UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS

BIOLOGÍA EVOLUTIVA

LA RELACIÓN ENTRE SUPERFETACIÓN, PLACENTACIÓN, RETENCIÓN DE ESPERMA Y TAMAÑO DEL EMBRIÓN EN PECES VIVÍPAROS DE LA FAMILIA POECILIIDAE

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

CLAUDIA OLIVERA TLAHUEL

TUTOR PRINCIPAL DE TESIS: DR. JOSÉ JAIME ZÚÑIGA VEGA
Facultad de Ciencias

COMITÉ TUTOR: DRA. MARICELA VILLAGRÁN SANTA CRUZ
Facultad de Ciencias

DRA. NORMA ANGÉLICA MORENO MENDOZA
Instituto de Investigaciones Biomédicas

Ciudad Universitaria, Cd. Mx., junio, 2017

UNAM
POSGRADO
Ciencias Biológicas

COORDINACIÓN

POSGRADO EN CIENCIAS BIOLÓGICAS
FACULTAD DE CIENCIAS
DIVISIÓN DE ESTUDIOS DE POSGRADO

OFICIO FCIE/DEP/376/2017

ASUNTO: Oficio de Jurado

Lic. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **13 de febrero de 2017**, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** del (la) alumno (a) **OLIVERA TLAHUEL CLAUDIA** con número de cuenta **302068470** con la tesis titulada: "**La relación entre superfetación, placentación, retención de esperma y tamaño del embrión en peces vivíparos de la familia Poeciliidae**", realizada bajo la dirección del (la) **DR. JOSÉ JAIME ZÚÑIGA VEGA:**

Presidente:	DRA. PATRICIA RIVAS MANZANO
Vocal:	DR. ABRAHAM KOBELKOWSKY DÍAZ
Secretario:	DRA. MARICELA VILLAGRÁN SANTA CRUZ
Suplente:	DRA. CLAUDIA PATRICIA ORNELAS GARCÍA
Suplente:	DR. JOSÉ LUIS GÓMEZ MÁRQUEZ

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPIRITU"
Ciudad Universitaria, Cd. Mx., a 31 de mayo de 2017

DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA
COORDINADORA DEL PROGRAMA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

MCAA/MJFM/ASR/grf*

AGRADECIMIENTOS

- Al posgrado en Ciencias Biológicas de UNAM
- A CONACYT por proporcionarme la beca (368782/245650) para el desarrollo de la presente investigación.
- Al proyecto SEP-CONACYT 129675, “El significado adaptativo de la superfetación en peces vivíparos la familia Poeciliidae”, por proporcionar fondos para la presente tesis.
- Al apoyo PAEP por financiar parte de los materiales utilizados en esta tesis.
- Al Dr. José Jaime Zúñiga Vega por enseñarme cada día y formarme como investigadora. Gracias por todo.
- A la Dra. Maricela Villagrán Santa Cruz por abrirme las puertas a su laboratorio y apoyarme siempre. Gracias por sus valiosos comentarios a lo largo de la presente investigación.
- A la Dra. Norma Angélica Moreno Mendoza por permitirme unirme a su grupo de trabajo. Gracias por sus valiosos comentarios a lo largo de la presente investigación.
- A los miembros del jurado por sus comentarios y sus diferentes puntos de vista.
- A la M. en C. Eva Mendoza Cruz por su asistencia y apoyo incondicional en técnicas de histología.
- A Pedro Medina Granados por su asistencia y siempre apoyo en técnicas de microscopía electrónica.
- Al Dr. Fernando García Hernández por su asistencia y confianza en el manejo del microscopio electrónico.
- A los miembros del laboratorio de Especializado de ecología por su apoyo académico. En especial, a Patricia de Lourdes Frías Álvarez, Luis Felipe Vázquez Vega, Paulina Quetzalli García, Diana Karina Villa Meza, Israel Solano-Zavaleta, Pedro Eloy Mendoza Hernández y Mariana Hernández Apolinar. Gracias por su asistencia en trabajo de campo.
- A los alumnos del grupo de trabajo del Instituto de Investigaciones Biomédicas. En especial, a Alma Lilia, Rocío, Tanya y Adriana. Gracias por sus enseñanzas en técnicas de microscopia electrónica e inmuno.
- A José Luis Bortolini Rosales, Beatriz Zúñiga Ruíz, María Eugenia Muñiz Díaz de León, Ignacio Andrés Morales Salas y Estela Pérez Cruz. Gracias por su apoyo técnico para la estancia y cuidados de los poecílidos.
- A los alumnos del grupo de trabajo de Biología Tisular: Eva y Gladys. Gracias por su apoyo en técnicas de histología y todos sus consejos a lo largo del proyecto.

