An Examination of the Variation in Maternal Placentae Across the Genus *Poeciliopsis* (Poeciliidae)

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ABSTRACT Placentae show considerable diversity in a number of nonmammalian, viviparous organisms, including amphibians, reptilian sauropsids, teleost fish, and chondrichthyes. However, the evolutionary processes driving the evolution of placenta are still debated. In teleost fishes, the genus *Poeciliopsis* (Poeciliidae) offers a rare opportunity for studying placental evolution: extensive placentation has evolved three independent times within the last 750,000 years and there is substantial interspecific variation in the degree of embryonic, maternal nutrient provisioning and development of the placenta. In poeciliids, the placenta is composed of a hypertrophied maternal follicular epithelium apposed to a highly vascularized embryonic pericardial sac. To better understand placental evolution, we have undertaken a comprehensive comparative study of the maternal follicle in eight closely related *Poeciliopsis* species that span the range in postfertilization, embryonic, maternal nutrient provisioning (from lecithotrophs, to moderate matrotrophs, to extensive matrotrophs). Using light and scanning electron microscopy, we found that the species that provide extensive postfertilization maternal nutrient provisioning (extensive matrotrophs) have thicker follicles and more extensive folding of the follicular epithelium compared to the lecithotrophs and moderate matrotrophs. Follicle sections and histology revealed that epithelial folds of the extensive matrotrophs are comprised primarily of cuboidal and columnar cells and are richly supplied with capillaries. Among the extensive matrotrophs, enhancements of follicle traits corresponded with increases in the level of maternal nutrient provisioning. Hypertrophied maternal follicles with richly vascularized folds can serve to increase the surface area and, thus, facilitate the transfer of substances between the mother and developing embryo. Finally, we found egg envelopes in the lecithotrophs and moderate matrotrophs, but not in the extensive matrotrophs. Morphological studies, like this one, can provide a better understanding of the natural variation in the structure and functioning of maternal and offspring traits associated with matrotrophy and, thus, insights into the processes driving placental evolution. J. Morphol. 000:000–000, 2015.

KEY WORDS: maternal follicle; maternal provisioning; placental evolution; viviparity

INTRODUCTION

Placental structures have been characterized for several nonmammalian, viviparous organisms, including amphibians, reptilian sauropsids, teleost fish, and chondrichthyes (reviewed in Blüm, 1986; Wooding and Burton, 2008; Blackburn, 2014). These structures are defined as placentae based on a unifying physiological function of facilitating the exchange of substances (e.g., gases, nutrients, wastes) between the mother and developing embryo (Mossman, 1937; Turner, 1940; Wourms, 1981; Wourms et al., 1988; Pires et al., 2007; Wooding and Burton, 2008; Blackburn et al., 2010). Although there is a considerable degree of variation among taxa in placental structures, all share the basic feature of apposing parental and embryonic tissues (Mossman, 1937; Wooding and Burton, 2008). Recently, Wooding and Burton (2008) and Blackburn (2014) provided a comprehensive review of placenta in viviparous organisms, including fishes where placentae range from simple to highly complex structures (see also Wourms, 1981, Wourms et al., 1988). Interspecific comparative studies characterizing fish placentae are necessary to develop an understanding of placental evolution, yet, to our knowledge, there are only a dozen of such studies in teleost fishes (Wourms, 1981; Wourms et al., 1988; Meisner and Burns, 1997; Reznick et al., 2002, 2007; Pires

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et al., 2007, 2010, 2011; Banet and Reznick, 2008; Wooding and Burton, 2008; Banet et al., 2010; Pollux et al., 2014).

A unique opportunity for understanding placental evolution exists in the fish genus *Poeciliopsis* (Poeciliidae). Turner (1940) described the *Poeciliopsis* placenta (also referred to as the "follicular placenta"; e.g., Grove and Wourms, 1991, 1994) as being composed of a hypertrophied maternal follicular epithelium apposed to a highly vascularized embryonic pericardial sac. Within the genus, there is considerable variation in both the timing of embryonic, maternal nutrient provisioning, and development of the placenta. Moreover, extensive placentation has evolved three independent times in the group within the last 750,000 years (Reznick et al., 2002). (Following previous work in *Poeciliopsis* (Turner, 1940; Reznick et al., 2002, 2007; Pires et al., 2007, 2010, 2011; Banet and Reznick, 2008; Banet et al., 2010; Pollux et al., 2014), here, we define "placentation" as inferred from the level of matrotrophy (Reznick et al., 2002) and the structures involved in intrafollicular gestation (Turner, 1940). Note that exceptions and concerns to this inference have been raised (see Blackburn, 2014).) The described *Poeciliopsis* species are all viviparous, yet interspecific variation in the degree of postfertilization, embryonic, maternal nutrient provisioning ranges from zero (i.e., lecithotrophy) to nearly continuous (i.e., matrotrophy; Reznick et al., 2002). Consequently, as noted by Reznick et al., (2002), *Poeciliopsis* offers biologists the rare opportunity to study the evolution and diversity of placentation. This is something we cannot do in placental mammals, as the single common ancestor lived over 100 million years ago (Reznick et al., 2002; O'Neill et al., 2007).

Currently, there are three main hypotheses to explain the origin and evolution of matrotrophy and placentae in the Poeciliidae (reviewed in Pollux et al., 2009): a) locomotor cost hypothesis (Plaut, 2002; Ghalambor et al., 2004), b) resource-availability hypothesis (Trexler, 1997; Trexler and DeAngelis, 2003), and/or c) parent-offspring conflict hypothesis (Trivers, 1974; Zeh and Zeh, 2000; Crespi and Semeniuk, 2004). The first two hypotheses focus on the effects of ecology on the benefits of maternal provisioning. Parent-offspring conflict, on the other hand, focuses on the potentially antagonistic interactions between a provisioning parent and their offspring, once a placenta has evolved. Here, the maternal-fetal interface acts as a "battlefront" between the mother and developing embryo(s) and may lead to a co-evolutionary arms race between maternal and embryonic traits associated with postfertilization maternal provisioning (Zeh and Zeh, 2000; Crespi and Semeniuk, 2004). Although there is empirical support for the role of parent-offspring conflict in placental evolution (Lawton et al., 2005; O'Neill et al., 2007; Schrader and Travis, 2008, 2009), recent work has found contradictory results that, instead, point to parent-offspring coadaptation (Schrader et al., 2011, 2013; Schrader and Travis, 2012a).

To better understand the evolutionary processes driving the evolution of placenta in poeciliids, the next step is to quantify the natural variation in the structure and function of maternal and offspring traits associated with the placenta. Here, we conduct a comprehensive comparison of the placenta in eight closely related *Poeciliopsis* species, specifically focusing on the maternal follicle (also referred to as the "ovarian follicle"—e.g., Grove and Wourms, 1994). Among these species, two are lecithotrophs (no postfertilization maternal nutrient provisioning of the embryo), three are moderate matrotrophs (moderate postfertilization maternal nutrient provisioning), and three are extensive matrotrophs (extensive postfertilization maternal nutrient provisioning; defined by Reznick et al., 2002). To characterize and contrast the inner lining of the maternal follicle (i.e., the surface that is in close apposition to the embryo), we use light and scanning electron microscopy (SEM). We also contrast the thickness and tissue structures of the maternal follicle in these three groups using tissue sections. Given the proposed functional role of the maternal follicle in facilitating the exchange of substances between the mother and embryo, we predict that the follicle will be well defined and thicker for the extensive matrotrophs (e.g., increased surface area through thick, vascularized folds, and apical cell surface structures, such as microvilli) than for the moderate matrotrophs and lecithotrophs. We discuss our results in light of placental evolution and compare them to other well-studied teleost fishes with placenta-like tissues.

