The Journal of Experimental Biology 209, 1112-1121
Published by The Company of Biologists 2006
doi:10.1242/jeb.02119

Differential parental nutrient allocation in two congeneric pipefish species (Syngnathidae: *Syngnathus* spp.)

Jennifer L. Ripley* and Christy M. Foran
Department of Biology, West Virginia University, PO Box 6057, Morgantown, WV 26506, USA
*Author for correspondence (e-mail: jripley@mix.wvu.edu)

Accepted 23 January 2006

Summary

Male seahorses and pipefishes of the family Syngnathidae are heralded for their unique brood pouch structures for incubating embryos. There are three general types of brood pouch with increasing complexity: simple ventral gluing, two pouch flaps and a completely sealed sac. The diversity of functional roles within a type in providing nutrition, aeration and protection to offspring is unknown. Here we reveal significant differences in parental nutrient allocation to embryos for two closely related, sympatric pipefishes with similar brood pouch structure. We document differences in embryo attachment, depletion of pouch fluid nutrients over development and egg nutrient partitioning between *Syngnathus floridae* and *Syngnathus fuscus*. In *S. fuscus*, females produce nutritionally poor eggs and the males implant developing embryos in the brood pouch adjacent to blood vessels. A female-biased breeding population was observed, supporting the hypothesis that the cost of male parental care is high in this species. The loose connection between eggs and brood pouch tissues and the appearance of undeveloped eggs and lipid droplets in the pouch of *S. floridae* males suggest this species utilizes nutrient-rich eggs produced by females as nurse eggs to supplement embryonic development. A balanced sex-ratio for *S. floridae* further supports more equal parental contribution. This comparison provides evidence of a decline in female gametic investment and reveals the rapid diversification of syngnathid brood pouch function. Our results indicate gross classification of brooding structures into one of the three general pouch types does not predict the energetic investment of males in parental care. But rather, physiological characterization of the relative investment by each sex to offspring is essential to understanding the functional significance of the brood pouch.

Key words: brood pouch, nutrient allocation, nurse eggs, sex ratios, Chesapeake bay.

Introduction

In mammals and other vertebrate taxa females tend to provide substantial parental care. Part of the explanation for this role lies in the gamete dimorphism that defines the sexes. Females invest in large, nutritious eggs whereas males produce small, mobile sperm (Parker et al., 1972; Alexander and Borgia, 1979). Anisogamy, gametes of two different sizes from the male and female, underlies the evolution of sex differences in morphology and behavior including parental care (Maynard-Smith, 1978). Evolutionary theory predicts that in species with female parental care, males will compete for access to females (Trivers, 1972). When males exhibit paternal care, they incur a loss of fitness due to increased predation, decreased foraging time and territory and limited mating opportunities relative to males of other species (Williams, 1966; Williams, 1975; Gross and Shine, 1981; Svennsson, 1998; Vincent et al., 1992). Caring males are accordingly expected to be more discriminating in their choice of mates, and females will compete for access to receptive males. Sex roles are therefore historically defined by mating competition (Emlen and Oring, 1977). Species exhibiting greater competition by females than males for mates are said to be sex-role reversed (Gwynne, 1991). The occurrence of sex-role reversal offers an opportunity to explore the evolution of sex differences. For example, an extreme case of sex-role reversal has been suggested to result in complete sex reversal where anisogamy is inverted as indicated by a loss of nutritional investment from female gametes (Pagel, 2003).

Pipefishes and seahorses of the family Syngnathidae are characterized by a unique mode of ovoviviparous reproduction in which the male carries the developing embryos in a special organ referred to as the brood pouch (Lockwood, 1867). The degree of brood pouch closure varies considerably and has classically been grouped into three types: ventral gluing, two pouch flaps that meet midline and a completely sealed sac (Duncker, 1915; Herald, 1959). A female oviposits her eggs directly onto the brooding skin where they are fertilized. ‘Internal’ fertilization in males assures paternity and is

probably one of the selective pressures behind the evolution of this structure (Helfman et al., 1997; Jones and Avise, 1997; Jones et al., 1998; Jones et al., 1999). The developing embryos remain in the brood pouch for a lengthy incubation period before being released as independent young without a yolk sac.

The brood pouch is believed to protect, aerate, osmotically buffer and nourish the embryos (for a review, see Azzarello, 1991; Carcupino et al., 1997; Drozdov et al., 1997; Carcupino et al., 2002). An investigation on the pipefish *Syngnathus acusimilis* demonstrated that the dorsal epithelial lining of the brood pouch is well vascularized and composed of thick cuboidal cells similar to those known to have a secretory function (Drozdov et al., 1997). The syngnathid brood pouch is expected to be an epithelochordal placenta, because eggs are deposited with a large amount of yolk and a yolk sac is still visible once the fry hatch in the pouch. Therefore, the syngnathid brood pouch resembles that of squamate lizards, conferring inorganic ion exchange (Stewart, 1992). Several authors have suggested an ionic exchange function that regulates the osmolality of the brood pouch fluid to that of paternal blood, facilitating embryonic development (Linton and Soloff, 1964; Quast and Howe, 1980; Watanabe et al., 1999). A nourishment function for the brood pouch has been suggested in *Syngnathus scovelli*, because smaller eggs are absorbed by the pouch epithelium and thought to serve as 'nurse eggs' (Ahnesjo, 1996). The pouch has also been suggested to transfer steroid or growth hormones to the embryos, but this function has yet to be fully investigated (Haresign and Shumway, 1981; Azzarello, 1991; Mayer et al., 1993).

The extent to which the various pouch types perform these physiological roles is unknown. For instance, in two related pipefish species in which fry measure 11–13 mm total length (*TL*) at release, removal of *S. scovelli* embryos at least 4 mm *TL* resulted in normal development (Azzarello, 1991). Conversely, *S. acusimilis* young could not survive outside the brood pouch until 11–12 mm *TL* (Drozdov et al., 1997). Molecular phylogenic analyses indicate the syngnathid brood pouch underwent rapid diversification evidenced by repeated evolution of a number of pouch types (Wilson et al., 2001). Rapid diversification of placental function in females has been documented in another teleost system (Morrell, 2002; Resnick et al., 2002). These studies suggest the function of the paternal brood pouch is a fairly plastic trait and that syngnathid groups may differ in brood pouch physiology.

Regardless of the degree of closure, all syngnathid brood pouches were once assumed to provide developing embryos with protection, osmoregulation and nutrients (Vincent et al., 1992; Jones and Avise, 1997). However, sex-role reversal as predicted by extensive male investment was not consistently reported within this taxa (Vincent et al., 1992). Recent ultrastructural comparisons of three syngnathid species representing each of the brood pouch types refuted the hypothesis of uniform functionality by suggesting the epithelium has different functions in each type of enclosure. Specifically, more enclosed pouches were observed to contain greater anatomical complexity and secretory function (Carcupino et al., 2002). Based on this positive correlation between the degree of pouch closure and male parental care (Berglund et al., 1986; Masonjones, 2001; Carcupino et al., 2002), the frequency of sex-role reversal across Syngnathidae was examined as a measure of female competition. The predicted relationship of frequent sex-role reversal in taxa with more enclosed brood pouches was not observed (Carcupino et al., 2002; Wilson et al., 2003).