A Dario, por ser mi estrella favorita y mi guía en cada momento.

A Luz, por siempre apoyarme y ser mi mejor amiga.

A Rubén, por todos tus consejos y ánimos.

A Manuel, por ser mi Shuhari.

ÍNDICE

RESUMEN

La superfetación es la capacidad de la hembra para llevar dentro de su ovario simultáneamente múltiples camadas de embriones en diferentes estadios de desarrollo. Este modo reproductor predomina en algunos géneros de peces vivíparos de la familia Poeciliidae (*Poeciliopsis*, *Heterandria* y *Neoheterandria*). La matrotrofía es un modo de aprovisionamiento materno en la cual la energía destinada al desarrollo del embrión es provista por la madre después de la fertilización y durante todo el desarrollo embrionario. En peces poecílidos, entre mayor es el nivel matrotrófico la superfetación aumenta. Tanto para la superfetación como para la matrotrofía existen hipótesis al respecto de su origen evolutivo. Sin embargo, son escasos los estudios que se enfocan en demostrar las consecuencias evolutivas (cambios o efectos en sus atributos de historias de vida) de la superfetación y la matrotrofía. Por lo que en la presente tesis probé diferentes hipótesis de las consecuencias evolutivas de la superfetación y la matrotrofía sobre características de historia de vida (tamaño de la cría) y reproductoras (nivel de placentación y retención de esperma) en algunas especies de la familia Poeciliidae.

En el primer capítulo, analicé la relación entre la matrotrofía y la superfetación con diferentes ultraestructuras placentarias. Esperaba que debido a que las hembras con superfetación requieren una regulación diferencial de nutrientes de embriones en distintos estadios de desarrollo, estas especies que superfetan tendrían una placenta ultraestructuralmente más compleja comparada con la placenta de especies que no superfetan. Esta hipótesis se cumplió parcialmente, ya que observé sólo una tendencia de algunas características morfológicas a ser más complejas (mayor número de microvellosidades en la célula folicular y mayor área del folículo materno) en las especies que superfetan en comparación con las especies que no superfetan. Con respecto a la matrotrofía, esperaba que debido a que ésta implica la transferencia activa de nutrientes por parte de la madre hacia sus embriones a lo largo de todo el desarrollo, las hembras de especies con altos niveles de matrotrofía tendrían una placenta más compleja (mayor valor en las diferentes ultraestructuras de la placenta) en comparación con las hembras de especies con bajos niveles de matrotrofía. Esta hipótesis se cumplió, porque observé que especies con altos niveles de matrotrofía tienen placentas más complejas (altos valores de las características ultraestructurales). Por lo que el área del folículo materno, el número de microvellosidades, el número de vesículas en la célula folicular y el tamaño de estas vesículas tienden a aumentar conforme aumenta el índice matrotrófico. Estas características ultraestructurales de la placenta posiblemente favorecen la transferencia activa de nutrientes entre la madre y el embrión.

En el segundo capítulo examiné la relación que existe entre las especies que superfetan y las que no superfetan con la capacidad de almacenar esperma. La hipótesis se debe a que las hembras de las especies que superfetan tienen que fertilizar constantemente a los ovocitos, se esperaban más y más grandes espermatecas en especies que superfetan en comparación con especies que no superfetan. Encontré espermatecas en ovarios de nueve de las doce especies que examiné (*Gambusia panuco, Xiphophorus hellerii, Poecilia mexicana, Poeciliopsis prolifica, P. infans, P.*

viriosa, P. presidionis, P. gracilis y Heterandria formosa). Las espermatecas están compuestas de epitelio ovárico y en general de forma ovalada o circular que rodean completamente a los espermatozoides para protegerlos. Las cabezas de los espermatozoides se encontraron en contacto con el epitelio ovárico y las colas hacia la luz de la espermateca. Además, encontré que existe una relación entre la presencia de superfetación con el número y tamaño de las espermatecas. Las especies que presentan superfetación tienen mayor número y más grandes espermatecas en comparación con las especies que no superfetan. Por lo tanto, las especies superfetadoras destinan una mayor proporción del volumen total del ovario a la producción de espermatecas en comparación con las especies sin superfetación.