MATERIAL AND METHODS

Study Species

Eight *Poeciliopsis* (Poeciliidae) species were used in this study (Table 1). Species were chosen based on two criteria: a) they represented a range in the extent of postfertilization maternal provisioning, and b) museum or lab populations were available for dissection and tissue collection. Maternal nutrient provisioning is indirectly measured and ranked based on the matrotrophy index (MI), which is the dry mass of offspring at birth divided by the dry mass of the egg at fertilization (Wourms et al., 1988; Reznick et al., 2002). The eight species ranged in MI from 0.66 to 117 and each falls into one of three groups based on the level of postfertilization maternal nutrient provisioning (Reznick et al., 2002). Two of the eight species are lecithotrophs ($MI < 0.7$; *Poeciliopsis turrubarensis, P. gracilis*), three species are moderate matrotrophs ($0.7 < MI < 5$; *P. latidens, P. viriosa, P. occidentalis*), and three species are extensive matrotrophs ($MI > 5$; *P. prolifica, P. turneri, P. retropinna*). In the lecithotrophs, females provide resources (i.e., a considerable amount of yolk) prior to fertilization, but very little or none after fertilization, and embryos lose mass during development (30–40%) to metabolic costs (Wourms et al., 1988; Reznick

TABLE 1. Poeciliopsis specimens sampled, in order of increasing MI values

Species	MI*	Level of provisioning†	Locality	Date	Specimen type	Specimen reference‡
P. turrubarensis	0.66	Lecithotroph	Chiriqui Province, Panama, Central America	December 01, 1961	Museum	The Academy of Natural Sciences of Drexel University (ANSP104379)
P. gracilis	0.69	Lecithotroph	Guatemala, Central America	March 02, 1925	Museum	The Academy of Natural Sciences of Drexel University (ANSP64734)
			Rio Jones Tributary, between km 145 and 146 on Carretara Jacobo Arbenz Guzman, Guatemala (GPS: 15.071381°N and -89.509580°W)	June 24, 1993	Lab	
P. latidens	0.86	Moderate matrotroph	San Lorenzo, Veracruz, Mexico	June 30, 1939	Museum	Royal Ontario Museum (ROM25298)
P. viriosa	0.93	Moderate matrotroph	Nayarit, Mexico	Not available	Museum	Canadian Museum of Nature (CMNFI 1959-0190.1)
P. occidentalis	1.12	Moderate matrotroph	Dexter National Fish Hatchery, Dexter, New Mexico	September 21, 1981	Museum	University of New Mexico's Museum of Southwestern Biology (MSC68313)
P. prolifica	5.4	Extensive matrotroph	Rio El Palillo, km 16 on highway 74 from San Blas to Tepic, Nayarit, Mexico (GPS: 21° 38.498 N, 105° 8.489 W)	January 11, 2004	Lab	
P. turneri	41.4	Extensive matrotroph	Rio Purification drainage, near Cosimiro Castillo Jalisco, Mexico (GPS: 19° 36.74 N, 104° 25.524 W)	January 19, 2004	Lab	
P. retropinna	117	Extensive matrotroph	Chiriqui Province, Panama, Central America	December 16, 1961 December 02, 1961 January 27, 1983	Museum	The Academy of Natural Sciences of Drexel University (ANSP104453, 104478, 151259)

*MI value, the dry mass of offspring divided by the dry mass of the egg at fertilization, was obtained from Wourms et al. (1988) and Reznick et al. (2002).
†Lecithotrophs, moderate matrotrophs, and extensive matrotrophs provide no, moderate, and extensive postfertilization maternal nutrient provisioning, respectively.
‡References for lab specimens are available on request.

et al., 2002). In the moderate and extensive matrotrophs, females provide resources to embryos throughout postfertilization development, offsetting the metabolic costs, and, thus, embryos increase in weight during development (Reznick et al., 2002). Each of the three extensive matrotrophs in this study represent an independent event in placental evolution (Reznick et al., 2002), and span the considerable range in MI values in this group, from 5.4 (*P. prolifica*) to 41.4 (*P. turneri*) to 117 (*P. retropinna*; Reznick et al., 2002).

Six of the eight *Poeciliopsis* species were on loan from museums (Table 1). All museum specimens were stored in 70 or 75% ethanol and may have been fixed in an unknown fixative prior to ethanol storage. Three of the eight *Poeciliopsis* species were from a breeding stock population at Ohio Wesleyan University (Table 1). These populations were established between 2011 and 2012 from adult and juvenile breeding populations, kindly provided by Reznick at the University of California, Riverside. Fish handling and euthanasia (in MS-222 and decapitation) were performed following an approved Institutional Animal Care and Use Committee protocol.

Dissection and Embryonic Stage Classification

Pregnant females were dissected and the entire ovary (containing the embryos, which were within the maternal follicle) was removed from the abdominal cavity. Embryos were then carefully removed from the ovary and separated from one another. Using a modified 11-stage developmental scheme from Haynes (1995), the embryos were classified and sorted into four developmental stages (1, 2, 3, and 4), corresponding to Stages 1–5, 6–7, 8, and 9–11, respectively.

For the museum specimens, the embryos were either maintained within the follicles or dissected out of the follicles with forceps. The embryos and follicles were then stored in 70–75%

ethanol until preparation for SEM or tissue sectioning. For the fresh specimens, the ovary was removed from the abdominal cavity of sacrificed females, and the embryos (within the follicles) were removed from the ovary and fixed overnight in 2–3% paraformaldehyde–gluteraldehyde. The fixed samples were then either carried through an ethanol dehydration series for SEM (see below) or directly preserved in 70% ethanol for tissue sectioning.

Microscopy Approaches

Light and scanning electron microscope. Light microscope images of the embryo with and then without the maternal follicles (i.e., removed) were captured. For each female, up to 10 embryos per developmental stage were imaged using an Olympus® SZ61 light microscope, with a Scion Corporation® CFQ-1612C camera, at ×1.5 and ×2.5 magnifications.

A SEM was used to characterize and contrast the anatomical surface structures of the maternal follicle, specifically the follicular epithelium, at different developmental stages for the lecithotrophs, moderate matrotrophs, and extensive matrotrophs. All museum samples were rehydrated through a descending graded series of ethanol (70–10%), rinsed several times with distilled water, and then dehydrated through an ascending graded series of ethanol (10–100%). Lab specimen samples were dehydrated with a graded series of ethanol (10–100%) after overnight fixation (2–3% paraformaldehyde–gluteraldehyde). All samples were critical point dried using a Samdri® 750 Critical Point Dryer (Tousimis, Rockville, MD). Dried samples were attached to aluminium stubs with double sided mounting tape. Follicles were mounted by gently prying open the follicle, removing the embryo, and exposing as much of the inner surface of the follicle as possible. All samples were gold-coated with a SPI® Module Sputter Coater (SPI Supplies/Structure Probe, West Chester, PA) for 60–80 s. When not in use, the gold-coated samples were stored in a sealed desiccator with desiccant. We used a Zeiss® EVO LS10 SEM (Carl Zeiss Microscopy, LLC, Peabody, MA) at 15–25 keV and a working distance of approximately 5–5.5 mm and 10–30 mm for smaller and larger samples, respectively. The probe current was kept at 150 pA. For each species and developmental stage, at least two samples were viewed with SEM. Images were then digitally recorded and compiled using Adobe Photoshop® Elements software (version 4).

Tissue sectioning. To study a) the relationship between the level of postfertilization maternal nutrient provisioning and thickness of the maternal follicle and b) the follicle epithelium and connective tissue morphologies, we sectioned the follicle tissue. Three developmental stages (2, 3, and 4) were sampled for each species, when possible. Stage 1 was excluded from the analysis because of the difficulty of maintaining the integrity of the follicle during removal from such small-sized samples. For each species and developmental stage, three maternal follicles were collected from three embryos from the same female (i.e., siblings, or half-siblings in species with multiple paternity). When this was not possible (e.g., limited number of embryos), we collected from other females from the same museum catalogue or lab population.

