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REPRODUCTIVE BIOLOGY OF THE OPOSSUM PIPEFISH, *MICROPHIS BRACHYURUS LINEATUS*, IN TECOLUTLA ESTUARY, VERACRUZ, MEXICO

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ABSTRACT The reproductive biology of the opossum pipefish, *Microphis brachyurus lineatus*, was investigated in Tecolutla estuary, Veracruz, Mexico, to determine sex ratio, size at maturity, gonadal and brood pouch histology, reproductive seasonality, and fecundity of this little-known syngnathid. A total of 345 fish were collected between 1995–1998, with an overall sex ratio of 1:1.35 favoring females. Brooding males made up 45% of the population, resulting in an operational sex ratio of 1:2.45 heavily favoring females. All males > 90 mm SL were considered sexually mature, as this was the size at which the brood pouch was clearly developed. Females > 110 mm SL were sexually mature, and had asynchronous oocyte development. Opossum pipefish appear to have a year-round reproductive season in Tecolutla estuary, as females with vitellogenic oocytes and males with eggs in the brood pouch were captured during every month of the year. The number of eggs in the brood pouch was positively correlated with male SL ($P < 0.001$), and in all but 2 cases males brooded embryos from a single female. The brood pouch is not enclosed by a membrane, and each egg is embedded in a septum consisting of epithelial tissue and numerous blood vessels. Evidence from this study suggests *M. brachyurus lineatus* may be a sequentially polygamous species with sex-role reversal reproductive behavior, although additional research is necessary to confirm the reproductive ecology and behavior of the species.

RESUMEN Fue investigada la biología reproductiva del pez pipa *Microphis brachyurus lineatus*, en el estuario de Tecolutla, Veracruz, México, para determinar la proporción sexual, el tamaño de maduración, la histología gonádica y de la bolsa incubadora, la estacionalidad reproductiva y la fecundidad de este signátido poco conocido. Fueron colectados un total de 345 peces, entre 1995 y 1998, con una proporción sexual de 1:1.35 a favor de las hembras. Los machos incubadores comprenden el 45% de la población, con el resultado de una proporción sexual operacional de 1:2.45 a favor de las hembras. Todos los machos mayores a 90 mm de longitud patron fueron considerados sexualmente maduros, ya que este fue el tamaño al cual la bolsa incubadora estuvo claramente desarrollada. Las hembras mayores a 110 mm de longitud patron estuvieron sexualmente maduras y tuvieron un desarrollo asincrónico de los ovocitos. El pez pipa parece tener una estación reproductiva durante todo el año en el estuario de Tecolutla, ya que las hembras con ovocitos vitelogénicos y los machos con huevos en el saco incubador fueron capturados durante todos los meses del año. El número de huevos en la bolsa incubadora estuvo positivamente correlacionado con la longitud patron del macho ($P < 0.001$) y en todos, menos en 2 casos, los embriones de la bolsa incubadora del macho fueron de una sola hembra. La bolsa incubadora no está encerrada por una membrana, y cada huevo está depositado en un septo, que consiste de tejido epitelial y numerosos vasos sanguíneos. La evidencia de este estudio sugiere que *M. brachyurus lineatus* puede ser una especie secuencialmente polígama, con una conducta reproductiva de reversión sexual, aunque es necesario estudios adicionales para confirmar la ecología reproductiva y comportamiento de la especie.

INTRODUCTION

Various species of fish have developed a brood protection strategy to elevate the probability of survival and reproductive success. One family widely known for parental care is the Syngnathidae (seahorses and pipefishes), in which the males exclusively brood and care for the young. There has been much recent interest in the reproductive ecology of the syngnathids in an effort to more fully understand the evolution of sexual differences and the mating system. Within the syngnathids, there are about 300 species of pipefish divided into 35 genera (Nelson

1994). Species differ in the external morphology of the brood pouch, which varies from a series of lateral dermal plates to a pouch that is completely closed. In some species, no pouch is present, and the eggs are adhered to the ventral side of the male (Dawson 1982). The reproductive behavior of syngnathids exhibits similar variation, ranging from monogamy, typical of seahorses (Jones et al. 1998) and some pipefishes (Berglund et al. 1989, Vincent et al. 1995), to polygamy, as seen in a variety of pipefishes (Berglund et al. 1988, 1989, Berglund and Rosenqvist 1990, Vincent et al. 1995, Jones and Avise 1997, Jones et al. 2000, 2001). Finally, there appears to be some correla-

tion between monogamy and “conventional” sex roles (males competing for female mates) versus polygamy and “sex-role reversal” (females choosing male mates), in the syngnathids studied (Vincent et al. 1992). Furthermore, sex-role reversal often seems to be related to a female-biased operational sex ratio (OSR; Vincent et al. 1992, Kvarnemo and Ahnesjö 1996).