En el tercer capítulo investigué la relación que existe entre la combinación de matrotrofía-superfetación con el tamaño de la cría. La hipótesis es que la evolución conjunta de la superfetación y la matrotrofía en la familia Poeciliidae ha facilitado la evolución de crías más grandes. Las hembras superfetadoras producen varias camadas pequeñas y más frecuentemente en diferentes estadios de desarrollo, en consecuencia con distinto tamaño, por lo que deberían liberar un poco de espacio dentro de su ovario y producir crías más grandes. Por otro lado, la matrotrofía implica que los embriones son pequeños en los primeros estadios de desarrollo y más grandes en los últimos estadios del desarrollo. Por lo que, una combinación de la presencia de superfetación y alta matrotrofía podría promover el incremento del tamaño de las crías al nacer. Sin embargo, los resultados de métodos comparados filogenéticos mostraron que altos niveles superfetación y matrotrofía no tienen una relación con el tamaño de las crías al nacer. Adicionalmente, los resultados de métodos comparados filogenéticos permitieron evaluar el nivel de heredabilidad filogenética del tamaño de la cría al nacer, mostrando que el tamaño de las crías en todas las especies que se analizaron tiene baja heredabilidad filogenética. Esto indica que la evolución del tamaño al nacer en poecílidos se debe a procesos de adaptación local.

ABSTRACT

Superfetation is the ability of females to simultaneously carry multiple broods of embryos at different developmental stages. This mode of reproduction predominates in some genera of viviparous fishes of the family Poeciliidae (*Poeciliopsis*, *Heterandria* and *Neoheterandria*). Matrotrophy is a mode of maternal provisioning in which females provide the nutrients to developing embryos after fertilization and during the embryo development. In poeciliid fishes, superfetation increases when the matrotrophy index is high. There are hypothesis with respect to the evolutionary origin of superfetation and matrotrophy. However, there are a few studies that considerer to prove evolutionary consequences (changes or effects on life history traits) of superfetation and matrotrophy. Hence, in this thesis I proved different hypothesis of evolutionary consequences of superfetation and matrotrophy on life history traits (offspring size) and reproductive characteristics (level of placentation and sperm storage) in some species of the family Poeciliidae.

In chapter one, I analyzed the relationship between matrotrophy and superfetation with different placental ultrastructures. I expected that because females with superfetation require differential regulation of embryo nutrients in distinct developmental stages, these superfetating species would have an ultrastructurally more complex placenta compared with placentas of non-superfetating species. This hypothesis was partially fulfilled, since I only observed a trend of some morphological characteristics to be more complex (greater microvilli number of the follicular cell and greater area of maternal follicle) in superfetating species compared with non-superfetating species. With respect to matrotrophy, I expected that because matrotrophy involves active transfer of nutrients from the mother to their embryos during the whole development, species with high matrotropy would require a more specialized placenta (greater value in the different ultrastructures of the follicular placenta) compared with low matrotrophy. This hypothesis was fulfilled, because I observed that species with high degrees of matrotrophy have more complex placentas (high values of the ultrastructural characteristics). Therefore, area of maternal follicle, number of microvilli, number of vesicles, and size of these vesicles tend to increase with the matrotrophy index. These ultrastructural characteristics of the placenta possibly favor the active transfer of nutrients from the mother to the embryo.

In chapter two, I examined the relationship between superfetating species and non-superfetating species with the ability of sperm storage. The hypothesis is due to the fact females with superfetation having to fertilize their oocytes constantly, more and larger spermathecae were expected in species with superfetation compared to non-superfetating species. I found spermathecae in the ovaries of nine of the twelve species that I examined (*Gambusia panuco, Xiphophorus hellerii, Poecilia mexicana, Poeciliopsis prolifica, P. infans, P. viriosa, P. presidionis, P. gracilis* and *Heterandria formosa*). The spermathecae are composed of ovarian tissue and are in generally oval or circular structures that completely surround spermatozoa to protect them. The spermatozoa heads were found in contact with ovarian tissue and the tails towards the lumen of the spermatheca. Also, I found that there is a relationship between presence of superfetation with

number and size of the spermathecae. Superfetating species have a greater number and size spermathecae compared with non-superfetating species. Thus, species with superfetation allocate a greater proportion of the total volume of the ovary to spermathecae production compared with non-superfetating species.