To examine the follicle thickness, the follicle was sectioned and measured. To ensure that the maternal follicle was flat when sectioned (i.e., did not fold onto itself), we set the follicle in agar solution (3%) prior to the preparation. The samples were dehydrated through an ascending graded series of ethanol (70–100%), and then through several series of ethanol-resin (3:1 to 1:1 to 1:3 to 100% Spurr's resin, modified recipe) for embedding in moulds filled with 100% resin. The samples were dried in an oven (15–20 °C), dyed with toluidine blue and methylene blue, and sectioned with a Leica® EM UC6 (1 μm thick). Images of the maternal follicle tissue sections were captured using a Leica® DMI3000 B light microscope, with a Leica® DFC420 camera, at ×10, ×20, and ×40 magnifications. To quantify follicle thickness, we measured all the layers of the maternal follicle (i.e., epithelium and underlying connective tissues) collectively for each tissue section. Two independent sets of follicle thickness measurements were made (by the same individual, LK) to test for repeatability (i.e., intraclass correlation coefficient; Lessells and Boag, 1987; Sokal and Rohlf, 1995). For each set, 10 measurements (μm) were made where the width of the maternal follicle was greatest and the mean was calculated. ImageJ® software (version 1.44o; Rasband 1997-2011) was used for all measurements.

Following the follicle thickness analysis, a subset of the stained tissue sections was examined to characterize follicle epithelial cell morphology and connective tissue layers for each species and developmental stage (when possible). Tissue sections were viewed and images were captured on the transmitted light channel of an Olympus® FV300 confocal microscope or a Zeiss Axioskop, with a QICLICK-F-M-12 CCD camera (QImaging) equipped with an RGB liquid crystal color filter module, at ×20, ×40, ×60, and ×100 magnifications. Figures were compiled using Adobe Photoshop Elements software (version 4).

Statistical Analysis

Light and scanning electron microscope. The observations from the light microscope and SEM were qualitative in nature, so no statistical analyses were required.

Tissue sectioning. For the maternal follicle thickness, there was a high repeatability of 0.98 between the two sets of measurements on follicle width; we, therefore, used the average of the two sets. Data were log-transformed for normality. An ANOVA was used to determine the effects of the level of postfertilization maternal nutrient provisioning (lecithotroph, moderate matrotroph, and extensive matrotroph), developmental stage (2, 3, and 4), and their interaction on the thickness of the maternal follicle.

We also examined the differences in follicle thickness among the extensive matrotrophs: *P. prolifica, P. turneri*, and *P. retropinna*. Due to the limited number of museum samples for *P. retropinna* (Stage 2 follicles were unavailable for sectioning), we were unable to test the effect of developmental stage. So, here, an ANOVA with only the effect of species on follicle thickness was performed. All analyses were done using the statistical software R (version 2.14.2; R Development Core Team 2012).

For the maternal follicle epithelium and connective tissue morphology, the observations from the confocal and light microscope were qualitative in nature, so no statistical analyses were required.

RESULTS

For simplicity, we only present the results from a subset of the eight *Poeciliopsis* species which are representative of the overall trends and patterns. Results for the remaining species can be found in the Supporting Information (Table S1; Figs. S1–S4).

Light and Scanning Electron Microscope

Light microscopy and SEM observations revealed a thin and transparent maternal follicle in the lecithotrophs (*P. turrubarensis*: Fig. 1A,B; see also Supporting Information Fig. S1) and moderate matrotrophs (*P. occidentalis*: Fig. 1C,D; see also Supporting Information Fig. S1). The inner surface of the maternal follicular epithelium was flat, with a cellular layer covered by a noncellular, porous membrane apparent in most samples (Fig. 2; see also Supporting Information Table S1 and Fig. S2).

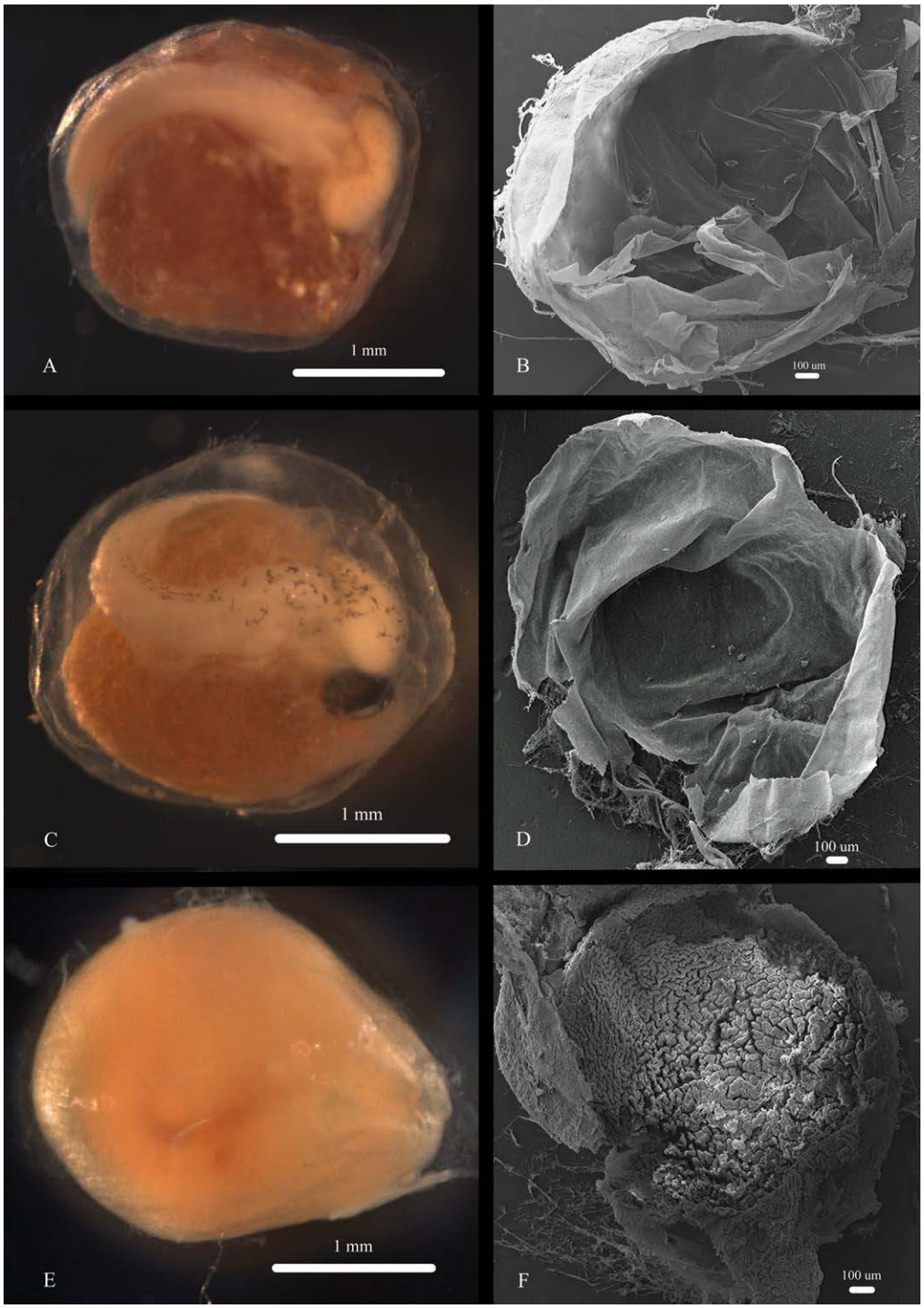

Fig. 1. Light microscope images of the developing embryos within the maternal follicle (left panel) and representative SEM images of the inner surface of the maternal follicular epithelium (right panel) from (**A**,**B**) *P. turrubarensis* (lecithotroph), (**C**,**D**) *P. occidentalis* (moderate matrotroph), and (**E**,**F**) *P. retropinna* (extensive matrotroph) at Stage 3.