Overall, paternal care, development, gonadal histology, importance of the brood pouch, and mating systems have been well documented for common syngnathid species in the western North Atlantic such as *Sygnathus typhle* (Berglund et al. 1988, Berglund 1991, Ahnesjö 1992, Vincent et al. 1994, Jones et al. 2000), *S. fuscus* (Anderson 1968, Hareseign and Shumway 1980, Roelke and Sogard 1993, Campbell and Able 1998), *S. scovelli* (Quast and Howe 1980, Begovac and Wallace 1987, 1988, Azzarello 1991, Jones and Avise 1997) and *Hippocampus erectus* (Linton and Soloff 1964, Selman et al. 1991). However, there is little available information on the opossum pipefish, *Microphis brachyurus lineatus*. This species ranges from south of New Jersey, USA, to Sao Paulo, Brazil, including the Gulf of Mexico (GOM), Cuba, and the Antilles, and has been found in the west Pacific Ocean only off Panama (Dawson 1982). The opossum pipefish is considered threatened in Florida and Texas and was designated as a candidate species on the federal threatened and endangered species list in 1999 (Federal Register 1999, Music et al. 2001). Permanent populations are limited to tropical and subtropical areas, and breeding adults typically occur in shallow, freshwater areas with emergent vegetation (Gilmore and Gilbert 1992), although larvae and juveniles are found in higher salinity areas (Gilmore 1977). There is little reproductive information available on the species, and nothing is known regarding its reproductive ecology. In Jucú River, Brazil, summer and winter reproductive periods have been found (Perrone 1989), while reproduction typically occurs in the GOM and Caribbean Sea from May through November during the rainy season (Frias-Torres 2002). Therefore, the objectives of the present study are to better describe certain aspects of the reproduction of *M. brachyurus lineatus* in Mexico. Specifically, we determine the sexual proportion of the species, size for external differentiation between females and males, the number of eggs in the brood pouch, and provide macroscopic and histological descriptions of the ovary, testis, and brood pouch.

MATERIALS AND METHODS

Periodic sampling was performed between 1995 and 1998 and covered an annual cycle in Tecolutla estuary,

Veracruz, Mexico (20°30'N, 97°01'W). Specimens were captured during daylight hours in shallow (1.5 m) grassbeds along the bank of Larios estuary using a beach seine (2.5 m x 1.20 m, mesh size 500 µm). The samples were placed in bags and preserved in 10% buffered formalin prior to transportation to the laboratory. Species identification was verified with Castro-Aguirre (1978) and Dawson (1982). All specimens were washed in running water, and measured for standard length (SL, mm) and wet weight (ww, 0.0001 g). The presence or absence of a brood pouch was noted; this information was used to determine both sexual proportions as well as the size at sexual maturity for males following Gilmore and Gilbert (1992). To determine fecundity, the number of eggs in the brood pouch were counted using dissection needles and a stereo microscope. Additionally, mature oocytes from the ovaries of 13 females were counted in the same manner.

Gonads of males and females were removed, measured, weighed, and processed for histological analysis following standard techniques, with the substitution of amyl alcohol for clearing the tissues. Tissues were embedded in paraffin and sectioned at 5 µm. Ovarian tissue from 11 females (93–184 mm TL) was stained with hematoxylin-eosin (H-E) and Masson trichrome to classify ovarian development following Brown-Peterson (2003). Testes from 16 males (93–174 mm TL, of which 37.5% were egg carriers) were stained with H-E, ferric hematoxylin and Masson trichrome and classified for testicular and spermatogenic development following Hyder (1969) and Grier (1981). One 160 mm TL male, with eggs in the brood pouch, was treated with disodium EDTA for 15 d to soften the bony plaques prior to standard histological processing. Sections were cut from 2 different levels, distal and proximal to the anus, and stained using H-E and Masson trichrome.