In chapter three, I explored the relationship that exists between the combination of matrotrophy and superfetation with offspring size. The hypothesis is that the combined evolution of superfetation and matrotrophy in the family Poeciliidae has facilitated the evolution of larger offspring. Females with superfetation produce smaller broods and do so more often in different development stages, consequently with different size, so females with superfetation should release some space inside their ovary and produce larger offspring. On the other hand, matrotrophy involves that embryos are small in early development stages and large in late development stage. Therefore, a combination of superfetation and high degree of matrotrophy could promote the increase in offspring size. However, the results of phylogenetic comparative methods showed that high degree of superfetation-matrotrophy is not related with offspring size. Additionally, the results of phylogenetic comparative methods allowed to evaluate the level of phylogenetic heritability of the offspring size, showing that offspring sizes in all analyzed species have low phylogenetic heritability. That indicates that offspring size evolution in poeciliids is due to local adaption processes.

INTRODUCCIÓN GENERAL

Superfetación

La superfetación es la capacidad de una hembra para llevar dentro de su ovario y de forma simultánea múltiples camadas de embriones en diferentes estadios de desarrollo (Turner, 1937, 1940; Scrimshaw, 1994). En mamíferos, la superfetación ocurre en pocos grupos como mustélidos y lepóridos (e.g. *Lepus europaeus, Meles meles* y *Neovison vison*; Yamaguchi *et al*., 2004, 2006; Roellig *et al*., 2011; Corner *et al*., 2015). Sin embargo, existen casos esporádicos en humanos, aunque esto sólo sucede como consecuencia de tratamientos de fertilidad (Pape *et al*., 2008). Estos dos fetos simultáneos mantuvieron una diferencia de 4 semanas en su desarrollo (Tuppen *et al*., 1999). Por otro lado, los moluscos como los bivalvos de la familia Sphaeriidae exhiben incubación secuencial (*secuencial brooding*) que es equivalente a la superfetación. Lo anterior se debe a que estos organismos presentan subconjuntos discretos de desarrollo en la camada (*brood masses*), con cada subconjunto encapsulado en su propio saco (Cooley y Foighil, 2000).

La superfetación es el modo reproducción que predomina en peces vivíparos (Turner, 1937, 1940; Scrimshaw, 1944; Thibault y Schultz, 1978; Zúñiga-Vega *et al*., 2010) y ha evolucionado de manera independiente en tres familias (i.e. Clinidae, Poeciliidae y Zenarchopteridae; Reznick y Miles, 1989; Gunn y Thresher, 1991; Reznick *et al*., 2007). Varios géneros de peces vivíparos de la familia Poeciliidae exhiben superfetación como *Poeciliopsis*, *Heterandria* y *Neoheterandria*, mientras que otros géneros no la presentan (e.g. *Alfaro*, *Brachyrhaphis* y *Xiphophorus*; Reznick y Miles, 1989; Zúñiga-Vega *et al*., 2010).

