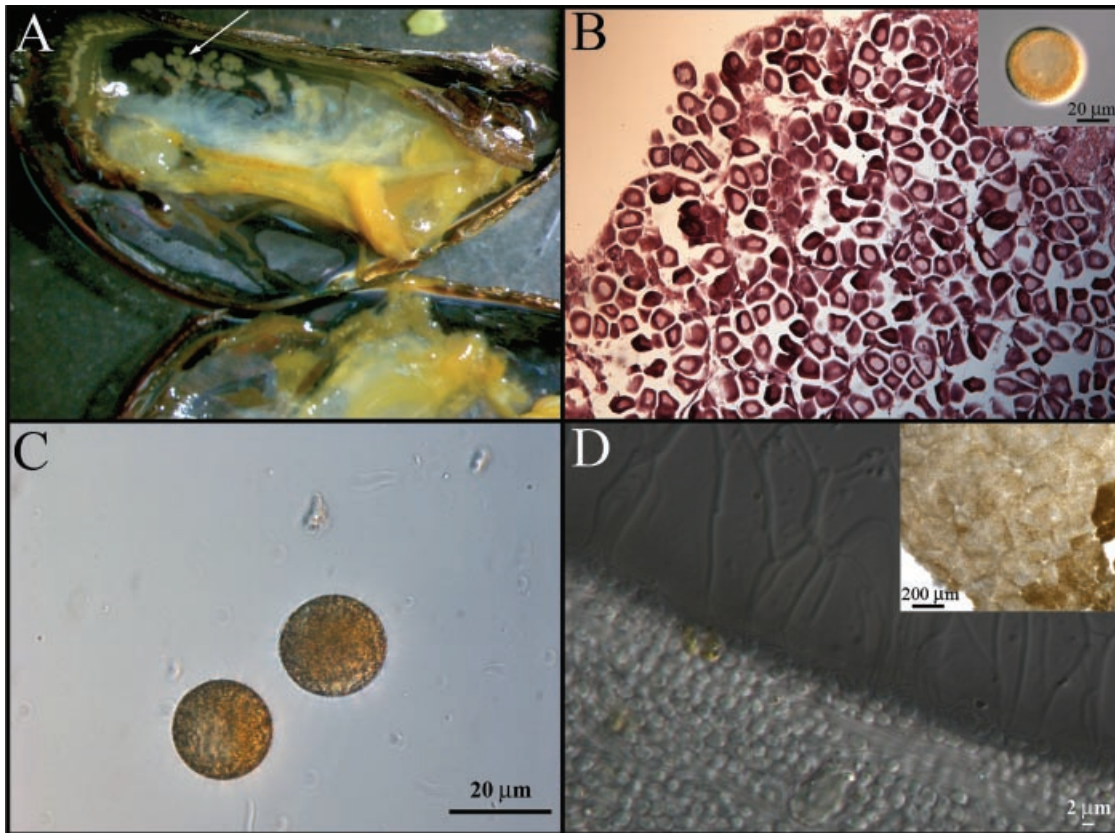


Figure 1. *M. charruana* gonads and gametes. **A:** Internal view of an adult female *M. charruana*. Valves are open and gills were removed. The white arrow indicates the gonads embedded within the whole mantle. **B:** Histological section of female gonad stained with H&E; inset: optical section of an egg isolated from fresh tissue (DIC image). **C:** DIC image of spawned eggs. **D:** Sperm cells inside the sperm-sacs; inset: portion of a male mantle with acinous structures, which contain the sperm-sacs.