In contrast, the extensive matrotrophs had a thick and less transparent follicle (*P. retropinna*: Figs. 1E,F and 3A; see also Supporting Information Fig. S1). The inner surface of the maternal follicular epithelium was highly hypertrophied and extensively folded for both *P. retropinna* (Fig. 3B) and *P. turneri* (Fig. 3D); for *P. prolifica*, the follicle was thinner and the inner surface had a rippled

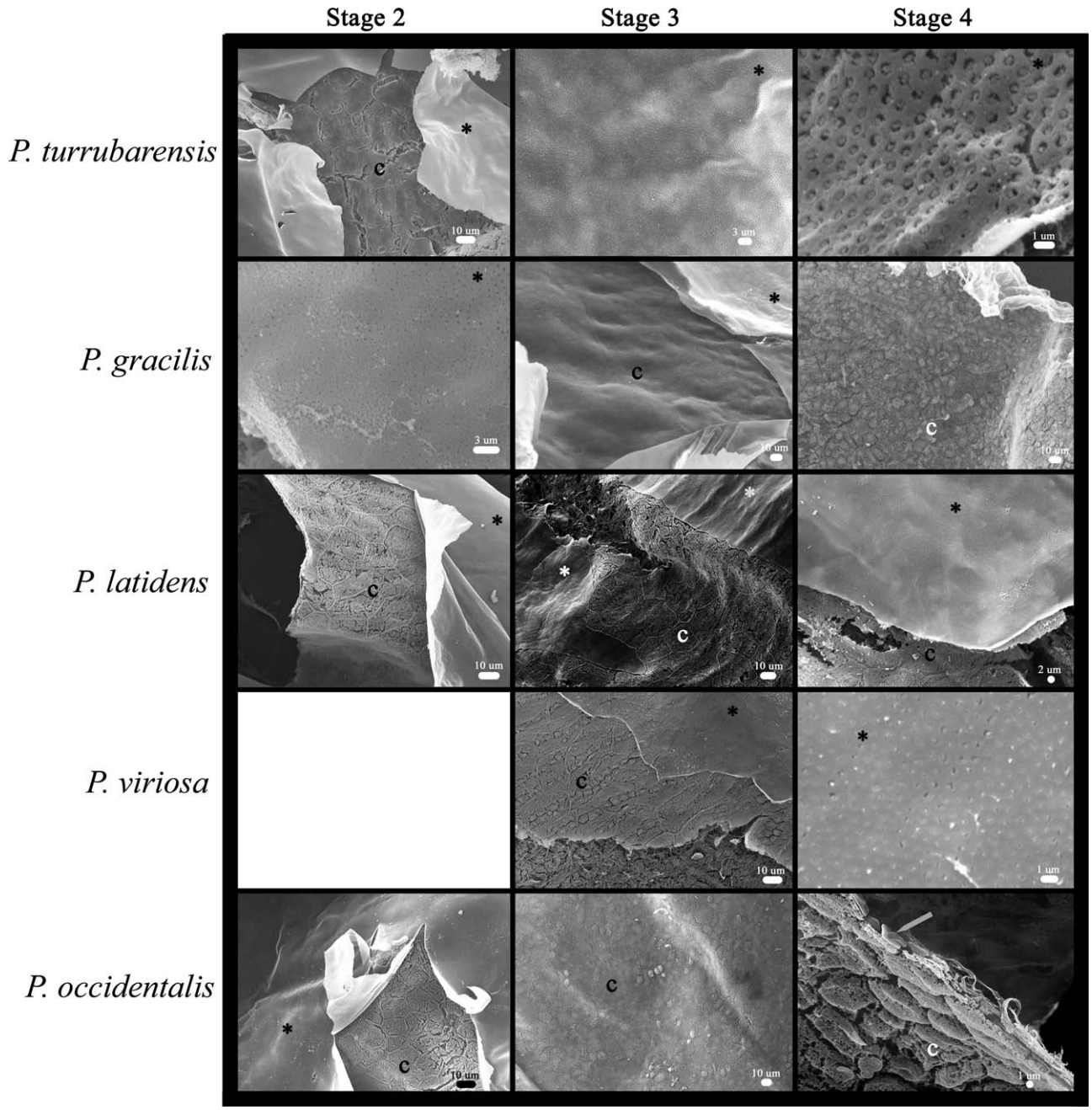

Fig. 2. Representative SEM images of the inner surface of the maternal follicle for the lecithotrophs (*P. turrubarensis*, *P. gracilis*) and moderate matrotrophs (*P. latidens*, *P. viriosa*, *P. occidentalis*) from Stages 2 to 4. A thin, noncellular, porous membrane (denoted by "*"), presumed to be the egg envelope, is present in most samples. The follicular epithelial cells (denoted by "c") are seen deep to the egg envelope. Gray arrows point to the presence of red blood cells, deep to the epithelial layer and close to the outer surface of the follicle, which was apparent in some samples.

appearance instead of extensive folding (Figs. 3E,F). The apical surface of the epithelial cells that line the inner surface was covered with microvilli in both *P. turneri* (Fig. 3C) and *P. prolifica* (Fig. 3E). In *P. retropinna*, however, the museum preservation of the samples prevented us from clearly determining if microvilli were present, but there was an abundance of preserved material on the apical surface, presumably mucus granules (Fig. 3A).

Tissue Sectioning

Maternal follicle thickness. Quantification of the thickness of the maternal follicles supports the light microscopy and SEM observations. There was a significant effect of the level of