Our comparison of two closely related, sympatric species, the northern pipefish *Syngnathus fuscus* and the dusky pipefish *Syngnathus floridae*, supports the rejection of brood pouch functional uniformity in syngnathids, as well as providing a hypothesis for the lack of relationship between sex-role reversal and pouch structure. We propose that pouch closure is not indicative of the degree of physiological allocation of nutrients by brooding males to embryos, but rather, brood pouch physiology varies between related species with similar brood pouch appearance. Brood enclosure for *S. fuscus* and *S. floridae* is intermediate within syngnathids with two pouch folds sealing along the midline but not permanently fusing. Our species comparison of nutrient concentrations in mature, unfertilized eggs and newly released fry, brood pouch morphology and nutrient levels of fluid from inside the brood pouch and blood plasma were used to evaluate this hypothesis. Further, characterization of parental nutrient allocation to offspring examines the proposition of the progression of some species of syngnathids toward complete sex reversal (Pagel, 2003).

Materials and methods

Fish collection and population sex ratios

We conducted *Syngnathus fuscus* (Storer 1839) and dusky pipefish *Syngnathus floridae* (Jordan and Gilbert 1882) collections by seining in the seasonally abundant shallow eelgrass (*Zostera marina*) beds of the Chincoteague Bay, VA, USA from May to September 2003–2004. Seining events covered a distance of 25 m in approximately 1 min using a 3.7 m×1.2 m net with a 1.5 m^2 bag and 0.4 cm^2 mesh (Fish Net Company, Jonesville, LA, USA). Collected organisms were immediately sorted on a submerged platform and sex and species of pipefish were noted. We calculated sex ratios for monthly collections yielding over 50 individuals by dividing the total number of males caught by the number of females for each species. All males with brood pouches and females larger than the recorded size for sexual maturation were included in this calculation (Teixeira and Musick, 1995). Initially, all adult males and females were retained for laboratory dissections, and juveniles were held briefly in aerated tanks onboard the boat and returned before leaving the site. Once the data set neared completion, the developmental state of the brood was estimated by examining the embryos through the translucent pouch flap, and only animals filling gaps in the data were collected from catches. Pipefish were held for a maximum of 3 days at the field station in aerated tanks with daily water

changes. Adults were transported in Kordon® breathing bags (Novalek Inc., Hayward, CA, USA) chilled with ice to the laboratory for tissue collection.

Tissue collection and nutrient analysis

In the laboratory, pipefishes were held in same sex groups of 10–12 fish in filtered 37.8 l tanks maintained at 24±1°C on a 14 h:10 h light:dark cycle (on 6:00 h: off 20:00 h). Pipefish were given a recovery period of at least 16 h in the laboratory with food withheld for a minimum of 36 h during travel and acclimation. Fish were not fed prior to tissue collection to avoid influences on nutrient levels from food intake, or the lack thereof. All tissues were collected within 2 days of laboratory arrival. We anesthetized pipefish with 3-aminobenzoic acid ethyl ester (MS222; Sigma Aldrich, St Louis, MO, USA) in saltwater until opercle movement ceased and the fish failed to respond to pinching the caudal peduncle. Pouch fluid from brooding and non-brooding males was collected with microcapillary tubes (Drummond Scientific Co., Broomall, PA, USA) by inserting the tube along the junction of the pouch flaps at the anterior of the brood pouch by the anus. The capillary tube could then be used to separate the pouch flaps and collect fluid from all areas of the brood pouch. In non-brooding males the pouch flaps are not fused and saltwater could mix with any secretions produced by the male. Pouch fluid was collected from these animals to determine how different this fluid is from the surrounding saltwater. Pouch fluid was immediately stored at –80°C until analysis. Blood was collected from the heart of brooding and non-brooding males and females with heparinized microcapillary tubes. Blood samples were transferred to microcentrifuge tubes containing 2 μl of a 6.5 mg ml^{-1} solution of sodium heparin salt in water, and centrifuged at 21 000 ***g*** for 10 min at 4°C. The plasma fraction was removed and stored in a microcentrifuge tube containing phenylmethylsulfonyl fluoride (PMSF, 99% purity; Sigma Aldrich) at –80°C until analysis. Brooding embryos and eggs were collected directly from the brood pouch or ovary, respectively, with fine point forceps. Developing embryos were anesthetized with MS222 in saltwater. Before fry release, brooding males were isolated so that newly released fry could be collected with nets. They were then anesthetized in MS222 in saltwater. For each brooding male in the study, 20 embryos or newly released fry (within 12 h) were pooled and homogenized with 40 μl of homogenization buffer (0.1 mol l^{-1} Tris base, 1 mmol l^{-1} EDTA) for 60 s in the case of yolk sac embryos or 120 s for late stage fry. Females with immature follicles that could not be removed intact from the ovary were termed immature females to distinguish them from gravid females with mature eggs that could be separated. From gravid females, 20 eggs were removed from the posterior section of the ovary. The pipefish ovary has been classified as an asynchronous type with follicles in all stages of development (Wallace and Selman, 1981; Begovac and Wallace, 1987). By collecting those closest to the ovipositor, the sample contained mature eggs ready for fertilization. Eggs were homogenized in buffer for 60 s and the homogenate centrifuged at 21 000 ***g*** for 30 min at 4°C. The supernatant was removed and stored at –80°C until analysis.

An additional sample of five eggs, embryos or fry from each fish were viewed with an Olympus SZ40 dissecting microscope (Melville, NY, USA) and photographed with a Spot Insight color camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA; model 3.2.0). These samples were used to determine the stage of development of fertilized embryos. Mature, unfertilized eggs were considered stage 0. Embryonic development was divided into seven stages based on the presence of the embryo stripe (state 1), development of eye spots and cups (state 2), detachment of the tail from the yolk sac (i.e. hatched; state 3), heart development (state 4), presence of fins (state 5), development of striped pigmentation (state 6) and complete absorption of the yolk sac (state 7). Newly released fry were classified as state 8. Both *S. fuscus* and *S. floridae* embryos fitted these developmental stages with the distinction of *S. floridae* fry exhibiting longer snout lengths in states 6–8. In this study, these embryonic development classifications were considered distinct stages in place of specific time periods from the date of fertilization because observations indicated factors such as the number of embryos in the pouch, water temperature and male size influenced brooding periods (Berglund et al., 1989; Ahnesjo, 1992). Individuals of both species at every stage were included in our analysis. Egg diameter and standard length of newly released fry were measured with the software Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA). We averaged the five measures for a single data point for each individual. Any excess eggs, embryos or fry collected were frozen or fixed in formalin for analysis in concurrent studies.

Nutrients, specifically proteins, lipids and carbohydrates, were measured in tissue samples as follows. Protein content was measured from dilutions of blood plasma (1:99), pouch fluid (1:99) and the supernatants from homogenized eggs (1:199) and fry (1:99). Total protein concentrations were determined using a Bio-Rad protein dye reagent and a standard curve of bovine serum albumin. The samples were transferred to a 96-well plate and read at 490 nm using a Tecan GENios (Durham, NC, USA). Lipid content was determined by acidifying methanol/chloroform extracts, adding a vanillin reagent and comparing the sample to known amounts of soybean oil (Wheeler and Buck, 1992; Lotufo et al., 2000). Samples were analyzed at 595 nm. Carbohydrates, diluted 1:3 for all samples, were quantified at 490 nm using an anthrone assay and sucrose standards (Van Handel, 1985; Wheeler and Buck, 1992). For each nutrient analysis, the sample was measured in triplicate and averaged for a single data point. Normality and homogeneity of data sets were assessed prior to the use of parametric statistics. All statistical analyses were performed with JMP 5.1. Variability measures calculated from these data can be used in power analyses to determine required sample sizes for future experiments.