Differences between males and females in SL, ww, and size at sexual maturity were evaluated using the Student t-test. Linear regression was used to determine the relationship between the number of eggs in the brood pouch as the dependent variable and male SL as the independent variable. In all cases, differences were considered significant if $P \leq 0.05$.

RESULTS

Sexual proportion, length-weight relationship, and fecundity

A total of 345 *Microphis brachyurus lineatus*, ranging in size from 64–205 mm SL and 0.0910–4.8203 g, were collected. The minimum size in which the brood pouch was clearly developed was 90 mm SL; thus, all fish <90

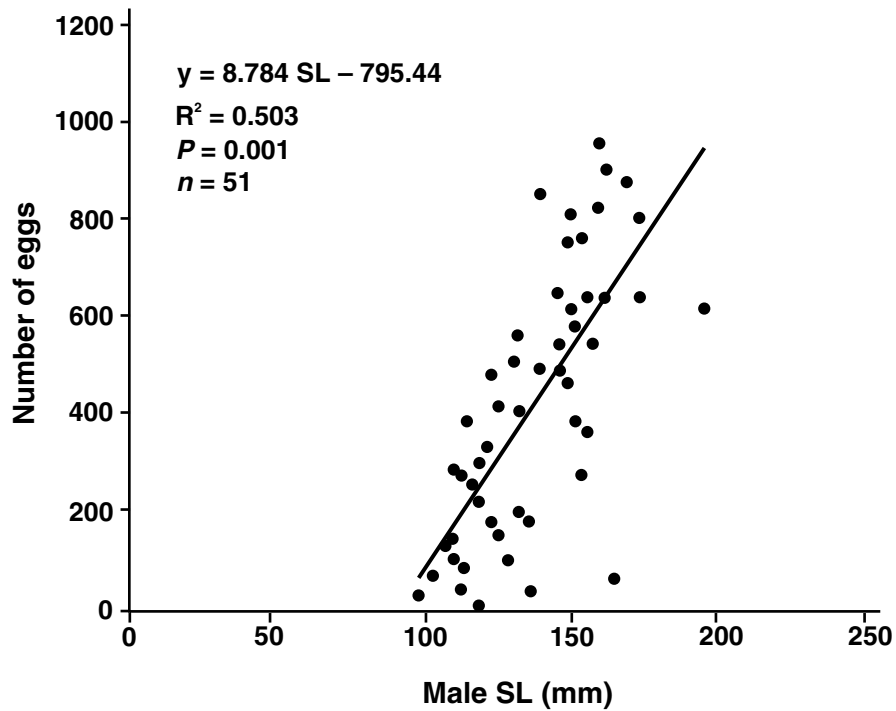


Figure 1. Linear regression of the number of eggs in the brood pouch vs. SL of male *Microphis brachyurus lineatus*.

mm SL were considered juveniles. Overall, 159 females (46%; 90–205 mm SL), 118 males (34%; 90–200 mm SL) and 68 juveniles (20%, 65–90 mm SL) were captured. Only 45% of males ranging in size from 99–196 mm SL had eggs in the brood pouch. Overall, the sex ratio was 1:1.35 favoring females. However, the ratio of reproductively available males (those with no eggs in the brood pouch) to females was 1:2.45, resulting in an OSR of 29%, heavily favoring females.

There was a significant difference in both SL ($t = -31.40$; $P < 0.001$, $n = 259$) and ww ($t = -15.76$; $P < 0.001$,

$n = 259$) between males and females when all sizes were combined. Among sexually mature males and females, there was also a significant difference in SL ($t = -21.31$; $P < 0.001$, $n = 189$) and ww ($t = -14.77$; $P < 0.001$, $n = 189$), with males heavier at a given length.