TABLE 1. Size Analysis of Mussels Collected at Different Locations

Site	Collection	Males	Females	Hermaphrodites	No gametes
1	7/12/2007	3.32 ± 0.65 (11)	3.15 ± 0.97 (48)	n.a.	3.17 ± 0.76 (7)
1	7/24/2007	3.80 ± 0.79 (3)	3.49 ± 0.47 (18)	3.48 ± 0.65 (7)	2.75 ± 0.49 (11)
2	8/6/2007	n.a.	3.32 ± 0.81 (27)	n.a.	1.81 ± 0.59 (14)
2	9/9/2007	4.06 ± 0.31 (23)	4.07 ± 0.38 (61)	n.a.	3.85 ± 0.41 (5)
2	10/15/2007	3.98 ± 0.51 (36)	4.15 ± 0.32 (11)	n.a.	4.13 ± 0.17 (13)
1	11/14/2007	n.a.	n.a.	n.a.	1.01 ± 0.33 (55)
1	11/21/2007	2.88 ± 0.39 (7)	2.92 ± 0.17 (7)	n.a.	1.12 ± 0.49 (56)
3	12/12/2007	3.71 ± 0.29 (15)	3.66 ± 0.28 (38)	n.a.	3.45 ± 0.92 (17)
3	1/15/2008	3.56 ± 0.63 (7)	3.87 ± 0.36 (26)	n.a.	3.71 ± 0.48 (16)
3	2/10/2008	3.42 ± 0.29 (3)	3.48 ± 0.44 (44)	n.a.	1.92 ± 0.88 (11)
3	2/25/2008	3.76 ± 0.29 (16)	3.64 ± 0.26 (25)	n.a.	3.03 ± 1.10 (19)
3	3/21/2008	3.41 ± 0.26 (17)	3.47 ± 0.41 (16)	n.a.	3.16 ± 0.54 (16)
3	4/6/2008	3.64 ± 0.48 (11)	3.49 ± 0.62 (25)	n.a.	3.08 ± 0.79 (25)
3	5/1/2008 ^a	3.61 ± 0.30 (17)	3.26 ± 0.30 (29)	3.78 ± 0.42 (3)	3.20 ± 0.26 (4)
All combined ^b		3.70 ± 0.50 (166)	3.60 ± 0.52 (375)	3.63 ± 0.56 (18)	2.25 ± 1.26 (269)

Mean ± SD shell length of males, females, hermaphrodites, and mussels without gametes according to the location and date of collection. The number of mussels in each category is indicated in parenthesis; n.a. = groups having mussel number <3. Significantly different data analyzed by one-way ANOVA are indicated as follow.

^aHighly significant average size difference between females and males ($P \leq 0.001$).

^bSignificant average size difference between females and males ($P \leq 0.05$).

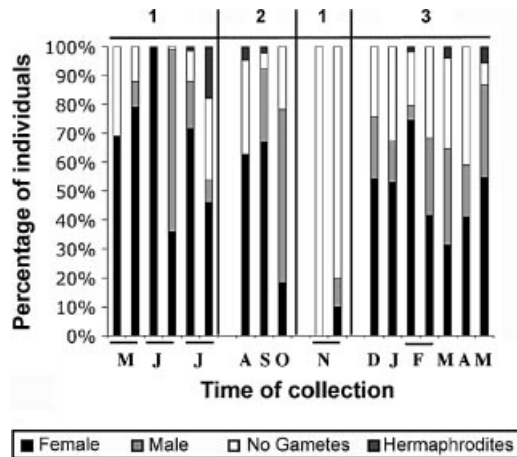


Figure 2. Sex ratio analysis of *M. charruana* populations over 12 months. The percentages of females, males, mussels without gametes and of hermaphrodites in each collection are reported. Mussels were collected from three different sites (indicated at the top of the graph): site 1, Sisters Creek Joe Carlucci—Jacksonville, FL; site 2, St. Marys, GA; site 3, Lion's Club Marina—Jacksonville, FL (see Table 2 for number of sexed mussels per collection).

male was 1.78 cm (from Jacksonville, FL in December 2007). The largest mussel was a 4.87 cm female (St. Marys, GA in September 2007). When monthly collection samples were compared, we did not observe a significant difference in size between females and males with the exception of the 5/1/08 collection, in which males were significantly larger than females. Only when all collections were combined was the difference between females and males significant, with males being on average 0.1 cm larger than females

(Table 1). Hermaphrodites were observed very rarely (1.9% of all collections combined). We observed hermaphrodites in 7 out of the 18 collections (range: 1.5–5.6%), with 1 collection having 18% hermaphrodites (July 2007; Fig. 2). The minimum shell length of a hermaphrodite was 2.16 cm (Jacksonville, FL, July 2007).