Results

Seines conducted in the Chincoteague Bay, VA, USA produced abundant catches of *S. fuscus* and *S. floridae* pipefishes. Sex ratio data indicate a heavily female-biased population for *S. fuscus*. The ratio of females to males differed significantly from 1:1 for *S. fuscus* (χ^2=147.717, P<0.001; Fig. 1) but not for *S. floridae* (χ^2=0.949, P=0.917; Fig. 1).

Analyses reveal comparable nutrient levels in *S. floridae* (N=6) and *S. fuscus* (N=7) fry at release (Table 1). However, protein (Fig. 2A, Table 1), lipid (Fig. 2B, Table 1) and carbohydrate (Fig. 2C, Table 1) reserves in unfertilized eggs of *S. floridae* (N=26) were significantly higher than in *S. fuscus* (N=16). Even though *S. floridae* females partitioned elevated levels of nutrients in eggs compared with *S. fuscus* females, egg size (*S. fuscus*, 0.87±0.27 mm, N=8; *S. floridae*, 1.18±0.10 mm, N=12) was comparable between species. The standard length of fry at release (*S. fuscus*, 10.18±0.28 mm, N=11; *S. floridae*, 11.74±0.20 mm, N=10) diverged, with *S. floridae* producing significantly larger fry (two-way ANOVA, F=1482.06, P<0.0001; *post-hoc* Tukey HSD, Q=2.6898, P<0.05).

Morphological observations of developing embryos held within the brood pouch of *S. fuscus* and *S. floridae* show a previously undescribed close association between fry and the pouch lining. Unlike most fish species in which the outer membrane hardens following fertilization to protect the embryo from water loss and environmental adversities, these pipefish embryos lack a rigid chorion (Jobling, 1995). In *S. fuscus*, the two flaps of the male brood pouch seal independently to the ventral body surface forming two chambers (Fig. 3A). Embryos are embedded under a clear membrane on the vascularized flaps of the brood pouch until release (Fig. 3A,B). Although most embryos develop at the same rate, one row on either side of the midline seal frequently remains underdeveloped relative to the rest of the brood. Limited vascularization and epithelial tissue in this region are likely to decrease the connectivity between embryos and the brood pouch. For *S. floridae*, the two flaps forming the pouch seal at the midline resulting in a single large brood chamber (Fig. 3C). One side of the fertilized egg becomes strongly attached to the ventral flap of the pouch (Fig. 3C,D). As the yolk sac is absorbed, this connection dissolves and embryos are contained within the pouch. Undeveloped eggs and lipid droplets are found interspersed with embryos all at the same stage of development (Fig. 3D). When fry are released, a clear matrix similar in appearance to a honeycomb and approximately the size of the pouch is also released. Overall our observations reveal differences between *S. floridae* and *S. fuscus* in the arrangement of undeveloped eggs and the connectivity of embryos to paternal tissue.

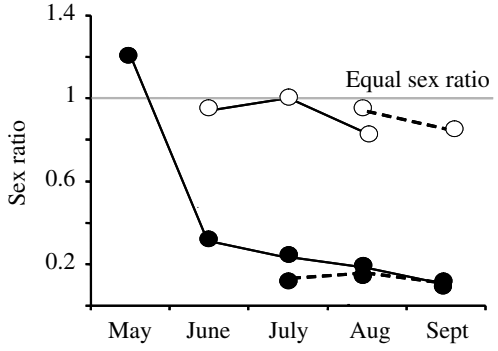

Fig. 1. Population sex ratios (number of males/number of females) for *Syngnathus fuscus* (black) and *Syngnathus floridae* (white) collected in the Chincoteague Bay, VA, USA during the 2003 (broken line) and 2004 (solid line) reproductive seasons. Sample sizes ranged from a minimum of 88 *Syngnathus fuscus* collected in September 2004 to 859 individuals of the same species counted in June 2004.

Nutrients potentially available for uptake by developing embryos were measured by determining protein, lipid and carbohydrate content of fluid collected from the pouch of brooding (N=21 per species) and non-brooding males (N=15 per species). When the two species are grouped together, brooding males with embryos in various developmental stages harbour pouch fluid rich in proteins (Table 2, Fig. 4A), lipids (Table 2, Fig. 4B) and carbohydrates (Table 2, Fig. 4C) relative to non-brooding males. Nutrient concentrations in the pouch fluid do not differ between males of the two species. However, in *S. floridae*, protein concentrations of the pouch fluid begin high for newly fertilized broods and decline rapidly over development (Table 3, Fig. 4A). A significantly more gradual decline characterizes the depletion of lipid from *S. floridae* pouch fluid (Table 3, Fig. 4B). Whereas carbohydrate content of pouch fluid for *S. floridae* and *S. fuscus* significantly decreased over embryonic development, species differences in the rate of decline were not observed (Table 3, Fig. 4C). To examine control of pouch fluid content in non-brooding males, nutrient levels were compared to saltwater containing MS222. Pouch fluid from non-brooding males of both species contained significantly higher protein, lipid and carbohydrate concentrations than saltwater with MS222 (Table 2).

Blood plasma concentrations of total protein, lipids and

Table 1. *Species comparisons of nutrient content of unfertilized eggs and newly released fry*

Nutrient (mg embryo^{-1})	Eggs	Fry	Two-way ANOVA
Protein	*S. fuscus*<*S. floridae*	*S. fuscus*≈*S. floridae*	F=78.8973, P<0.0001
Lipid	*S. fuscus*<*S. floridae*	*S. fuscus*≈*S. floridae*	F=24.8992, P<0.0001
Carbohydrate	*S. fuscus*<*S. floridae*	*S. fuscus*≈*S. floridae*	F=9.2084, P<0.0001

For all nutrient comparisons, the *post-hoc* Tukey HSD test was utilized (Q=2.6559, P<0.05).

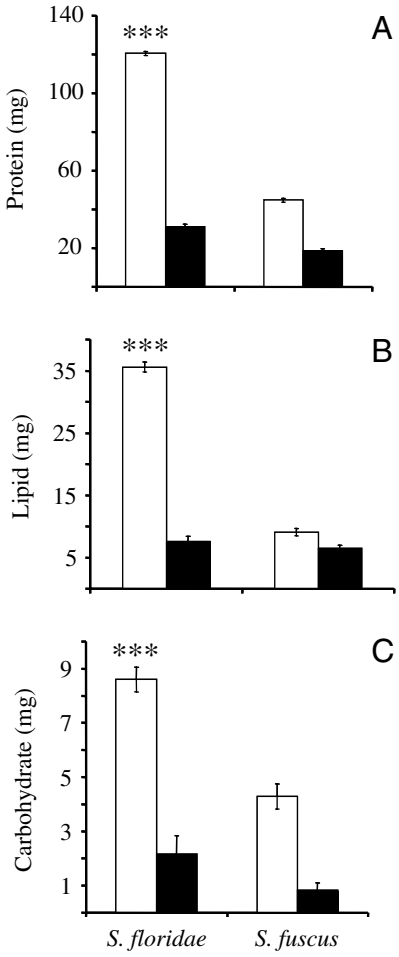

Fig. 2. (A) Protein, (B) lipid and (C) carbohydrate content of mature eggs (white) and released fry (black) of *Syngnathus floridae* (eggs, *N*=26; fry, *N*=6) and *Syngnathus fuscus* (eggs, *N*=16, fry, *N*=7). Values are mean ± s.e.m. For all comparisons, interspecific differences (asterisks) exist between eggs but not fry ($P<0.0001$).