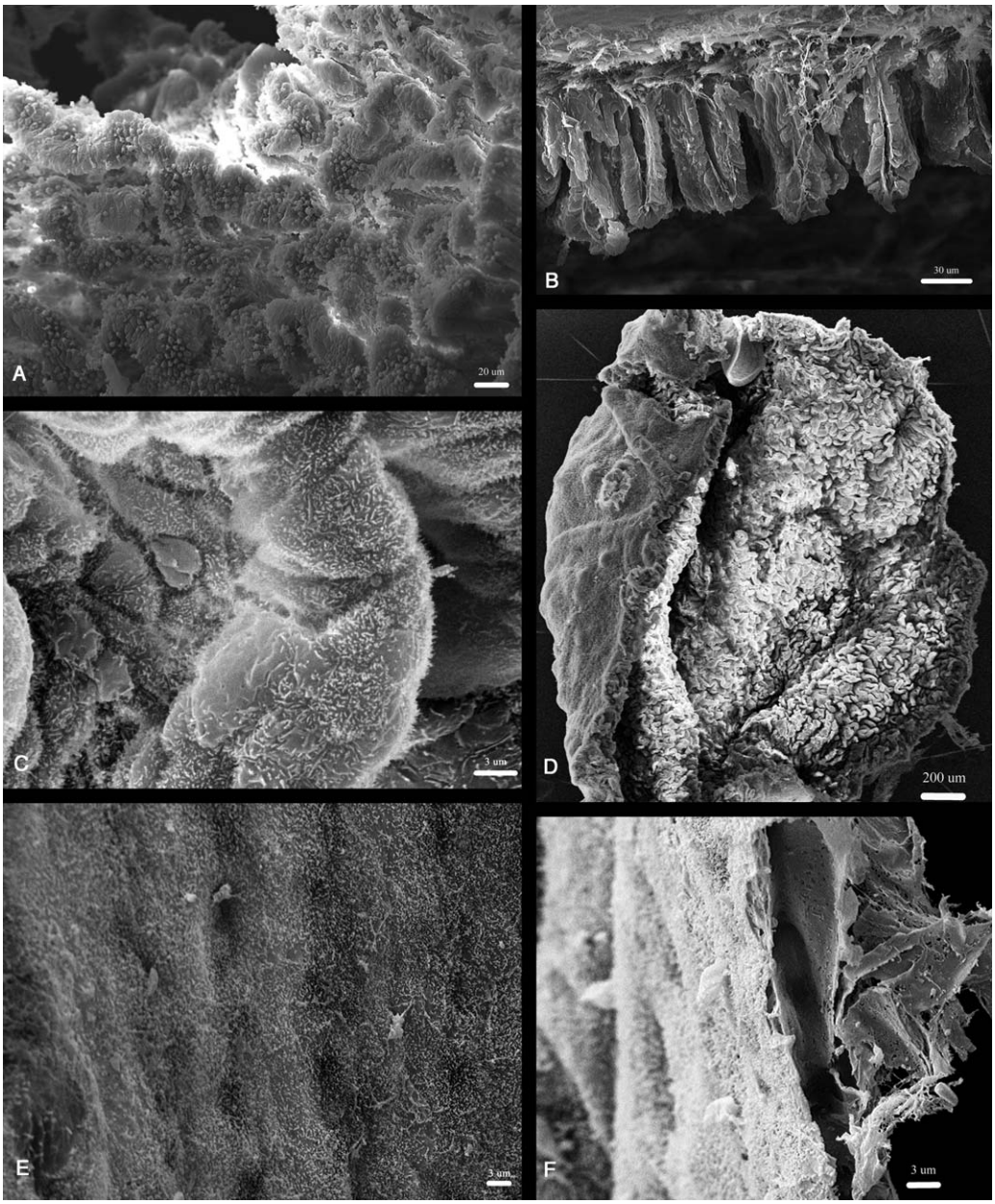

Fig. 3. Representative SEM images of the inner surface of the maternal follicular epithelium from the three extensive matrotrophs: (**A**,**B**) *P. retropinna* (cross section of epithelial folds), (**C**,**D**) *P. turneri* (D is a view of the entire inner surface of the follicular epithelium), and (**E**,**F**) *P. prolifica* (F is a side view) at Stages 3 and 4.

postfertilization maternal nutrient provisioning on the log-transformed thickness of the maternal follicles (ANOVA: $F_{2,53} = 48.67$, $P < 0.001$; Fig. 4; see also Supporting Information Fig. S3). Post hoc Tukey tests revealed a significant difference in two of the three comparisons among the three levels of provisioning, with the extensive matrotrophs having the thickest follicle (Fig. 4): extensive matrotrophs > moderate matrotrophs ($P < 0.001$), extensive matrotrophs > lecithotrophs ($P < 0.001$), and moderate matrotrophs = lecithotrophs ($P = 0.948$). There was neither a significant effect of developmental stage (ANOVA: $F_{2,53} = 0.97$, $P = 0.387$) nor of the interaction term between the level of postfertilization maternal nutrient provisioning and developmental stage (ANOVA: $F_{4,53} = 1.31$, $P = 0.280$) on follicle thickness.

Among the extensive matrotrophs, there was a significant effect of species on the log-transformed thickness of the maternal follicles (ANOVA: $F_{2,19} = 55.60$, $P < 0.001$; Fig. 5; see also Supporting Information Fig. S3). Post hoc Tukey tests

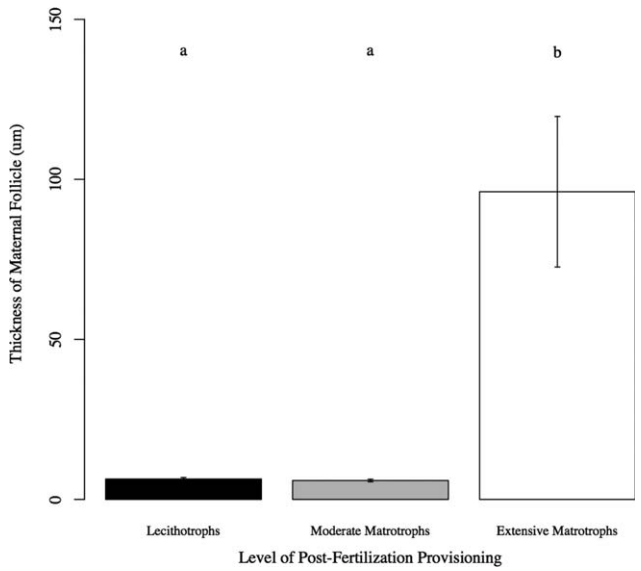

Fig. 4. Mean (±1SE) thickness of the maternal follicle from eight *Poeciliopsis* species, classified by the level of postfertilization maternal nutrient provisioning. Post hoc Tukey tests revealed that follicles of the extensive matrotrophs were significantly thicker than the lecithotrophs and moderate matrotrophs ($P < 0.05$). There, however, was not a statistical difference in the follicle thickness between the lecithotrophs and moderate matrotrophs ($P > 0.05$). Means that are not connected by the same letter (a, c) are significantly different.

revealed significant differences for all comparisons among the three species, with *P. retropinna* having the thickest follicle (Fig. 5): *P. retropinna* > *P. turneri* ($P = 0.001$), *P. retropinna* > *P. prolifica* ($P < 0.001$), and *P. turneri* > *P. prolifica* ($P < 0.001$).

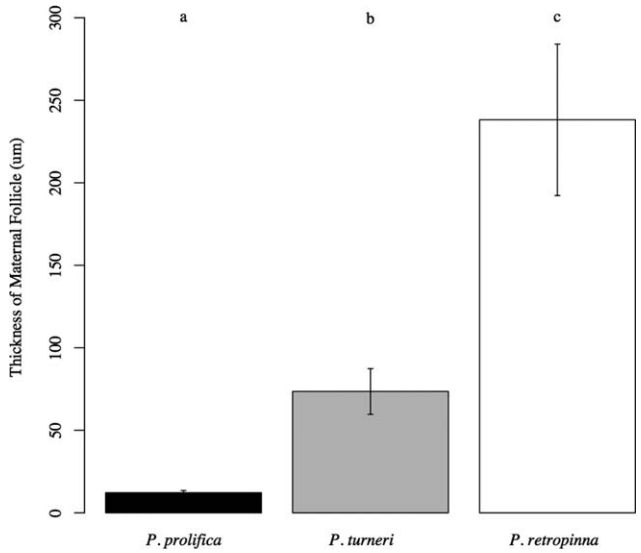

Fig. 5. Mean (±1SE) thickness of the maternal follicle from the extensive matrotrophs. Post hoc Tukey tests revealed a significant difference in all comparisons among the three species ($P < 0.05$). Means that are not connected by the same letter (a, b, and c) are significantly different.

Maternal follicle epithelium and connective tissue morphology. For the lecithotrophs and moderate matrotrophs, the cross sections of the follicle revealed a flat, simple epithelium, which corroborated the SEM observations (Fig. 6; see also Supporting Information Fig. S3). Qualitatively, there appeared to be no substantial differences in the follicle structures among species or developmental stages. However, preservation may have compromised cell integrity and, for some sections, made it difficult to clearly define the tissue layers of the follicle (as has been done in other studies, e.g., Jollie and Jollie, 1964b). The follicles were comprised primarily of epithelial cells that appeared to have either a flat or round nucleus and connective tissue (Fig. 6; see also Supporting Information Fig. S3). In most of the sections examined, the presence of the noncellular membrane seen in SEM (Fig. 2) was apparent as a darkly blue stained, thin ribbon structure apposed to the inner surface of epithelial cells and connective tissue (Fig. 6; see also Supporting Information Table S1). Finally, for both the lecithotrophs and moderate matrotrophs, red blood cells were apparent among the connective tissue in many of the sections, suggesting the presence of capillaries (Fig. 6). The presence of follicular blood vessels has been observed in other lecithotrophic poeciliids (e.g., *Poecilia (Lebistes) reticulata*) and it has been suggested that they facilitate gas exchange (Jollie and Jollie, 1964b). It has also been suggested that lecithotrophs with intrafollicular gestation have a non-nutritive placenta that primarily functions in gas exchange (Blackburn, 2014).