With the exception of the November 2007 collections, on average the mussels with no gametes were above the minimum observed reproductive size, suggesting they recently spawned. Interestingly, in the November 2007 collections from Jacksonville, most of the mussels were not gravid and their average size (~1 cm) was below the observed minimum size for reproduction (Table 1). Therefore we concluded that these November collections were composed mostly of immature mussels. If we exclude the November 2007 collections, the percentage of *M. charruana* predicted to have recently spawned varied from 0% to 41% (Fig. 2). From May to mid-July 2007, in September 2007 and May 2008 the percentage was below 12%. During the rest of the year, the percentage was above 20% (Fig. 2).

The population sex ratio of adult *M. charruana* changed with every collection (Table 2). The female to male sex ratio was significantly different from 1:1 for all collections with the exception of November 2007 and March 2008 (per collection Chi-square *P*-values are listed in Table 2). The female to male sex ratio ranged from 1:0 (100% females) to 1:3.3 (Table 2). Most of the collected samples had a sex ratio highly biased toward females. The annual sex ratio was 2.55:1 (female/male), which was significantly different from 1:1 (Table 2). Moreover, through time the sex ratios varied widely and rapidly (e.g., within 2 weeks; Table 2). At site 1 between June and July, the sex ratio reversed twice at 2-week intervals going from 1:0 to 1:1.8 and then to 4.4:1 (Table 2). At site 2 between September and October, it changed from 2.7:1 to 1:3.3 within a month (Table 2).

TABLE 2. Sex Ratio of Collected Mussel Samples

Site	Collection (no. of collected)	Female/male (no. of sexed)	Chi-square <i>P</i> -value
1	5/24/2007 (51)	1:0 (42)	<0.0001
1	5/31/2007 (80)	8.8:1 (80)	<0.0001
1	6/11/2007 (35)	1:0 (35)	<0.0001
1	6/26/2007 (50)	1:1.8 (50)	0.0051
1	7/12/2007 (70)	4.4:1 (67)	<0.0001
1	7/24/2007 (40)	6:1 (39)	<0.0001
2	8/6/2007 (120)	1:0 (43)	<0.0001
2	9/9/2007 (140)	2.7:1 (91)	<0.0001
2	10/15/2007 (150)	1:3.3 (60)	<0.0001
1	11/14/2007 (55)	n.a. (55)	n.a.
1	11/21/2007 (70)	1:1 (70)	1
3	12/12/2007 (130)	2.5:1 (70)	<0.0001
3	1/15/2008 (100)	3.7:1 (49)	<0.0001
3	2/10/2008 (110)	14.7:1 (59)	<0.0001
3	2/25/2008 (120)	1.6:1 (60)	0.0278
3	3/21/2008 (110)	1:1.1 (51)	0.6892
3	4/6/2008 (120)	2.3:1 (61)	<0.0001
3	5/1/2008 (53)	1.7:1 (53)	0.0093
All combined (1,604)		2.55:1 (1,035)	<0.0001

The female/male ratio is listed according to the location and date of collection. The total number of individuals collected on each date is indicated in parenthesis. The female/male ratio was analyzed for each collection date separately, and for all collection dates combined by Chi-square and the *P*-value is reported. The number of individuals sexed is indicated in parenthesis. n.a., not applicable (no females or males in the sample).