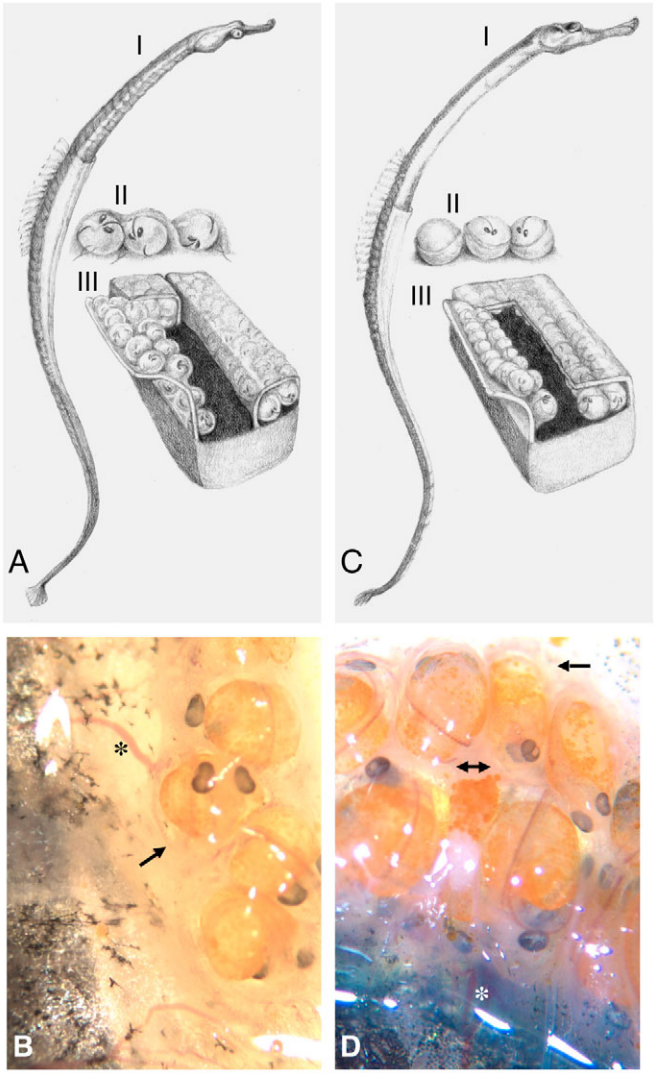

Fig. 3. Illustrations and photomicrographs depicting morphological differences in the *Syngnathus fuscus* (A,B) and *Syngnathus floridae* (C,D) male brood pouch. (A) *Syngnathus fuscus* are distinguished by their shorter snout length (I) and enclosure of developing embryos underneath a pouch-derived epithelium (II). Males form an enclosed brood pouch by the attachment of each flap to the ventral surface of the animal (III). (B) Blood vessels (asterisk) transverse the pouch flaps in close proximity to embedded developing embryos (arrow). (C) *Syngnathus floridae*, with the longer snout (I), attach embryos on only one face (II) to the pouch flap. The two flaps that form the pouch seal at the midline (III). (D) Lipid droplets (double-sided arrow) are distinguishable in the pouch secretions, and blood vessels (asterisk) pass below the embryo connection to the pouch flap (arrow).

carbohydrates are similar between brooding males and gravid females in both species. Considering species and reproductive state, significant differences in protein content were not detected (2-way ANOVA, F=1.9132, P=0.0983; Table 4). Furthermore, when plasma protein concentration was examined over embryonic development in brooding males, trends did not emerge in either species (*S. fuscus*, R^2=0.1017, P=0.1480, slope=–1.5663; *S. floridae*, R^2=0.0868, P=0.1529, slope=–1.7106). There was a significant difference in lipid content of blood plasma, with gravid females circulating lower lipid levels than non-brooding males (2-way ANOVA, F=3.2819, P=0.0087; *post-hoc* Tukey HSD, Q=2.3791, $P<0.05$; Table 4). In addition, *S. floridae* overall contained higher concentrations of plasma lipids than *S. fuscus* (*post-hoc* Student's *t*-test, t=1.9840, $P<0.05$). Significant changes in plasma lipid content over the brooding period were not evident (*S. fuscus*, R^2=0.0493, P=0.2970, slope=–0.0701; *S. floridae*, R^2=0.0475, P=0.3300, slope=–0.0710). Examination of plasma carbohydrate concentrations revealed brooding *S. floridae* males had higher levels compared with brooding *S. fuscus* (two-way ANOVA, F=3.1526, P=0.0110; *post-hoc* Tukey HSD, Q=2.9057, $P<0.05$; Fig. 5, Table 4). Again, examining changes over embryonic development yielded no trends in plasma carbohydrate levels (*S. fuscus*, R^2=0.0188, P=0.5324, slope=–1.2927; *S. floridae*, R^2=0.0108, P=0.6533, slope=0.7158).

Table 2. *Relative nutrient content of the pouch fluid of brooding and non-brooding males*

Comparison	[Protein] (mg ml^{-1})	[Lipid] (mg ml^{-1})	[Carbohydrate] (mg ml^{-1})
Brooding *vs* Non-brooding	Brooding>Non-brooding *S. fuscus*≈*S. floridae*	Brooding>Non-brooding *S. fuscus*≈*S. floridae*	Brooding>Non-brooding *S. fuscus*≈*S. floridae*
Two-way ANOVA	F=5.9721, P=0.0011	F=3.7717, P=0.0143	F=4.3950, P=0.0069
Post-hoc Student's t-test	t=1.9935, P<0.05	t=1.9944, P<0.05	t=1.9950, P<0.05
Non-brooding *vs* Saltwater	Non-brooding>Saltwater	Non-brooding>Saltwater	Non-brooding>Saltwater
Student's t-test			
S. fuscus	t=10.7035, P<0.0001	t=3.8613, P=0.0026	t=8.4348, P<0.0001
S. floridae	t=6.7976, P<0.0001	t=7.4050, P<0.0001	t=6.1250, P<0.0001

Saltwater with MS222 was compared to the pouch fluid of non-brooding males to determine how different the fluid inside the pouch was from the surrounding seawater.