De la misma manera, en la familia Zenarchopteridae algunos géneros como *Hemirhamphodon, Nomorhamphus* y *Dermogenys* poseen superfetación, mientras que otros géneros no (e.g. *Tondanichthys* y *Zenarchopterus*; Reznick *et al*., 2007). Por lo que la distribución discontinua de la superfetación en las historias filogenéticas de las familias superfetadoras, sugiere que esta estrategia reproductiva ha evolucionado de forma independiente en diferentes linajes (Zúñiga-Vega *et al*., 2010). Siendo posible medir el grado de superfetación en una hembra, definido como el número de camadas simultáneas dentro del ovario de la hembra, y el cual puede variar dependiendo de la especie. Por ejemplo, especies como *Heterandria formosa, Poeciliopsis prolifica* y *Poeciliopsis turneri* pueden tener un alto grado de superfetación, siendo el extremo de la misma las hembras de *Heterandria formosa*, las cuales pueden tener hasta ocho camadas de diferente estadio de desarrollo dentro de su ovario (Travis *et al*., 1987). En contraste, *Poeciliopsis baenschi*, *P. viriosa y P. latidens* presentan un menor grado de superfetación, donde, hembras de *P. viriosa* pueden llevar solamente 2 camadas simultáneas de diferente estadio de desarrollo dentro de sus ovarios (Olivera-Tlahuel *et al*., 2015). Esta variación en el grado de superfetación se puede presentar entre poblaciones de una misma especie (*Heterandria formosa;* Travis *et al*., 1987; *Poeciliopsis prolifica,* Pires *et al*., 2007; *P. turrubarensis,* Zúñiga-Vega *et al*., 2007). Por ejemplo, en *Poeciliopsis turrubarensis,* las hembras de diferentes poblaciones pueden variar en su grado de superfetación, desde 2 a 4 camadas de diferente estadio de desarrollo dependiendo de las condiciones del ambiente (i.e. ambientes costeros o dulceacuícolas; Zúñiga-Vega *et al*., 2007).

Superfetación y placentación

Al igual que la superfetación, la viviparidad ha evolucionado en múltiples ocasiones en los vertebrados (Blackburn, 1992). Durante la evolución de la oviparidad a la viviparidad, el aparato reproductor de la hembra desarrolló estructuras más complejas para la trasferencia de nutrientes hacia el embrión de manera eficiente. Por lo que distintos tipos de placentas han evolucionado en distintas ocasiones a través del tiempo (Pollux *et al*., 2009; Pollux *et al*., 2014). En mamíferos, la placenta y sus características han sido ampliamente estudiadas (Mossman, 1937; Perry, 1981; Moll, 1985; Blackburn, 1999; Swain y Jones, 2000; Vogel, 2005; Carter y Mess, 2007). Sin embargo, en peces son relativamente pocos los estudios sobre la evolución de la placenta y sus características (e.g. los géneros *Heterandria* y *Poeciliopsis*; Rosen y Bailey 1963; Hrbek *et al*., 2007; Pollux, *et al*., 2009; Pollux *et al*., 2014). Por ejemplo, en *Poeciliopsis gracilis* se ha mostrado que el incremento en la actividad del epitelio folicular que rodea al embrión y su alta vascularización facilita el desarrollo embrionario (Wourms, 1981; Wourms *et al*., 1988; Constanz, 1989; Uribe *et al*., 2004). Similarmente, en hembras de *Heterandria formosa* y de algunas especies de *Poeciliopsis* se observó la presencia de estructuras con función de absorción e intercambio de nutrientes en el epitelio folicular (i.e. microvellosidades, sistemas complejos de vesículas, complejos aparatos de Golgi con 4 o 5 cisternas y un extenso retículo endoplásmico; Turner 1940; Grove y Wourms, 1991; Pollux *et al*., 2009).

En esta investigación, propongo la hipótesis de que las hembras de especies con superfetación desarrollaron características placentarias más complejas (valores elevados de las características ultraestructurales) a fin de mantener camadas simultáneas de diferentes

estadios de desarrollo dentro de su ovario. Hasta ahora, no se ha estudiado la posible relación entre las características placentarias y la presencia o ausencia de superfetación en peces vivíparos. La primera parte de esta tesis se enfocará en examinar morfológicamente las placentas de distintas especies de la familia Poeciliidae y la posible relación entre la complejidad placentaria y la presencia de matrotrofía y superfetación.