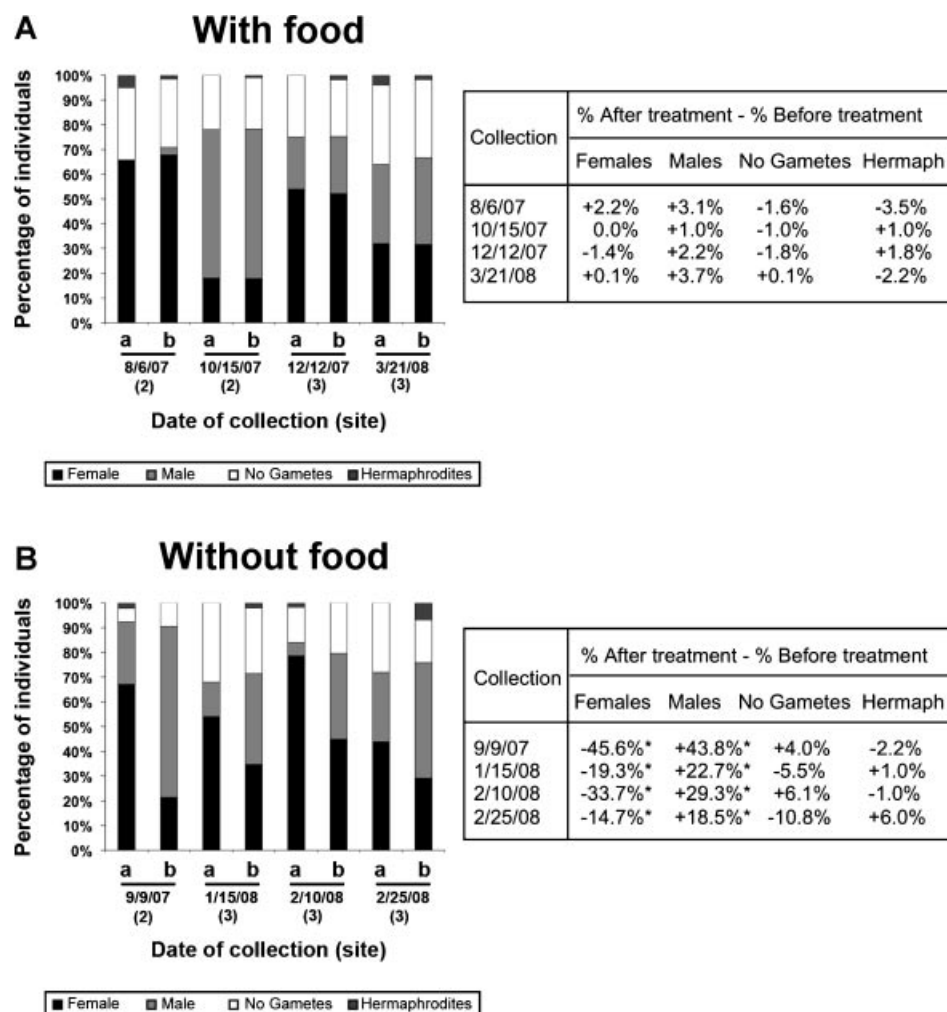


Figure 3. Analysis of starvation effect on *M. charruana* sex ratio. Data shown are the percentages of females, males, mussels without gametes and of hermaphrodites for each collection/treatment. Individuals below the minimum size for reproduction were excluded. The collection sites are indicated in parenthesis below the collection dates. **A:** Data were obtained from four different collections; for each collection a fraction of mussels was dissected upon arrival in the laboratory (a) and a second fraction was dissected 1 month later after being in an aquarium with food provided (b). The number of mussels sexed out of the total collected were: 8/6/07, 41 in (a) and 65 in (b) out of 111 total; 10/15/07, 60 in (a) and 60 in (b) out of 130 total; 12/12/07, 70 in (a) and 57 in (b) out of 128 total; 3/21/08, 51 in (a) and 57 in (b) out of 110 total. The total includes individuals dead during the trials. The table lists, for each collection date, the mussel percentage increase (+) or decrease (–) for each sex after a month in an aquarium with food provided. The percentage of females, males, mussels without gametes or hermaphrodites did not significantly change after 1 month in the tank (one-way ANOVA: $P = 0.98$, 0.91, 0.75, 0.70 respectively). **B:** The data shown were obtained from four different collections; for each collection a fraction of mussels was dissected upon arrival in the laboratory (a) and a fraction was dissected 1 month later after being in an aquarium with no food provided (b). The number of mussels sexed out of the total collected were: 9/9/07, 91 in (a) and 42 in (b) out of 145 total; 1/15/08, 49 in (a) and 49 in (b) out of 109 total; 2/10/08, 56 in (a) and 49 in (b) out of 111 total; 2/25/08, 57 in (a) and 58 in (b) out of 115 total. The total includes individuals dead during the trials. The table lists for each collection date the mussel percentage increase (+) or decrease (–) for each sex after a month in an aquarium with no food provided. The percentage of females or males were significantly different after 1 month in the tank (indicated with asterisks; one-way ANOVA: $P = 0.021$ and 0.023, respectively). In