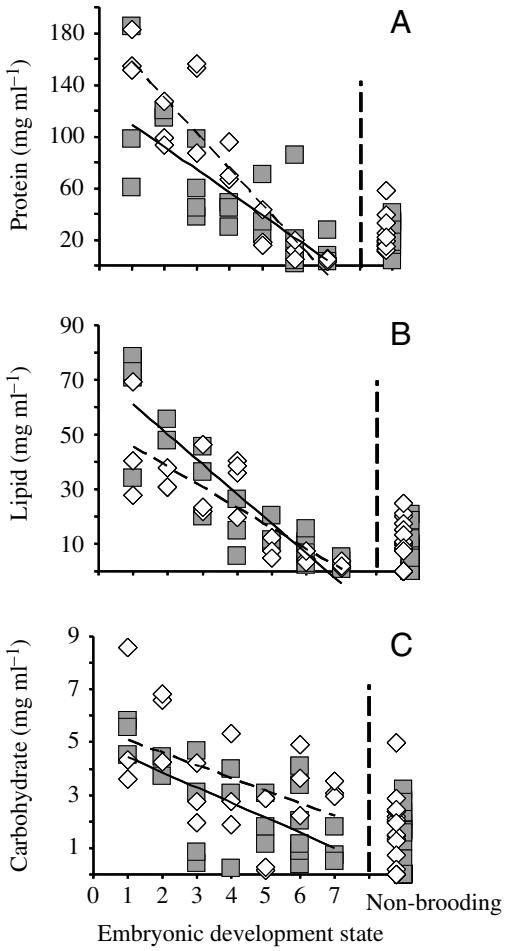

Fig. 4. Changes in (A) protein, (B) lipid and (C) carbohydrate concentrations in the pouch fluid of *Syngnathus fuscus* (gray squares; solid line) and *Syngnathus floridae* (white diamonds; broken line) across the progression of embryonic development. For both species, nutrient levels significantly decline in pouch fluid over the seven stages of development (P<0.05). The decrease in protein occurs at a steeper rate for *Syngnathus floridae*, whereas lipid concentrations drop more quickly in *Syngnathus fuscus* (both P<0.05).

Discussion

Our comparison reveals *S. fuscus* and *S. floridae* possess different balances in the dependence on maternal and paternal resources for embryonic development. Nutrients are normally lost during development because of the incomplete conversion of proteins and lipids into body tissue as they are used as metabolic fuels (Jobling, 1995). Indeed, lower concentrations of proteins, lipids and carbohydrates were measured in fry than in eggs of both species. Still, significant species differences in the nutrient reserves of eggs indicate that *S. floridae* embryos are more adequately supplied by the egg. *S. fuscus* females produce eggs with considerably less nutrients than *S. floridae*, which must be balanced by elevated paternal provisioning. These observations of *S. fuscus* follow predictions of evolutionary theory and the progression to complete sex reversal (Pagel, 2003).

The paternal brood pouch serves as a source of nutritional supplementation during development for both species, by bathing embryos in a fluid rich in macronutrients. Since the chorion is absent, nutrients and hormones may be readily absorbed. Differences between seawater and pouch fluid indicate concentrations of proteins, lipids and carbohydrates in the pouch can be regulated physiologically. Pouch fluid nutrients are nearly depleted over the brooding period. These decreases may reflect changes in the male's contribution, providing less to fry as they approach release, or a change in utilization of the pouch fluid by the embryos, increasing intake in relation to reduced yolk reserves. In placental sharks, embryos are initially reliant on yolk reserves sequestered in the egg until maternal supplementation is activated (Hamlett et al., 1987; Hamlett et al., 1993). A comparable series of events probably transpires in pipefish. Our study shows that species differ in the rate of decline of pouch fluid nutrients. The presence of yolk droplets floating in the pouch of *S. floridae* suggests utilization of 'nurse eggs', or release from fertilized embryos, provides a nutrient source in pouch fluid. Conversely, paternal contribution is more pronounced in *S. fuscus* with males encompassing relatively nutrient-poor eggs in epithelia

Table 3. *Regressions of nutrient concentrations in brood pouch fluid over the stages of embryonic development*

	Syngnathus fuscus			*Syngnathus floridae*			*F*-test*	
Nutrient (mg ml^{-1})	R^2	*P*	Slope	R^2	*P*	Slope	*F*	*P*
Protein	0.8433	<0.0001	–17.5761	0.5783	<0.0001	–27.5092	5.4697	0.0243
Lipid	0.8073	<0.0001	–10.8902	0.6679	<0.0001	–7.4870	4.3495	0.0430
Carbohydrate	0.4103	0.007	–0.5707	0.2329	0.0267	–0.4807	0.1362	0.7139

**F*-test for homogeneity of regression slopes.

Table 4. *Blood plasma concentrations of nutrients from adults collected in the Chincoteague Bay, VA, USA*

	Concentration (mg ml^{-1})					
Group	Protein	*N*	Lipid*	*N*	Carbohydrate†	*N*
Syngnathus fuscus						
Gravid females	33.51±0.64	16	10.75±0.60	15	3.04±0.44	16
Females w/o mature eggs	28.48±0.83	18	12.07±0.77	16	3.72±0.29	16
Brooding males	34.53±0.66	25	12.12±0.64	24	2.06±0.21	23
Non-brooding males	31.65±0.70	18	14.80±0.64	15	2.31±0.32	15
Syngnathus floridae						
Gravid females	24.96±0.85	15	11.68±0.65	15	2.81±0.28	16
Females w/o mature eggs	18.07±0.96	14	11.24±0.63	12	3.35±0.39	11
Brooding males	31.17±0.71	22	16.61±0.62	22	3.47±0.26	21
Non-brooding males	31.44±0.80	15	19.08±0.84	15	2.54±0.23	15

Values are means ± s.e.m.; *N*, sample number.

*Two-way ANOVA reveals gravid females have higher circulating lipid levels than non-brooding males. Overall, *S. floridae* plasma lipid concentrations are greater than that for *S. fuscus*.

†See Fig. 5.

supplied by brood pouch vasculature. The steep nutrient drop in the pouch fluid over embryonic development corresponds with differential use of protein from nurse eggs in *S. floridae* and lipids provided by the brooding male in *S. fuscus*. A level supply of protein across *S. fuscus* development is consistent with males continually secreting nutrients into the brood pouch. However the steeper drop in lipids may reflect dependence of *S. fuscus* embryos on brood pouch secretions for metabolic fuel. For both species, fry are released once paternal resources are exhausted.

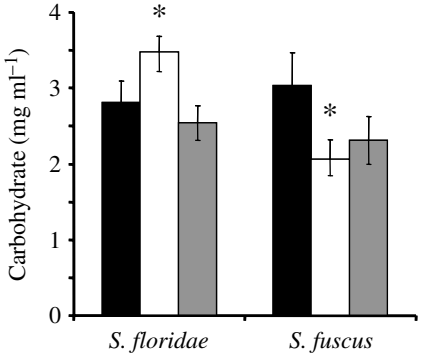

Fig. 5. Circulating carbohydrate levels in blood plasma of gravid females (black bars), brooding males (white bars) and non-brooding males (gray bars). Values are means ± 1 s.e.m. An asterisk indicates a significant difference between groups (P<0.05).