In contrast, for *P. turneri* and *P. retropinna* (the two most extensive matrotrophs), the cross sections of the follicle revealed extensive folds on the inner surface of the maternal follicular epithelium, which were evident from the SEM observations (Fig. 7; see also Supporting Information Figs. S3 and S4). In *P. retropinna*, the folds were well-formed, finger-like projections, and composed primarily of cuboidal and columnar cells (Fig. 7E,F; see also Supporting Information Fig. S4F,G). In *P. turneri*, the folds were relatively less finger-like, more stubby, and composed primarily of cuboidal and columnar cells (Fig. 7C,D; see also Supporting Information Fig. S4C,D). Furthermore, the staining pattern on the apical surface of the epithelial cells suggested the presence of microvilli in *P. turneri*. For both species, there were capillaries present within the folds, evident from stained red blood cells. The follicles of *P. retropinna* and *P. turneri* also have a substantial connective tissue layer below the epithelium. Within this connective tissue layer, there were several, scattered, cellular ring structures that are most likely small blood vessels (Fig. 7C–F; see also Supporting Information Fig. S4D,G). There did not appear to be evidence of well-formed, clustered, glandular

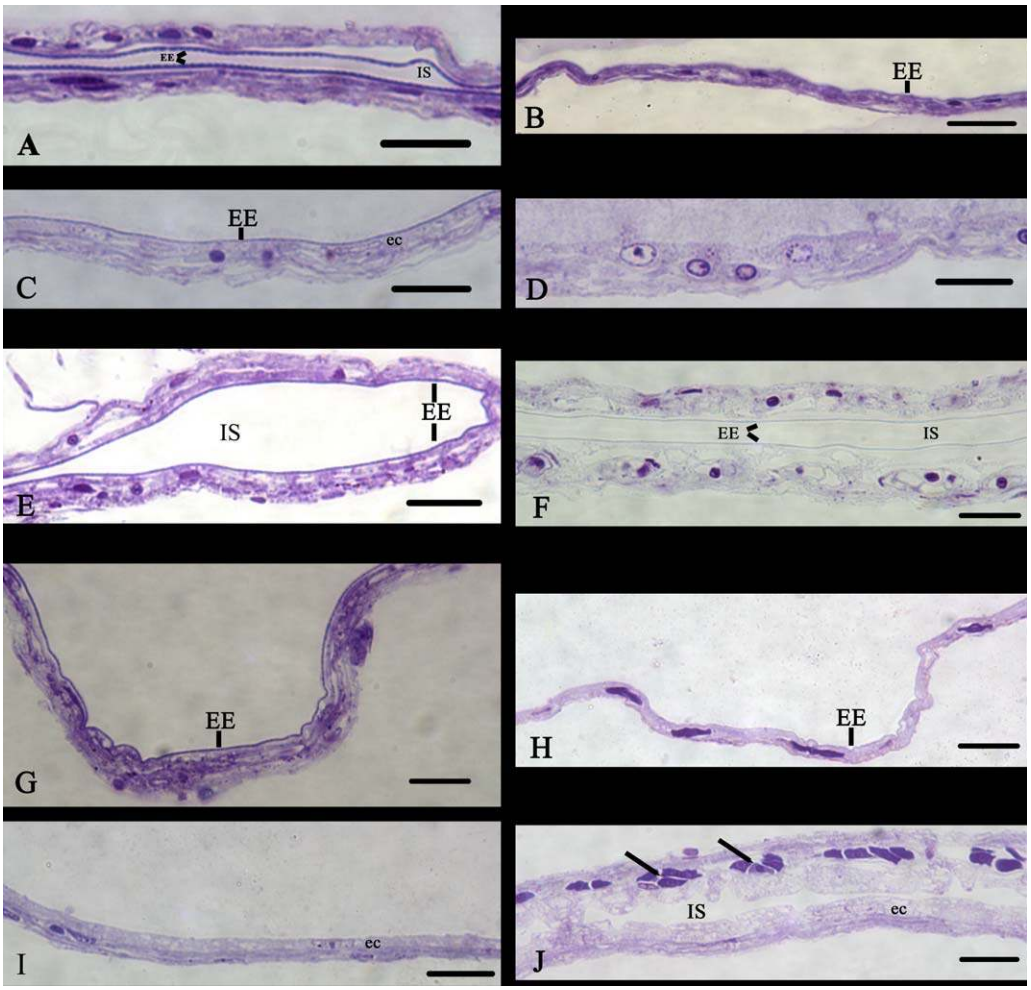

Fig. 6. Tissue cross-sections of the maternal follicle from the lecithotrophs and moderate matrotrophs, stained with toluidine blue and methylene blue: (**A**,**B**) *P. turrubarensis*, Stages 3 and 4, respectively, (**C**,**D**) *P. gracilis*, Stages 3 and 4, respectively, (**E**,**F**) *P. latidens*, Stages 3 and 4, respectively, (**G**,**H**) *P. viriosa*, Stages 3 and 4, respectively, and (**I**,**J**) *P. occidentalis*, Stages 3 and 4, respectively. Presumed epithelial cells are denoted by "ec" and the intrafollicular space, where the embryo would reside, is indicated with "IS." Arrows point to red blood cells. The presumed egg envelope, or fertilization membrane, is denoted by "EE" and a black line. "EE" also indicates the orientation of the follicle, with "EE" representing the inner most surface of the follicle (i.e., the egg envelope sits between the epithelial cell layer of the maternal follicle and the embryo). Scale bar equals 10 μm.

structures within this layer for either species. Qualitatively, the thickness of the connective tissue layer, shape of the folds, and density of the blood vessels varied depending on both the developmental stage and tissue section. Finally, for *P. retropinna* and *P. turneri*, the outer-most, superficial layer of the follicle was primarily a thin, loose layer of cells and fibrous tissue.

Similar to the SEM observations, the tissue sections of *P. prolifica* revealed a relatively flat epithelium, with no folds, that was composed primarily of large cuboidal cells (Fig. 7A,B; see also Supporting Information Fig. S4A,B). Dense microvilli associated with the apical surface of the epithelial cuboidal cells were present (Fig. 7A,B; see also Supporting Information Fig. S4A,B). Among the cuboidal cells, there were blood vessels (occasionally, pushed up close to the surface of the epithelium), evident from stained red blood cells; this is suggestive of a well-vascularized follicle. The connective tissue, deep to the epithelium, was dense, but thinner than that seen in *P. turneri* or *P. retropinna*. Finally, for *P. prolifica*, the outermost, superficial layer of the follicle was comprised of a thin, loose layer of cells and fibrous tissue.