Analysis of Sex Change Under Variable Food Regimes

The wide variation in the sex ratio of populations of *M. charruana* throughout the year led us to investigate whether food availability triggers sex change. All populations used for this experiment had different female/male sex ratios at the

time of collection, ranging from 1:0 (100% females) to 1:3.3 (Table 2). In our laboratory trials, the percentage of females and males did not significantly change when mussels were maintained in the aquarium and fed throughout the month (Fig. 3A). In contrast, the percentage of females and males was significantly different when the mussels were kept

without food (Fig. 3B). In the starvation treatment, a male-biased sex ratio was found, with the increase in the number of males similar to the decrease in the number of females (Fig. 3B). The percentage of mussels without gametes (above the minimum reproductive size) or the percentage of hermaphrodites did not significantly differ between collection time and the end of the month for both feeding and starvation conditions (Fig. 3). Date of collection had no effect on the proportion of mussels in each category (female, male, no gametes, hermaphrodite; logistic regression: $P = 0.302$). Mortality observed during all trials was very low ($\leq 10\%$). Although dead animals were not sexed due to rapid tissue decomposition, the low number of dead individuals did not likely influence final results.

DISCUSSION

The primary goal of this study was to investigate the reproductive biology of the marine mussel *M. charruana* to determine the potential reproductive success of this newly introduced species. We analyzed gonad morphology, gametogenesis, and the sex ratios of introduced populations at multiple time points throughout 12-month period. Upon discovery of variable sex ratios, we conducted an experiment to determine whether food availability contributed to this variability.

Gametes and Gametogenesis

Gonads of *M. charruana* were morphologically similar in females and males (Fig. 1). Both were composed of several acinous structures interconnected by gonad tubules, and both were embedded within the two mantles. This morphology is similar to other gonochoristic marine bivalves (Kasyanov, 2001). Mature eggs of *M. charruana* (20 μm) were below the range described for mature eggs of bivalves (40–360 μm ; Mackie, 1984; Kasyanov, 2001). This could be either a species-specific characteristic (egg sizes in native range are not known), or a consequence of an abiotic or biotic factor in the nonnative habitat limiting egg reserve accumulation.

The annual average percentage of hermaphrodites that we observed was low: 1.7%. Coe (1943) classified hermaphroditism at such low frequency as accidental hermaphroditism in a gonochoristic species. In *Mytilus edulis*, hermaphrodites were 2.2% of populations, while in other gonochoristic species, hermaphrodites usually comprise less than 1% of a population (Morton, 1991).

Spontaneous spawning occurred in our aquaria in June and the presence of unfertilized eggs in the water indicated that *M. charruana* undergoes external fertilization. External fertilization is the most common form of sexual reproduction in marine bivalves (Kasyanov, 2001). Our data on gamete size and fertilization strategy suggest that *M. charruana* is likely to have planktotrophic larval development. A pelagic larval phase would favor dispersal and therefore may contribute to the invasive potential of this species.

In regard to gametogenesis, most individuals we collected produced gametes throughout the year. Therefore, in the nonnative habitat *M. charruana* shows the same opportu-