Undeveloped nurse eggs in the *S. floridae* brood pouch are believed to have originated from overripe oocytes (Teixeira and Vieira, 1995). Other syngnathid species frequently possess undeveloped eggs as well. In *Syngnathus typhle*, these eggs and less developed embryos attach to the placenta-like structure in the pouch (Ahnesjo, 1992). The presence of lipid cells in the pouch epithelium of the seahorse *Hippocampus brevirostris* suggests egg yolk can be re-absorbed (Rauther, 1925). From these observations, the syngnathid pouch fluid is believed to be at least partially derived from nurse eggs. Specifically, the protein hormone, prolactin, is released into the pouch causing enzymatic breakdown of the egg to form a placental fluid (Boisseau, 1967; Ahnesjo, 1996). In pipefish, the presence of undeveloped eggs in the brood pouch is more likely to indicate that a nurse egg system is being employed rather than eggs simply not being fertilized. Internal fertilization in these species involves sperm released into the pouch, and therefore, the rate of fertilization is high (Fielder, 1954). Employing a nurse egg system in *S. floridae* and thus decreasing paternal nutrient allocation may translate into a more equal sex ratio, as well as higher circulating nutrient concentrations for brooding *S. floridae* males over *S. fuscus*.

The matrix construct observed with fry release in *S. floridae*

may be a structure produced by the male during brooding to support developing embryos. In two *Stigmatopora* pipefish, males incur an additional energetic cost in making membranous egg-holding compartments within the brood pouch. These specialized structures are not present in the non-brooding *S. floridae* males or *Stigmatopora*, implying this structure must be rebuilt every time a male broods (Berglund et al., 1986; Steffe et al., 1989). The functional significance of this matrix has yet to be explored.

A different embryonic supplementation mechanism revealed by our study involves a paternally derived, nutrient-rich pouch fluid. *S. fuscus* brood pouch flaps have epithelial coverings and extensive vascularization that are only visible when males are brooding. Histological examinations of several syngnathids show similar morphological changes in the pouch wall concurrent with brooding (Lockwood, 1867; Huot, 1902; Steffe et al., 1989; Carcupino et al., 1997; Drozdov et al., 1997; Carcupino et al., 2002). Most commonly noted is the development and growth of capillaries in the epithelium of the pouch walls (Gill, 1905; Thevenin, 1936; Carcupino et al., 1997; Drozdov et al., 1997). Carcupino et al. (Carcupino et al., 2002) hypothesized that nutrients may be transferred from the paternal blood to the pouch by transcytosis or may be synthesized or modified in the epithelial cells. In *S. abaster*, large intercellular spaces form at the bases of epithelial cells functioning as a freeway to facilitate the passage of molecules from capillaries to the lumen of the pouch (Carcupino et al., 1997). Accordingly, we believe profusion of blood vessels in the pouch flaps and implantation of embryos adjacent to these vessels is evidence for paternal provisioning in *S. fuscus*.

Blood plasma nutrient levels provide further evidence of greater paternal investment in *S. fuscus*. We document equivalent plasma nutrient levels in brooding males and conspecific gravid females, suggesting brooding males incur a cost for parental care in both species. A study on threespine sticklebacks, measuring lipid, glycogen and protein, found the concentrations of all these substances peaked at the beginning of the breeding season. At the end of the season, males that brooded had lower energy reserves and higher mortality rates than males that did not breed (Chellappa et al., 1989). Thus, low nutrient levels in spawning fish indicate allocation to gamete production and/or parental care. Our data reveals circulating carbohydrate concentrations are lower in brooding males of *S. fuscus* than *S. floridae*. Because the pipefish diet is nearly exclusively composed of proteins and lipids, carbohydrate levels are a measure of the availability of metabolic fuel (Ryer and Boehlert, 1983; Huh, 1986; Jobling, 1995). Lower concentrations in *S. fuscus* are indicative of the higher metabolic cost of brooding.

Our data concerning egg and fry size support the hypothesized paternal provisioning mechanisms suggested by the adult nutrient analyses. If changes in weight from egg to fry state are considered for species with various modes of development, viviparous organisms commonly loose up to 50% in weight, oviparous 20–30% and matrotrophic ovipararous gain 11–369% (Needham, 1942; Amoroso, 1960; Hamlett et al., 1993). Rather than measure weight changes, we examined drops in nutrient levels. The difference in nutrient stores between mature, unfertilized eggs and released fry was always greater for *S. floridae*. When compared to *S. fuscus*, the nutrient declines in *S. floridae* were greater in magnitude by 2.4 for protein, 9.6 for lipids and 0.9 for carbohydrates. Considering the brood period for both species overlap (personal observation) (Bigelow and Schroeder, 1953), *S. fuscus* would necessarily acquire more nutrients from the pouch fluid.

Brooding in syngnathids has been described as carrying a cost of parental care which exceeds that of most vertebrates (Breder and Rosen, 1966; Clutton-Brock and Vincent, 1991). Within this family, it has been stated that species with less complex brood pouches spend less energy brooding young than do males with more enclosed pouches (Berglund et al., 1986; Masonjones, 2001; Carcupino et al., 2002). Although the physiological cost of parental care is not equivalent to parental investment in offspring, energy expenditures may be positively correlated with parental care in many cases (Wilson et al., 2003). Higher costs of parental care are likely to be reflected in a lower frequency of potential mates, and therefore a skewed operational sex ratio in breeding populations (Trivers, 1975; Clutton-Brock and Vincent, 1991; Gwynne, 1991). Defined as the ratio of fertilizable females to sexually active males, the operational sex ratio is dependent upon several factors in addition to differences in parental care, including spatial and temporal clumping of the limited sex and life history differences between the sexes (Emlen, 1976; Emlen and Oring, 1977; Berglund and Rosenqvist, 1993; Andersson, 1994). Hence, if other factors are constant or very similar, paternal nutrient provisioning and the cost of reproduction for male syngnathids should be reflected in sex ratios. Breeding populations of the two species in this study overlap spatially and temporally. Although we cannot completely rule out other factors, a significant difference in the proportion of wild-caught adult males between *S. fuscus* and *S. floridae* breeding populations suggests higher relative paternal energy expenditure in *S. fuscus*. The costs of parental care and parental investment to brooding offspring in these species need to be tested through an examination of lifetime reproductive success.

If, as our data suggest, *S. fuscus* males contribute a greater proportion of parental care than *S. floridae* males, we predict a corresponding divergence in sex roles, mating competition and the evolution of secondary sexual characteristics between these species. Some of these predictions are supported by accounts in the literature. *S. fuscus* females are reported to roam a larger area and to develop dimorphic banding coloration to find and attract mates (Roelke and Sogard, 1993; Berglund et al., 1997; Bernet et al., 1998). More balanced investment in progeny between the sexes in *S. floridae* predicts comparable reproductive rates for males and females. The sexes would not be expected to evolve strongly dimorphic behaviors and traits in this species, and descriptions of *S. floridae* report no obvious secondary sexual characters (Jones and Avise, 2001). Regardless of these accounts, the

behavioural ecology of these species needs to be studied to support the above hypothesis.