DISCUSSION

Using light microscopy, SEM, and tissue sections, we have undertaken a comparative study of the thickness and tissue structures of the maternal follicle in eight closely related *Poeciliopsis* species that span the range in postfertilization maternal nutrient provisioning of offspring. As the level of maternal nutrient provisioning increased, the maternal follicles tended to be thicker, better

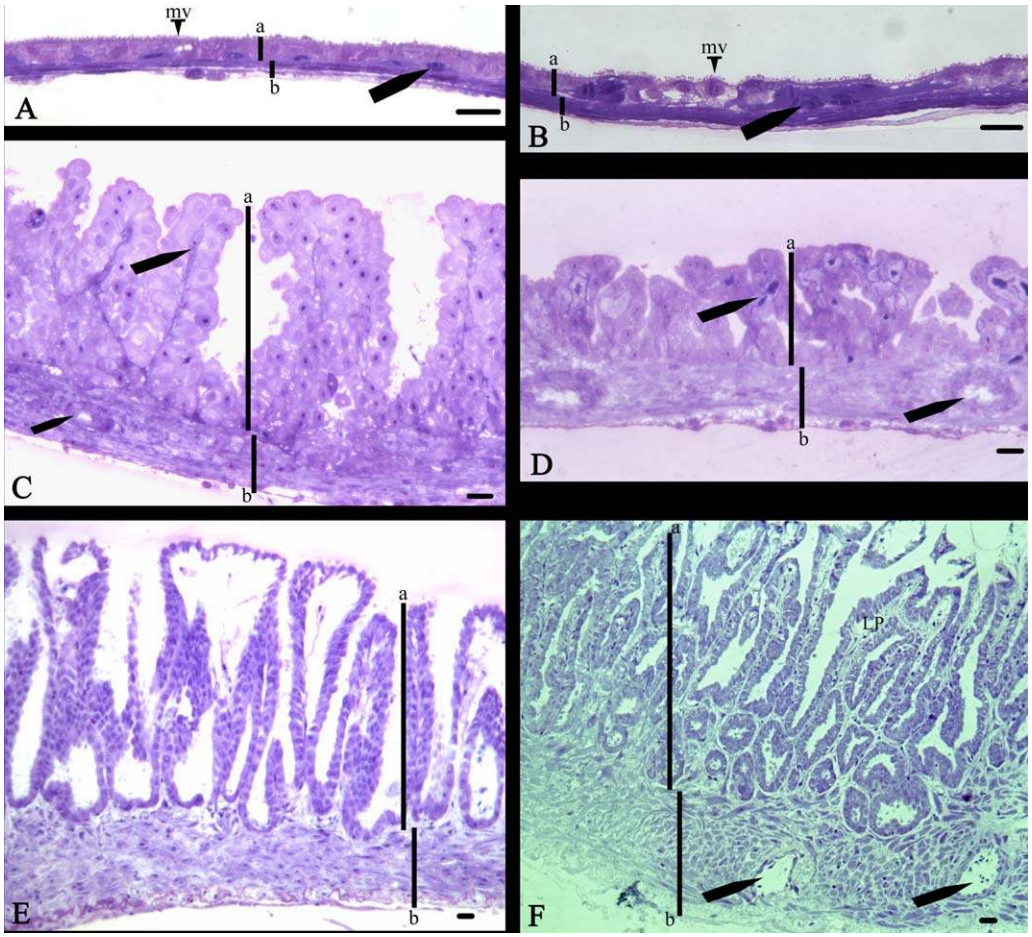

Fig. 7. Tissue cross-sections of the maternal follicle from the three extensive matrotrophs, stained with toluidine blue and methylene blue. (**A**,**B**) *P. prolifica*, Stages 3 and 4, respectively, (**C**,**D**) *P. turneri*, Stages 3 and 4, respectively, and (**E**,**F**) *P. retropinna*, Stages 3 and 4, respectively. Black arrows point to blood vessels, many evident from stained red blood cells. The inner epithelial layer that apposes the embryo is denoted by a black bar and the letter "a," while the connective tissue layers deep to the epithelium are denoted by the black bar "b." Apical cell surface microvilli are denoted by "mv" and an arrowhead. The lamina propria tissue, denoted by "LP," is dispersed within the folds and comprised primarily of capillaries in many cases. Scale bars equal 10 μm.

vascularized, and with more extensive folding of the follicular epithelium. Quantification of the follicle thickness revealed that the extensive matrotrophs have significantly thicker follicles than the lecithotrophs and moderate matrotrophs; however, perhaps surprisingly, there was neither significant effect of developmental stage nor an interaction between the level of postfertilization maternal nutrient provisioning and developmental stage on follicle thickness. Among the extensive matrotrophs, enhancements of the follicle thickness, degree of folding, and associated capillaries of follicular epithelium corresponded with an ascending MI value (provisioning) for *P. prolifica* (MI = 5.4), *P. turneri* (MI = 41.4), and *P. retropinna* (MI = 117). This pattern of increased epithelial folds, with closely associated blood vessels, suggests that these traits are required to support the increased exchange of substances between the mother and developing embryo in species with extensive postfertilization maternal provisioning.

Our work complements previous studies that have provided valuable drawings and descriptions of the maternal follicle of *Poeciliopsis* species and other teleost fishes based on light microscopy and ultrastructure images (i.e., transmission electron microscopy [TEM] and SEM; Turner, 1940; Wourms, 1981; Knight et al., 1985; Wourms et al., 1988; Grove and Wourms, 1991, 1994; Meisner and Burns, 1997; reviewed in Wooding and Burton, 2008, Blackburn, 2014). Here, we discuss our results in light of previous research on teleost fish placentae and placental evolution in this group.

Maternal Follicle of *Poeciliopsis* with No or Moderate Postfertilization Provisioning

Until this study, there were limited ultrastructure microscopy data on the maternal follicles of lecithotrophs (no postfertilization maternal nutrient provisioning) and moderate matrotrophs (moderate postfertilization maternal nutrient

provisioning) in the Poeciliidae. We found that the *Poeciliopsis* lecithotrophs and moderate matrotrophs have relatively thin follicles with epithelial cells that appear to lack apical microvilli. Similarly, in *P. reticulata*, a well-studied lecithotroph (MI = 0.70; see summary of MI values in Schrader and Travis, 2012b), TEM revealed an inner follicular epithelium that is also relatively thin and composed of flat squamous or low cuboidal cells that lack apical surface microvilli (Jollie and Jollie, 1964b; Grove and Wourms, 1994). It is possible, in our study, that the lack of microvilli in the lecithotrophs and moderate matrotrophs is a result of using museum specimens, where the quality and age of the specimen may have compromised the integrity of these fine cellular processes. There was also no significant difference in the follicle thickness between the *Poeciliopsis* lecithotrophs and moderate matrotrophs. Interestingly, from our SEM and tissue sectioning, we were unable to detect any substantial differences in placental traits between these two groups of *Poeciliopsis* species. One explanation may be that the range and difference in MI values between the lecithotrophs (MI = 0.66–0.69) and moderate matrotrophs (MI = 0.86–1.12) is functionally much smaller than for the extensive matrotrophs (MI = 5.4–117). As MI value is an indirect measure of the postfertilization maternal investment in embryonic development (Reznick et al., 2002), it is possible that there really are no substantial morphological differences in the maternal follicle between the lecithotrophs and moderate matrotrophs. Alternatively, morphological differences may occur between these two groups in the cellular ultrastructures of the maternal follicle and/or in the embryonic placental structures (i.e., embryonic pericardial sac). TEM of lecithotrophs and moderate matrotrophs would help to determine the cellular structures and specific follicle layer components [as presented for *P. reticulata* in Jollie and Jollie (1964b)] that were not clearly definable in this study.

Interestingly, our SEM and tissue section analyses revealed that the *Poeciliopsis* lecithotrophs and moderate matrotrophs have a noncellular membrane between the inner follicular epithelium and embryonic space. This noncellular membrane appeared uniformly thin, porous, and grainy in the SEM images and as a darkly blue stained, thin ribbon structure in the tissue sections. Exceptions were found for the follicles of *P. gracilis* from Stage 4 and *P. occidentalis* from Stages 3 and 4. In these samples, the membrane was not apparent in SEM and difficult to clearly detect in the tissue sections. Here, it is unclear if the membrane exists for these species in the later developmental stages and was lost during sample preparation, or disappears between the early and late stage follicles. The follicle of *P. reticulata* is also characterized as having a relatively thin, dense, noncellular membrane between the inner follicular epithelium and the embryo (Jollie and Jollie, 1964b; Grove and Wourms, 1994). Jollie and Jollie (1964a,b) classified this membrane as the "fertilization membrane," which is reported to be equivalent to the egg or vitelline envelope of unfertilized oocytes (Grove and Wourms, 1994). In *P. reticulata*, the fertilization membrane was present throughout development (1–4 weeks postfertilization), but it was absent from regions where the apposition between the embryonic yolk sac and inner maternal follicular epithelium occurred in the later stages (Jollie and Jollie, 1964b).