The number of eggs in the brood pouch ranged from 10–953, with a mean of 409 eggs. In contrast, the mean number of mature oocytes in the ovary was 765. The fertilized egg is oval, with a diameter along the long axis of 0.8–1.1 mm. Each egg in the pouch was surrounded by spongy tissue (see descriptions below). There was a significant,

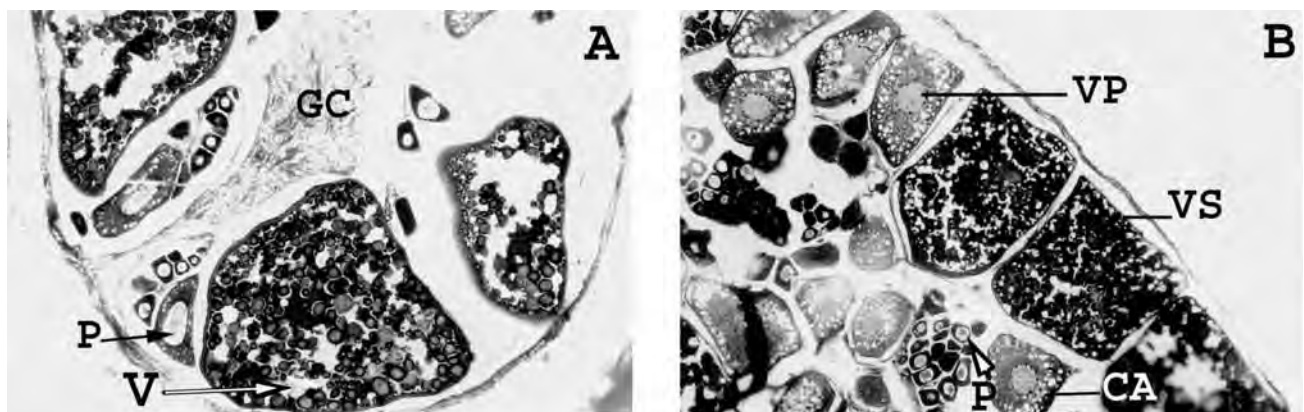


Figure 2. Histological sections from the ovary of *M. brachyurus lineatus*. A) Transverse section of the ovary, showing the U-shaped projection from the germinal cord. Germinal cord (GC), perinucleolar oocyte (P), vitellogenic oocyte (V), 100X. B) Longitudinal section of the ovary showing asynchronous oocyte development. Perinucleolar oocyte (P), cortical alveolar oocyte (CA), primary vitellogenic oocyte (VP), secondary vitellogenic oocyte (VS), 400X.

TABLE 1

Oocyte stages found in ovaries of *Microphis brachyurus lineatus* from Tecolutla estuary, Veracruz, Mexico.

Oocyte Stage	Diameter (μm)	Characteristics
Oogonia	2.6	Spherical; 1–3 nucleoli
Chromatin nucleolar	6–29	Large nucleus; 3–16 nucleoli
Perinucleolar	29–80	Basophilic; 9–29 nucleoli around nucleus
Cortical alveolar	80–150	Chorion visible; small lipid droplets; 9–20 nucleoli
Vitellogenic (Primary, Secondary, Tertiary)	150–480	Well defined chorion; vitellogenin spheres central (primary), throughout (secondary), or fused (tertiary); nucleoli peripheral (primary, secondary) or throughout nucleus (tertiary)
Mature	800	Trapezoid shape

positive relationship between male SL and egg number ($R^2 = 0.503$, $P < 0.001$, $n = 51$; Figure 1). In organisms with the brood pouch only partially full of eggs, the anterior portion of the pouch was always empty, suggesting oviposition occurs first in the posterior portion of the pouch.

Macroscopic and microscopic description of the ovary

The ovaries are paired structures that are fused in the terminal part of the body and occupy 3/4 of the trunk length of the female. The ovaries of a mature female (mean = 158 mm SL) measured 35 mm, weighed 0.200 g and were longitudinally asymmetrical. In 57% of the females studied, the right ovary was slightly longer than the left (0.3–2.8 mm difference).

Histologically, the germinal cord of *M. brachyurus lineatus* runs along the medial dorsal part of the ovary and is formed by connective tissue and germinal cells that later develop into oocytes. The previtellogenic oocytes form on each side of the germinal cord in a U-shaped projection directed toward the lumen of the ovary (Figure 2a). Oocyte development occurs sequentially across the cord and is termed the maturity line. The maturity line is defined by the position of vitellogenic oocytes which are ready for maturation and ovulation.