Our results highlight the importance of basic reproductive physiology in understanding the functional significance of the brood pouch. The location and enclosure of the male brood pouch defines primary taxonomic groupings (Dunker, 1915; Herald, 1959). In general, syngnathid phylogeny is largely based on the three paternal brood pouch types (Herald, 1959; Wilson et al., 2001). Our comparison reveals physiology differs considerably within a pouch type. Perhaps both phylogeny and taxonomy should be reinvestigated with more detailed reproductive physiological data. Syngnathids offer a plethora of opportunities to explore the evolution of placental-like structures and their role in development (Milius, 2000). Connectivity of embryos to the brood pouch of *S. floridae* and *S. fuscus* differs considerably, and the physiological mechanism for this distinction may provide a greater understanding of selective pressures for parental investment. The comparison of the eggs from these two species provides evidence of female restriction in gametic provisioning. Our results imply that female allocation to offspring in oviviparous animals can be altered with relative selective pressure. Since females are defined through the production of large, nutrient-laden gametes, a drop in egg nutrient content represents the logical progression from a sex-role reversed to a fully sex-reversed species (Pagel, 2003). The comparison of *S. fuscus* and *S. floridae* offers unique insight into the evolution of mechanisms of parental care, and provides an interesting mechanism to explore the distinction between the sexes based on gamete production.

We thank the Marine Science Consortium of Wallops Island, Virginia for the use of their facility and equipment. Brood pouch sketches were drawn by A. Payne. We also thank D. Hardee, J. Marshall, W. Sites and T. Stueckle for assistance in field collections. Pipefish collection was approved by the Commonwealth of Virginia Marine Resources Commission (permits 03–36 and 04–22). All animal care and methods complied with regulations enforced by the West Virginia University Animal Care and Use Committee (protocol number 03-0501, November 2003). This research was supported by Oak Ridge Associated Universities, the West Virginia University Research Corporation and the West Virginia University Department of Biology.

References

Ahnesjo, I. (1992). Consequences of male brood care: weight and number of newborn in a sex-role reversed pipefish. *Funct. Ecol.* **6**, 274-281.

Ahnesjo, I. (1996). Apparent resource competition among embryos in the brood pouch of a male pipefish. *Behav. Ecol. Sociobiol.* **38**, 167-172.

Alexander, R. D. and Borgia, G. (1979). On the origin and basis of the male-female phenomenon. In *Sexual Selection and Reproductive Competition in Insects* (ed. M. F. Blum and N. Blum), pp. 417-440. New York: Academic Press.

Amoroso, E. C. (1960). Viviparity in fishes. *Symp. Zool. Soc. (Lond.)* **1**, 153-181.

Andersson, M. (1994). *Sexual Selection*. Princeton, NJ, USA: Princeton University Press.

Azzarello, M. Y. (1991). Some questions concerning the Syngnathidae brood pouch. *Bull. Mar. Sci.* **49**, 741-747.

Begovac, P. C. and Wallace, R. A. (1987). Ovary of the pipefish, *Syngnathus scovelli*. *J. Morphol.* **193**, 117-133.

Berglund, A. and Rosenqvist, G. (1993). Selective males and ardent females in pipefishes. *Behav. Ecol. Sociobiol.* **32**, 331-336.

Berglund, A., Rosenqvist, G. and Svensson, I. (1986). Reversed sex roles and parental energy investment in zygotes of two pipefish (Syngnathidae) species. *Mar. Ecol. Prog. Ser.* **29**, 209-215.

Berglund, A., Rosenqvist, G. and Svensson, I. (1989). Reproductive success of females limited by males in two pipefish species. *Am. Nat.* **133**, 506-516.

Berglund, A., Rosenqvist, G. and Bernet, P. (1997). Ornamentation predicts reproductive success in female pipefish. *Behav. Ecol. Sociobiol.* **40**, 145.

Bernet, P., Rosenqvist, G. and Berglund, A. (1998). Female-female competition affects female ornamentation in the sex-role reversed pipefish *Syngnathus typhle*. *Behaviour* **135**, 535.

Bigelow, H. B. and Schroeder, W. C. (1953). Fishes of the Gulf of Maine. *US Fish. Bull.* **53**, 1-597.

Boisseau, J. (1967). *Les Regulations Hormonales de l'Incubations Chez un Vertebre Male: Recherches Sur la Reproduction de l'Hippocampe.* DSc thesis, University of Bordeaux.

Breder, C. M. and Rosen, D. E. (1966). *Modes of Reproduction in Fishes*. New York: Natural History Press.

Carcupino, M., Baldacci, A., Mazzini, M. and Franzoi, P. (1997). Morphological organization of the male brood pouch epithelium of *Syngnathus abaster* Risso (Teleostea, Syngnathidae) before, during, and after egg incubation. *Tissue Cell* **29**, 21-30.

Carcupino, M., Baldacci, A., Mazzini, M. and Franzoi, P. (2002). Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a morphological and ultrastructural comparative study on three anatomically different pouches. *J. Fish Biol.* **61**, 1465-1480.

Chellappa, S., Huntingford, F. A., Strang, R. H. C. and Thomson, R. Y. (1989). Annual variation in energy reserves in male three-spined stickleback, *Gasterosteus aculeatus* L. (Pisces, Gasterostidae). *J. Fish Biol.* **35**, 275-286.

Clutton-Brock, T. H. and Vincent, A. C. (1991). Sexual selection and the potential reproductive rates of males and females. *Nature* **351**, 58-60.

Drozdov, A. L., Kornienko, E. S. and Krasnolutsky, A. V. (1997). Reproduction and development of *Syngnathus acusimils*. *Rus. J. Mar. Biol.* **23**, 265-268.

Duncker, G. (1915). Revision der Syngnathidae. *Jahrbuch der Hamburgischen Wissenschaftlichen Antalten* **32**, 9-120.

Emlen, S. T. (1976). Lek organization and mating strategies in the bullfrog. *Behav. Ecol. Sociobiol.* **1**, 283-313.

Emlen, S. T. and Oring, L. W. (1977). Ecology, sexual selection, and the evolution of mating systems. *Science* **197**, 215-223.

Fiedler, K. (1954). Vergleichende Verhaltensstudien an Seenadeln, Schangennadeln und Seepferdchen (Syngnathidae). *Z. Tierpsychol.* **11**, 358-416.

Gill, T. (1905). The life history of the sea-horses (Hippocampids). *Proc. US Natl. Mus.* **28**, 805-814.

Gross, M. R. and Shine, R. (1981). Parental care and mode of fertilization in ectothermic vertebrates. *Evolution* **35**, 775-793.

Gwynne, D. T. (1991). Sexual competition among females: What causes courtship role reversal? *Trends Ecol. Evol.* **6**, 118-121.

Hamlett, W. C., Schwartz, F. J. and DiDio, L. J. A. (1987). Subcellular organization of the yolk syncytial-endoderm complex in the preimplantation yolk sac of the shark, *Rhizoprionodon terraenovae*. *Cell Tissue Res.* **247**, 275-285.

Hamlett, W. C., Eulitt, A. M., Jarrell, R. L. and Kelly, M. A. (1993). Uterogestation and placentation in elasmobranches. *J. Exp. Zool.* **266**, 347-367.

Haresign, T. W. and Schumway, S. E. (1981). Permeability of the marsupium of the pipefish *Syngnathus fuscus* to [14C]-alpha animo isobutyric acid. *Comp. Biochem. Physiol.* **69A**, 603-604.

Helfman, G. S., Collette, B. B. and Facey, D. E. (1997). *The Diversity of Fishes.* Malden, MA, USA: Blackwell Science.

Herald, E. S. (1959). From pipefish to seahorse – a study of phylogenetic relationships. *Proc. Calif. Acad. Sci.* **29**, 465-473.