nistic strategy for gametogenesis that had been observed in its native range (Cruz and Villalobos, 1993; Cardenas and Aranda, 2000). Each of our monthly collections had a proportion of individuals without gametes with shell lengths greater than the minimum size for reproduction (Fig. 2; Table 1). These individuals likely represent recent spawners. This suggests that the spawning period of *M. charruana* along the southeastern US coast is extended compared to native populations on the Pacific coast of Mexico, with an annual spawning period from July to October (Cardenas and Aranda, 2000). The lack of gametes could also be explained by gamete re-absorption. Gamete re-absorption in bivalves and other marine invertebrates is usually caused by nutritional stress (Kasyanov, 2001). Since most mussels appeared healthy and contained gametes, it is more likely that individuals that lacked gametes spawned them before collection and did not re-absorb them. Plasticity in reproductive patterns is common in mussels; gametogenesis and spawning periods are variable across many mussel species and environments. A study of the reproductive pattern of mollusks in multiple locations along the Pacific coast of Mexico showed that gametogenesis and spawning patterns can be quite variable within a species when living in different habitats (Cardenas and Aranda, 2000). Similarly, the closely related species *P. viridis*, a marine mussel native to the Indo-Pacific and recently introduced to Florida, was conservative in gametogenesis in some native and introduced populations (Lee, 1988; Barber et al., 2005). However, in the Philippines, *P. viridis* shows an opportunistic type of gametogenesis with spawning occurring throughout the year (Walter, 1982). Environmental factors known to be important in regulating gametogenesis and spawning periods in bivalves are temperature and food (e.g., phytoplankton) availability (Bayne, 1975, 1976; Sastry, 1979). For example, *M. edulis* (the blue mussel) can adopt both conservative and opportunistic strategies during its life cycle based on food availability (Rodhouse et al., 1984). Water temperature in one known native region of *M. charruana* ranges annually from 28.5 to 33°C (Cardenas and Aranda, 2000), while in Florida and Georgia, water temperature ranges from 16 to 30°C (FDEP, 2009; <http://www.dep.state.fl.us/northeast/RAAG/JaxMetroPark.htm>). The lower winter water temperature in Florida and Georgia could be contributing to the different pattern of gametogenesis and spawning of *M. charruana* in the nonnative environment. No data are available on relative phytoplankton abundances.

Overall, our data suggest that the southeastern US is a favorable habitat for *M. charruana*. Opportunistic gametogenesis, extended spawning period and a likely pelagic larval phase favor the steady, continued spread of this mussel.

Minimum size for reproduction and sex ratio. The mean shell length of all mussels collected showed a slight difference between females and males, with the male shell 2.7% longer than the female one (Table 1). Even though *M. charruana* shell length appears to be sexually dimorphic, the difference is too small to be employed to distinguish females from males. Sexual dimorphism is rare in gonochoristic bivalves and has been observed

mostly in freshwater species (Mackie, 1984). The largest *M. charruana* we found was a female of 4.87 cm; it was larger than the largest individual previously recorded (4.5 cm; Szefer et al., 1998).

The minimum size for reproduction in *M. charruana* (1.25 cm) is comparable to closely related mussels. *M. guayanensis* is sexually mature at 1.8 cm shell length (Cruz and Villalobos, 1993). *M. edulis*, which belongs to the same family (Mytilidae), has been found to be sexually mature at 1.8 cm when growing subtidally, and 3 cm when growing in the intertidal zone (Sprung, 1983). *Geukensia dimissa*, another confamilial mussel living in the same area where we collected *M. charruana*, has a minimum size for reproduction of 1.2 cm (Franz, 1996).

The annual female/male sex ratio we observed (2.55:1) in the nonnative environment was different from the 1:1 annual sex ratio observed for *M. charruana* in their native environment (Cardenas and Aranda, 2000). It was also different from the 1:1 sex ratio observed in other gonochoristic marine bivalves (Morton, 1991). The female-biased sex ratio may also favor the spread of *M. charruana* in the nonnative environment. In fact, group productivity is known to positively correlate with a female-biased sex ratio (Harvey, 1985). A female-biased sex ratio could result from a combination of consecutive sexuality (e.g., protandry) and a collection bias toward larger individuals in the populations we analyzed. However, this does not appear to be true for *M. charruana* based on our mussel size data (Table 1): if size correlated with age, we would have observed smaller (younger) males and larger (older) females, but in each collection we did not observe any significant difference in size between females and males (Table 1). Most likely the female-bias was due to differences in environmental factors unique to the nonnative environments. A female-biased sex ratio has been observed most often in freshwater bivalve species, which brood their larvae (Morton, 1991; McIvor and Aldridge, 2007). *M. charruana* is a euryaline species, but it lives optimally in brackish waters (Yuan et al., 2009). Our data do not suggest a correlation between water salinity and sex ratio since we observed a female-biased sex ratio in locations with salinities ranging from 6 to 30 ppt. Alternatively, a female-biased sex ratio may be caused by aquatic pollutants. For example, exposure of the oyster *C. gigas* at 7–8 days post-fertilization to nonylphenol, a plastic additive, led to a female-biased sex ratio in adult populations and a higher level of hermaphroditism (17–30%; Nice et al., 2003).