Microphis brachyurus lineatus show asynchronous oocyte development (Figure 2b). We identified 6 oocyte stages in the specimens we examined (Table 1). Oogonia, chromatin nucleolar, and perinucleolar oocytes were found in the ovaries of all females, regardless of reproductive season or SL. Ovarian recrudescence begins with the appearance of cortical alveolar oocytes. All 3 stages of vitellogenic oocytes were commonly seen, although final oocyte maturation was not observed in any samples. No females < 110 mm SL were observed with oocytes in the cortical alveolar, vitellogenic, or mature stages, suggesting sexual maturity is not reached until this size.

Macroscopic and microscopic description of the testis

The testes are paired structures in a ventral position and can be divided into 2 regions. The first is distal to the anus, occupies 3/4 of the gonad, is extremely thin, and has a cylindrical shape. The second part is a clearly expanded region proximal to the anus, consisting of the remaining 4th of the testis. The testis can occupy more than 3 quarters of the trunk. An adult male (mean = 140 mm SL) had testes of 25 mm and 0.0035 g. The length of the right testis was slightly greater in 63% of the organisms studied.

Histologically, the testes of the opossum pipefish are the lobular, restricted spermatogonial type. Spermatogonia were found only at the distal, “blind” end of the testis, and spermatocysts were not obvious in that section. Each primary spermatogonium was surrounded by connective tissue. In the proximal portion of the testis, spermatocysts were easily observed. Spermatozoa were found both in cysts and in the lumen of the lobule. The diameter of the spermatozoa was about 8.3 μm ; the nucleus (4.48 μm) was often off-center in spermatozoa in the lumen of the lobule, but the nucleolus (1.6 μm) remained centric. There was no evidence of flagella on the spermatozoa, although a small, pointed protrusion was often visible on one side of the cell.

Reproductive season

Opossum pipefish in the Tecolutla estuary appear to have a year-round reproductive season. Females with vitellogenic oocytes were collected during every sampling event, which covered all 12 months of the year. Additionally, we captured males with recently spawned eggs, eggs in advanced phases of development, and embryos almost ready to hatch during each sampling event. However, within the same organism, all eggs present were in the same development stage, with the exception of 2 males containing eggs in different phases of development.

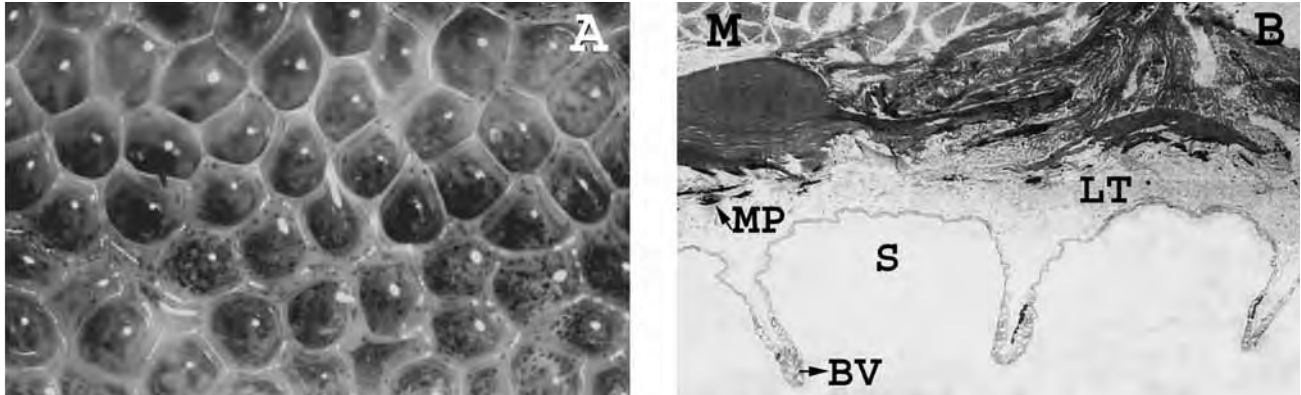


Figure 3. The brood pouch of male *M. brachyurus lineatus*. A) Internal tissue of the brood pouch, containing developing embryos in individual cells in a honeycomb-like arrangement, 15X. B) Transverse histological section, showing the internal septum (S) where each egg lodges, muscle (M), melanophores (MP), loose connective tissue (LT), and blood vessels (BV), 100 X.