Maternal Follicle of *Poeciliopsis* with Extensive Postfertilization Provisioning

The maternal follicles of several teleost fishes with extensive matrotrophy (extensive postfertilization maternal nutrient provisioning) have been previously examined using light microscopy, SEM, and TEM (Fraser and Renton, 1940; Turner, 1940; Wourms, 1981; Knight et al., 1985; Grove and Wourms, 1983, 1991, 1994; Meisner and Burns, 1997). Here, our microscopy work revealed extensive folding (villi) of the inner follicular epithelium, which was richly vascularized with capillaries and red blood cells, for two of the three *Poeciliopsis* extensive matrotrophs: *P. turneri* and *P. retropinna*. Follicular epithelial villi are thought to increase surface area for the transport of substances at the maternal-fetal interface (Turner, 1940; Grove and Wourms, 1994), and have been reported for several other *Poeciliopsis* (Poeciliidae; Turner, 1940) and *Anableps* (Anablepidae; Knight et al., 1985) species. Turner (1940) further highlighted the variation in the degree of follicular folding in several *Poeciliopsis* species: *P. elongatus* and *P. retropinna* (referred to as *Aulophallus*) have "finger-like and unbranched" villi, while an unnamed *Poeciliopsis* species had villi that were "low and branched" (note: *P. elongatus* has a MI value of 68.9 and is closely related to *P. retropinna*; Reznick et al., 2002). Similarly, our SEM and tissue section analyses showed extensive "finger-like and unbranched" villi in *P. retropinna*. The well-defined epithelial villi and more hypertrophied follicular epithelium of *P. retropinna* likely explain the threefold difference in follicle thickness between *P. retropinna* and *P. turneri*. Interestingly, the follicle for the remaining extensive matrotroph, *P. prolifica*, was quite different from *P. turneri* and *P. retropinna*. First, the follicle was 6× and 19× thinner than *P. turneri* and *P. retropinna*, respectively. Second, rather than extensive folds or villi, the *P. prolifica* follicular epithelium had only a slight rippled and corduroy-like appearance. Underlying the epithelial cells, nevertheless, there was a rich supply of blood

vessels with erythrocytes. *P. prolifica* has an MI value of 5.4, which is on the lower end of the described extensive matrotrophs and considerably lower than both *P. turneri* (MI = 41.4) and *P. retropinna* (MI = 117; Reznick et al., 2002). Recall that MI value is an indirect measure of the postfertilization maternal investment (Reznick et al., 2002); the interspecific differences in follicular epithelium folding and follicle thickness may reflect the heterogeneity in embryonic, maternal nutrient provisioning among the three *Poeciliopsis* species. Future research should determine if other poeciliids that provide extensive postfertilization maternal nutrient provisioning display the same or similar interspecific variation observed here in *Poeciliopsis*.

For two of the three extensive matrotrophs, *P. turneri* and *P. prolifica*, our SEM analysis revealed an extensive number of microvilli on the apical surface of follicular epithelial cells in three developmental stages (2–4). We were unable to determine if such cell structures are present in the moderate matrotrophs (*P. latidens*, *P. viriosa, P. occidentalis*) or *P. retropinna* because of the preservation quality of the museum specimens. In another analysis of a well-studied, extensive matrotroph, *Heterandria formosa* (MI = 41.9–66.4; see summary of MI values in Schrader and Travis, 2012b), TEM observations revealed specialized follicular epithelial cells involved in molecular transport at the maternal-fetal interface in mid-stage embryos (corresponds to Stage 3 here; Grove and Wourms, 1994). The follicular epithelial cells are described as cuboidal, highly microvilliated, and contain coated endocytotic pits, which may facilitate both follicular fluid secretions and absorption at the interface (Grove and Wourms, 1994). The basal surface of the *H. formosa* follicle cells is folded and increases surface area for transport of molecules into the cell from the closely apposed maternal blood vessels (Grove and Wourms, 1994). Consequently, the apical cell microvilli found in *P. turneri* and *P. prolifica* may be important traits associated with extensive matrotrophy.

Finally, unlike the lecithotrophs and moderate matrotrophs, an egg envelope was not apparent in any of the three extensive matrotrophs. Interestingly, the follicle of *H. formosa* is characterized as having a noncellular egg envelope (vitelline or fertilization membrane) present at all developmental stages (Grove and Wourms, 1994). This egg envelope sits between the embryo and the maternal follicular epithelium and changes from a dense membrane early in development to a thin, porous membrane in mid-stage embryos (corresponds to Stage 3 here; Grove and Wourms, 1994). A couple of explanations are possible for the lack of an egg envelope in the *Poeciliopsis* extensive matrotrophs that have been examined in this study. First, the membrane may be present, but becomes exceedingly thin (as it does in *H. formosa;* Grove and Wourms, 1994) and, thus, is easily lost during preparation for SEM and tissue sectioning. Alternatively, these extensive matrotrophs may lack an egg envelope after fertilization.

Placental Evolution

Our results show that, at higher MI values, the maternal follicle becomes thicker, vascularized, and its epithelial layer is arranged into folds (villi) or ripples and, in some species, the apical surface of the follicular epithelial cells is covered with microvilli (most apparent in the extensive matrotrophs). These traits can all serve to increase the surface area and, thus, facilitate the absorption and/or secretion of molecules (e.g., nutrients) at the maternal-fetal interface (Turner, 1940; Knight et al., 1985; Grove and Wourms, 1994; Meisner and Burns, 1997; Reznick et al., 2002; Pires et al., 2007, 2011). As postfertilization maternal nutrient provisioning increases, the structural changes of the maternal follicle suggest that there is a high degree of interaction between the mother and developing embryo. Such interactions may account for the associated high level of provisioning, but they also raise the spectre of parent-offspring conflict over provisioning (Zeh and Zeh, 2000; Crespi and Semeniuk, 2004). However, morphological studies, like this one, are not direct tests of the alternative hypotheses and can only provide insight into the potential interactions between the mother and embryo. As a next step in understanding the evolutionary processes driving placental evolution in poeciliids, in our future research, we will use a similar, microscopy approach to focus on the embryonic structures (i.e., embryonic pericardial sac) for the same eight *Poeciliopsis* species. Our results can then be used to determine whether there is an association between maternal-fetal morphology. Future work should look for associations between these morphologies and the ecological conditions and/or mating systems that are thought to drive the evolution of matrotrophy and placentation (Zeh and Zeh, 2000; Crespi and Semeniuk, 2004; Pollux et al., 2009, 2014).

CONCLUSION

Using a comparative approach and multiple techniques, we have performed a comprehensive comparison of the fish placentae of eight closely related *Poeciliopsis* species. We found substantial variation in several maternal follicle traits among lecithotrophs, moderate matrotrophs, and extensive matrotrophs. However, given the limited number of species and the structural integrity of the samples, it is difficult to infer past evolutionary events and phylogenetic relationships in the

evolution of placenta in *Poeciliopsis*. Future research will focus on creating a larger and more extensive database for both the maternal follicle and embryonic pericardial sac. More broadly, as noted by Losos (2011), we should work toward integrating morphological studies, like this one, with phylogenies and direct studies of the exchange of substances (e.g., gases, nutrients, wastes) to fully understand the processes driving the evolution of the *Poeciliopsis* placenta.

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