Sex change caused by an environmental trigger. We found that food availability played a significant role in sex ratio of *M. charruana*. From our data, we inferred that this mussel is capable of changing sex from female to male under starvation conditions. The ability to change sex classifies this species as having alternative sexuality (Coe, 1943). One explanation of our data is that females preferentially spawned within the holding tank and hence decreased in frequency during the trial. However, the change cannot be attributed to spawning events because the percentage of mussels with no gametes in their mantle remained unchanged after a month of starvation (Fig. 3B).

Further evidence of a controlled switch is that the mussels changed to the sex most energetically favorable under conditions of food deprivation. Producing eggs (yolk reserves) is energetically more expensive than producing sperm (Russell-Hunter, 1979). In mollusks, egg production requires 50% more energy than sperm production (Russell-Hunter, 1979). Mussel gonads are likely sensitive to energy reserve levels because of their structure. The gonads do not have an epithelial wall but they are surrounded by connective tissue and by mantle cells accumulating glycogen, which is mobilized to provide energy (reviewed in Bayne, 1976). Oogenesis and spermatogenesis only start when a considerable level of energy reserves has been accumulated (reviewed in Bayne, 1976). Therefore it is possible that gametogenic cell proliferation and/or differentiation are in some way regulated by energy reserve levels of the surrounding mantle cells. The ability to change sex within a short period of time could be due to the presence of both oogonia and spermatogonia in the gonads of marine bivalves, even if in very different relative amounts in females and males of gonochoristic species (Galtsoff, 1964). A previous study suggests that it is the proliferation rate of one type of gametogenic cell relative to the other (oogonia vs. spermatogonia) that ultimately determines the sex of marine bivalves (Lucas, 1975).

Several previous bivalve studies provided correlations between nutrition and sex ratio. Excision of gills in adult oyster *C. gigas* led to an increase of the percentage of males, predicted to be due to reduced food intake (Amemiya, 1935; Egami, 1953). The same effect was observed when *C. virginica*, the eastern oyster, underwent starvation in combination with shell injury (Bahr and Hillman, 1967; Davis and Hillman, 1971). A study on hermaphroditic bay scallops showed that oocytes do not develop and oogonia numbers decrease in the absence of food. In contrast, under a combination of low food and temperature, spermatozoa still developed (Sastry, 1968). A male-biased sex ratio has also been observed in association with parasitic infections of oysters that depleted their nutrient storage (Cox and Mann, 1992). In addition to nutritive stress, studies in oysters identified an influence on sex by other individuals living in close proximity, probably through secretion of pheromone-like compounds (Kennedy, 1983). Our study provides the first direct experimental evidence that food availability triggers sex change in mussels. Although possible, it is unlikely that other stressors might have induced the observed sex change. Water quality was monitored daily and maintained consistently during all trials (see the Materials and Methods Section). Animal mortality was minimal during the month-long trials and the dead were removed within 24 hr.

The ability to change sex could provide *M. charruana* with a great advantage over native species that reside within the same environment, such as the ribbed mussel, in which no such mechanism has been observed. *M. charruana* may be able to conserve their energy and live under limiting nutrient conditions and still maintain their potential to reproduce when environmental conditions become optimal.

From our analysis of the reproductive strategy of *M. charruana*, we conclude that this mussel species will likely

continue to spread along the east coast of the US and other areas with similar environmental conditions. Hence, human intervention is warranted as limiting the spread of this species is a worthy goal. Moreover, a better understanding into why this species has been successful provides important data for both invasion biologists and resource managers. As such, *M. charruana* should be used in future studies as a model to further understand the physiological, cellular and molecular basis of sex reversal and provide insights on its evolution.