Macroscopic and microscopic description of the brood pouch

The brood pouch begins at the first trunk ring, ends just anterior of the first anal ring, and is not enclosed by a membrane. In the interior of the pouch, the eggs are embedded in a honeycomb-like spongy tissue of hexagonal cells in 3 to 12 (generally 10) transverse rows in males 145–174 mm SL (Figure 3a). In transverse histological sections, the elongated extensions that form the septum of each internal cell in the brood pouch are distinct. The basal zone of the chamber where the eggs are deposited is delineated by an external layer of flat epithelial tissue that rests on a thick layer of loose connective tissue containing small blood vessels dispersed throughout (Figure 3b). The flat epithelial tissue of the elongations in the chamber is stratified, principally in the apical part where there are 4 layers of tissue. A large number of blood vessels of different diameters are present in each epithelial layer. Melanophores are present in the marginal portion of the epidermis and in the muscle of the body (Figure 3b).

DISCUSSION

Syngnathids are considered to be a true estuarine species, spending their entire life cycle in the estuary. We found opossum pipefish > 64 mm SL in every sample within the Tecolutla estuary but did not capture young juveniles. This is similar to findings by Gilmore (1977), who found no opossum pipefish < 60 mm SL in the Indian River Lagoon, Florida. The lack of young juveniles in the low salinity estuary of Tecolutla supports hypotheses that larvae migrate offshore to develop (Gilmore 1977, Frias-Torres 2002). It has been proposed that juveniles are transported back to tropical estuarine areas by the Caribbean, Loop and Antilles Currents (Frias-Torres 2002).

The presence of eggs in the brood pouch throughout the year indicates *M. brachyurus lineatus* does not have a specific reproductive season, confirming descriptions by Perrone (1989) for the same species in Brazil. Histological inspection of the ovaries found asynchronous oocyte development. Furthermore, females with vitellogenic oocytes were present throughout the year. In Indian River Lagoon, Florida, males with eggs in the brood pouch were reported during the wet season, May through November (Gilmore 1977). Brooding males were found only from July through September in Mississippi (Dawson 1970). Perhaps in truly tropical areas, such as Tecolutla estuary, at the Tropic of Cancer, and Espirito Santo, Brazil at the Tropic of Capricorn, the reproductive season is year-round, whereas it is shortened in warm-temperate and subtropical waters. Since egg maturation is dependent upon food availability in pipefishes (J. Wetzel, pers. comm., Presbyterian College, Clinton, SC), the shortened reproductive season in non-tropical regions may be a function of reduced food availability during the cooler dry season.

Male opossum pipefish from Tecolutla estuary appear to reach sexual maturity at a smaller size than previously reported for the species. The brood pouch could be distinguished in males as small as 90 mm SL in Tecolutla, and males as small as 99 mm SL carried eggs. This is similar to findings from Jucú River, Brazil, where the smallest male with a developed brood pouch was 95 mm SL, although the size at 50% sexual maturity was calculated to be 112 mm SL (Perrone 1990). In contrast, the smallest males with developed brood pouches were 105 mm SL (Loxahatchee, Florida and Biloxi, Mississippi), 106 mm SL (Mexico and Panama), to 110 mm SL (Sebastian River, Florida) (Frias-Torres 2002). This discrepancy in size at sexual maturity could be due to differences in sampling among areas.

The lobular, restricted spermatogonial testicular structure of opossum pipefish is commonly found in atherinomorph species (Grier 1981) and is recognized by the presence of spermatogonia at the distal end of the testis. The size of the spermatozoa in the terminal region of the testis is quite large, and is similar to the primitive spermatozoid described by Grier (1981) for species with external fertilization. In most teleosts, the germ cells become smaller as spermatogenesis progresses (Hyder 1969, Nagahama 1983). However, in *M. brachyurus lineatus*, the spermatozoa are larger than the spermatogonia. Furthermore, the spermatozoan of opossum pipefish is unique in that it does not have a flagellum, quite uncommon among teleosts (Mattei 1970). However, since the spermatozoa of the pipefish are expelled directly into the brood pouch to fertilize the eggs (Prein 1995), the requirement for mobility is reduced. Most likely, the spermatozoa move in an amoeboid fashion to fertilize the eggs, as has been shown in *Gymnarchus niloticus* (Mattei 1970), a species that also has spermatozoa without flagella.