Huh, S. H. (1986). Ontogenetic food habits of four common fish species in seagrass meadows. *J. Ocean. Soc. Korea* **21**, 25-33.

Huot, A. (1902). Recherches sur les poisons lophobranches. *Ann. Sci. Nat. (Zool.)* **8**, 197-288.
Jobling, M. (1995). *Environmental Biology of Fishes.* New York. NY, USA: Chapman and Hall.
Jones, A. G. and Avise, J. C. (1997). Microsatellite analysis of maternity and the mating system in the Gulf pipefish *Syngnathus scovelli*, a species with male pregnancy and sex-role reversal. *Mol. Ecol.* **6**, 203-213.
Jones, A. G. and Avise, J. C. (2001). Mating systems and sexual selection in male-pregnant pipefishes and seahorses: insights from microsatellite-based studies of maternity. *J. Hered.* **92**, 150-158.
Jones, A. G., Kvarnemo, C., Moore, G. I., Simmons, L. W. and Avise, J. C. (1998). Microsatellite evidence for monogamy and sex-biased recombination in the Western Australian seahorse *Hippocampus angustus*. *Mol. Ecol.* **7**, 1497-1505.
Jones, A. G., Rosenqvist, G., Berglund, A. and Avise, J. C. (1999). The genetic mating system of a sex-role-reversed pipefish (*Syngnthus typhle*): a molecular inquiry. *Behav. Ecol. Sociobiol.* **46**, 357-365.
Linton, J. R. and Soloff, B. L. (1964). The physiology of the brood pouch of the male seahorse, *Hippocampus erectus*. *Bull. Mar. Sci. Gulf Caribb.* **14**, 45-61.
Lockwood, S. (1867). The seahorse (*Hippocampous hudsonius*) and its young. *Am. Nat.* **1**, 225-234.
Lotufo, G. R., Farrar, J. D. and Bridges, T. S. (2000). Effects of exposure source, worm density, and sex on DDT bioaccumulation and toxicity in the marine polychaete *Neanthes arenaceodentata*. *Environ. Toxicol. Chem.* **19**, 472-484.
Masonjones, H. D. (2001). The effect of social context and reproductive status on the metabolic rates of dwarf seahorses (*Hippocampus zosterae*). *Comp. Biochem. Physiol.* **129A**, 541-555.
Mayer, I., Rosenqvist, G., Borg, B., Ahnesjo, I., Berglund, A. and Schulz, R. W. (1993). Plasma levels of sex steroids in three species of pipefish (Syngnathidae). *Can. J. Zool.* **71**, 1903-1907.
Maynard-Smith, J. (1978). *The Evolution of Sex.* Cambridge: Cambridge University Press.
Milius, S. (2000). Pregnant and still macho. *Sci. News* **157**, 168.
Morrell, V. (2002). Placentas may nourish complexity studies. *Science* **298**, 945.
Needham, J. (1942). *Biochemistry and Morphogenesis.* Cambridge: Cambridge University Press.
Pagel, M. (2003). Evolutionary biology: Polygamy and parenting. *Nature* **424**, 23-24.
Parker, G. A., Baker, R. R. and Smith, V. G. F. (1972). The origin and evolution of gamete dimorphism and the male-female phenomenon. *J. Theor. Biol.* **36**, 181-198.
Quast, W. D. and Howe, N. R. (1980). The osmotic role of the brood pouch in the pipefish *Syngnathus scovelli*. *Comp. Biochem. Physiol.* **67A**, 675-678.
Rauther, M. (1925). Die Syngnathiden des Golfes von Neapel. *Fauna Flora Golfo Napoli Monograph.* **36**.
Resnick, D. N., Mateos, M. and Springer, M. S. (2002). Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science* **298**, 1018-1020.
Roelke, D. L. and Sogard, S. M. (1993). Gender-based differences in the habitat selection and activity level in the northern pipefish (*Syngnathus fuscus*). *Copeia* **1993**, 528.
Ryer, C. H. and Boehlert, G. W. (1983). Feeding chronology, daily ration, and the effects of temperature upon gastric evacuation in the pipefish, *Syngnathus fuscus*. *Environ. Biol. Fishes* **6**, 301-306.
Steffe, A. S., Westoby, M. and Bell, J. D. (1989). Habitat selection and diet in two species of pipefish from seagrass: sex difference. *Mar. Ecol. Prog.* Ser. **55**, 23-30.
Stewart, J. R. (1992). Placental structure and nutritional provision to embryos in predominantly lecithotrophic vivparous reptiles. *Am. Zool.* **32**, 303-312.
Svensson, I. (1988). Reproductive costs in two sex-role reversed pipefish species (Syngnathidae). *J. Anim. Ecol.* **57**, 929-942.
Teixeira, R. L. and Musick, J. A. (1995). Trophic ecology of two congeneric pipefishes (Syngnathidae) of the lower York River, Virginia. *Environ. Biol. Fishes* **43**, 295-309.
Teixeira, R. L. and Vieira, J. P. (1995). The breeding population of the pipefish, *Syngnathus folletti* (Pisces: Syngnathidae) from Southern Brazil. *Atlantica* **17**, 123-134.
Thevenin, R. (1936). The curious life habits of the sea horse. *Nat. Hist.* **1936**, 211-222.
Trivers, R. L. (1972). Parental Investment and Sexual Selection. In *Sexual Selection and the Descent of Man, 1871-1971* (ed. B. Campbell), pp. 136-179. Chicago, IL, USA: Aldine-Atherton.
Trivers, R. L. (1975). *Social Evolution.* Menlo Park, CA, USA: Benjamin Cummings.
Van Handel, E. (1985). Rapid-determination of glycogen and sugars in mosquitoes. *J. Am. Mosq. Control Assoc.* **1**, 299-301.
Vincent, A., Ahnesjo, I., Berglund, A. and Rosenqvist, G. (1992). Pipefishes and seahorses: are they all sex role reversed? *Trends Ecol. Evol.* **7**, 237-241.
Wallace, R. A. and Selman, K. (1981). Cellular and dynamic aspects of oocyte growth in teleosts. *Am. Zool.* **21**, 325-343.
Watanabe, S., Kaneko, T. and Watanabe, Y. (1999). Immunocytochemical detection of mitochondria-rich cells in the brood pouch epithelium of the pipefish, *Syngnathus schlegeli*: structural comparison with mitochondria-rich cells in the gills and larval epidermis. *Cell Tissue Res.* **295**, 141-149.
Wheeler, D. E. and Buck, N. A. (1992). Protein, lipid and carbohydrate use during metamorphosis in the fire ant, *Solenopsis xyloni*. *Physiol. Entomol.* **17**, 397-403.
Williams, G. C. (1966). *Adaptation and Natural Selection.* Princeton, NJ, USA: Princeton University Press.
Williams, G. C. (1975). *Sex and Evolution.* Princeton (NJ): Princeton University Press.
Wilson, A. B., Vincent, A., Ahnesjo, I. and Meyer, A. (2001). Male pregnancy in seahorses and pipefishes (family Syngnathidae): rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny. *J. Hered.* **92**, 159-166.
Wilson, A. B., Ahensjo, I., Vincent, A. C. J. and Meyer, A. (2003). The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family Syngnathidae). *Evolution* **57**, 1374-1386.