MATERIALS AND METHODS

Animal Collections and Maintenance

Mussels were collected monthly or bi-monthly for 12 consecutive months for a total of 18 collections on the undersides of boat docks at the following three locations: Sisters Creek Joe Carlucci—Jacksonville, FL (site 1: Latitude/Longitude 30.4195/–81.4194) on 5/24/2007, 5/31/07, 6/11/07, 6/26/07, 7/12/07, 7/24/07, 11/14/07, and 11/21/07; St. Marys, GA (site 2: Latitude/Longitude 30.7333/–81.5386) on 8/06/2007, 9/09/2007, and 10/15/2007; Lion's Club Marina—Jacksonville, FL (site 3: Latitude/Longitude 30.3782/–81.6209) on 12/12/2007, 1/15/2008, 2/10/2008, 2/25/2008, 3/21/2008, 4/6/2008, and 5/1/2008. The latter two sites were included after the mussel population at the initial Jacksonville site was depleted by our collections.

On each date 35–150 mussels (depending on animal availability) were collected and returned to the University of Central Florida (UCF) in buckets containing water from the site. All mussels were maintained in a 75.7 L aquarium at 22°C at a salinity equal to the collection site (27 ppt for the Sisters Creek Joe Carlucci collections, 6 ppt for the Lion's Club collections and 30 ppt for St. Marys). Water in aquaria was a 1:1 (v/v) solution of natural water from the collection location and Instant Ocean salts™ with the final salinity matching the collection site salinity. Aquaria were provided with a biofilter and a carbon filter. Water pH, nitrate, nitrite, and ammonia content were monitored daily.

Characterization of Gonadal Tissue and Gametes

Samples of fresh gonadal tissue were retrieved from mussels within 24 hr of collection and the overall tissue organization was observed under a compound microscope. Histological preparations of female gonads were made from two collections (St. Marys, GA: 9/9/2007 and 10/15/2007). Samples of mantle tissue were fixed at 4°C for 48 hr in a solution of 4% paraformaldehyde, 32.5% filter-sterilized seawater, 32.5 mM MOPS pH 7.0 and 162.5 mM NaCl (Minokawa et al., 2004). The tissue was then dehydrated in xylene, imbedded in paraplast and cut into 10 µm sections using a microtome. The tissue sections were stained using Harris Hematoxylin and Eosin solutions (Sigma-Aldrich, Inc., St. Louis, MO) following manufacturer's procedures. Tissue sections were observed under a compound microscope.

Temporal Analysis of Sex Ratio and Minimum Size for Reproduction

We determined the sex of 1,035 of the 1,604 mussels we collected. Sex was determined within 24 hr of collection. Of sexed mussels, we measured the shell length of 826 individuals. First, mussel shell length was measured using vernier calipers. Next, sex was determined using the following protocol. The valves of the mussel were opened by cutting the adductor muscles with a scalpel. Samples of the gonadal tissue (mantle) were dissected from each mussel and observed under a compound microscope. Sex was

determined by the presence of eggs or sperm in the mantle. Mussel length data were analyzed with a one-way ANOVA to determine size differences between sexes and through time. Deviation from 1:1 of female/male ratio was tested by Chi-square test for each collection.

Analysis of Sex Ratio Under Variable Food Regimes

A total of 959 mussels were used to test whether sex ratio changed under starvation conditions. For this experiment, trials with food provided and trials with no food provided were performed on separate mussel collections due to the limited number of mussels available at each collection site. Whenever we could retrieve at least 100 mussels we tested for a nutrition effect on sex ratio (see Fig. 3 for collection dates). We used logistic regression to test if the date of collection had an effect on the proportion of mussels in each category. The four trials with food provided included a total of 479 mussels and the four trials with no food provided included a total of 480 mussels. The initial sex was determined within 24 hr of collection for approximately 50% of the mussels on each date (see Fig. 3 for number of collected and sexed mussels). The remaining mussels were placed in a 75.7 L aquarium for 30 days with either no food provided or food provided every 4 days. The aquarium was maintained under the temperature and salinity conditions described above. Mussels were fed with an algal paste (Spat formula, Innovative Aqua products, Skerry Bay, Lasqueti Island, BC, Canada) diluted 10× with deionized water and 0.5 ml/mussel was added to the aquarium water every 4 days in the food provided treatment. After the 30-day experimental period, the sex of the remaining live mussels was determined by dissection as described above. Mortality was monitored on a daily basis and dead animals were promptly removed and discarded. Differences between initial and final percentage of individuals in each category (female, male, no gametes, hermaphrodite) were analyzed by one-way ANOVA.

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