Despite the existence of several studies on the brood pouch in syngnathids (Linton and Soloff 1964, Quast and Howe 1980, Haresign and Shumway 1980), this is the first study to evaluate an “open” brood pouch that does not have a membrane to isolate the eggs from the external environment. In *H. erectus* and *S. scovelli*, the spongy material in the brood pouch is permeable and permits gas interchange (Wetzel and Wourms 1991) and metabolic interchange of ions such as Ca^{+2} and Na^{+} , with their corresponding effect on osmoregulation (Linton and Soloff 1964, Quast and Howe 1980, Haresign and Shumway 1980). Possibly, the brood pouch of *M. brachyurus lineatus* does not participate in osmoregulation and its function is only to restrict gas exchange, as suggested by Azzarello (1991). Further studies to help in the understanding of active transport within the brood pouch tissue of *M. brachyurus lineatus* are necessary, as histological analysis is insufficient to establish whether the male supplies the embryos with energetic requirements through metabolic exchange during their development.

The reproductive ecology and mating strategies of the opossum pipefish can be examined from our data. In general, male opossum pipefish appear to mate with only one female at a time, since all eggs in the brood pouch of 96% of brooding males were in the same stage of development, suggesting contribution from just one female. However, this does not imply that the species is monogamous. Opossum pipefish could practice sequential polygamy, as in *Nerophis ophidion*, where the female gives one entire clutch to a male (Vincent et al. 1992). Female *M. brachyurus lineatus* deposit their eggs in the posterior portion of

the brood pouch first, as is also the case with *S. typhle* (Berglund and Rosenqvist 1990), a species in which polygamy and multiple matings by the male are common (Berglund et al. 1988, Jones et al. 2000). We found some evidence of multiple matings by male *M. brachyurus lineatus*, as 2 males had 2 distinct batches of eggs in their brood pouches. It is possible that these males had first mated with smaller females, as male *S. typhle* that mate with small females are significantly more likely to mate with more than one female (Jones et al. 2000). Thus, the only way to definitively ascertain if *M. brachyurus lineatus* is polygamous or monogamous is through microsatellite DNA markers to verify maternity of the embryos carried by males (Jones and Avise 1997).

The positive relationship between the number of eggs in the brood pouch and male size has been reported previously for opossum pipefish (Frias-Torres 2002) and is similar to reports for *S. typhle* (Berglund et al. 1986) but in contrast to *Nerophis ophidion* (Rosenqvist 1990), both polygamous pipefish (Vincent et al. 1992). Since *M. brachyurus lineatus* males appear to mate with only one female at a time, perhaps small females spawn their eggs in brood pouches of small males, whereas the larger females prefer the larger males. Thus, the number of eggs are “matched” to the energetic resources of the male, as suggested by Berglund et al. (1986). There is evidence that clutch size is positively correlated with the length of the female pipefish (Berglund et al. 1986, Ahnesjö 1992), suggesting clutch size can be used to determine if a male mated with a large or small female.

An understanding of the sexual roles in *M. brachyurus lineatus* is more difficult to determine from the available data. Pipefish with “conventional” sex roles, such as *S. scovelli* and *Syngnathoides biaculeatus*, tend to be monogamous (Jones and Avise 1997, Takahashi et al. 2003). However, pipefish with a strongly female-biased OSR, as seen here for opossum pipefish, are often polygamous (Vincent et al. 1992). Furthermore, a female-biased population, as seen in both Tecolotla estuary and in Jucú River, Brazil (Perrone 1989, 1990), may result in female competition for available males, a classic sex-role reversal pattern. However, while sex-role reversed pipefish tend to show sexual dimorphism, as in *N. ophidion* and *S. typhle* (Vincent et al. 1992), sexual dimorphism was not clearly demonstrated in *M. brachyurus lineatus*. Overall, evidence from this study suggests that opossum pipefish may be a sequentially polygamous species with sex-role reversal behavior. Clearly, additional research on the sex roles and breeding behavior of *M. brachyurus lineatus* is necessary for a complete understanding of their mating system, as the available data can be interpreted in a contradictory fashion.

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