Superfetación y matrotrofía

Por otro lado, se ha demostrado que existe una relación positiva entre la superfetación y la matrotrofía. En peces poecílidos, entre mayor es el nivel matrotrófico la superfetación aumenta (Meredith *et al*., 2011; Pollux *et al*., 2014). La matrotrofía es un modo reproductor en la cual la energía destinada al desarrollo del embrión es provista por la madre después de la fertilización y durante todo el desarrollo embrionario (Wourms, 1981; Marsh-Matthews, 2011). Presentando, los embriones de especies matrotróficas escaso vitelo al momento de ser fertilizados (i.e. reserva de sustancias nutritivas). Por lo tanto, las especies matrotróficas se caracterizan por un aumento elevado del peso del embrión durante el desarrollo debido a que las madres les están trasfiriendo nutrientes de manera activa (e.g. hembras de *Poecilia branneri* y *P. bifurca*; Pires *et al*., 2010). En contraste a la matrotrofía, la lecitrotrofía es un modo reproductor en el cual la energía destinada al desarrollo del embrión es provista antes de la fecundación por medio del vitelo (Wourms, 1981; Marsh-Matthews, 2011). Por lo tanto, los embriones pierden peso durante su desarrollo embrionario debido a costos metabólicos (de 30 a 35 % de su peso; Wourms,

1981; Pires *et al*., 2010). En la familia Poeciliidae, existe mucha variación entre estas dos estrategias reproductoras, desde especies completamente lecitotróficas hasta especies con matrotrofía extensa (Pollux *et al*., 2014).

Al igual que lo que se espera con respecto a la superfetación, un organismo altamente matrotrófico debería tener una placenta mucho más desarrollada en comparación con especies con menor intensidad matrotrófica (Kwan *et al*., 2015). Esta hipótesis se basa en el hecho de que en las especies altamente matrotróficas la madre proporciona todos los nutrientes necesarios para el crecimiento del embrión de manera activa a lo largo de todo el desarrollo (Mossman, 1937, 1987). Entonces, una placenta con ultraestructuras más complejas podría facilitar el intercambio de nutrientes entre la madre y el embrión. Recientemente, Kwan y colaboradores (2015) demostraron que especies de poecílidos con diferentes niveles de matrotrofía variaban en el grosor de la placenta folicular, el plegamiento del epitelio folicular y su cercana asociación con los capilares sanguíneos sugiere que estas características son requeridas para soportar el incremento del intercambio de sustancias de la madre hacia el embrión. En especies que son altamente matrotróficas el grosor de sus placentas foliculares es mayor en comparación con especies con menor nivel de matrotrofía. Sin embargo, no existen más estudios sobre la relación entre la matrotrofía y las características morfológicas y fisiológicas de las placentas. La primera parte de esta tesis se enfocará en examinar morfológicamente las placentas de distintas especies de la familia Poeciliidae y la posible relación entre la complejidad ultraestructural placentaria con la presencia de matrotrofía y superfetación.

Superfetación y retención de esperma

La retención de esperma es la capacidad de las hembras para almacenar esperma viable dentro de su aparato reproductor por largos periodos (Orr y Zuk, 2012). Este interesante fenómeno se presenta en algunos grupos de insectos (Simmons, 2001), reptiles (Birkhead y Møller, 1993), anfibios (Seber, 2002), peces óseos (Potter y Kramer, 2000), peces cartilaginosos (Pratt, 1993) y mamíferos (Birkhead y Møller, 1993). El tiempo de retención del esperma puede variar entre estos grupos, desde sólo algunas horas hasta años después de que la hembra se aparea. Por ejemplo, los insectos (abejas y termitas reinas) pueden guardar el esperma durante varios años (Orr y Zuk, 2012). En contraste, las hembras de mamíferos (e.g. cerdos u ovejas) retienen el esperma sólo un par de horas (Orr y Zuk, 2012). Particularmente en peces óseos, la retención de esperma se ha documentado en las familias Cottidae, Embiotocidae, Sebastidae y Poeciliidae (Fraser y Renton, 1940; Darling *et al*., 1980; Koya *et al.*, 2002; Vila *et al*., 2007). En estos grupos de peces, el almacenamiento de los espermatozoides puede ocurrir en el ovario o en el gonoducto (Darling *et al*., 1980; Koya *et al*., 2002; Vila *et al*., 2007). En *Helicolenus dactylopterus* (Sebastidae) la retención de esperma ocurre en cámaras de almacenamiento que se encuentran en el ovario (Vila *et al*., 2007), para dichas familias, los espermatozoides se concentran en el interior de estas cámaras. Por otro lado, en *Xiphophorus maculatus* (Poeciliidae) se han observado espermatozoides asociados al epitelio del ovario y del gonoducto (Potter y Kramer, 2000).

En peces poecílidos la capacidad para almacenar esperma se ha estudiado relativamente poco y hasta la fecha sólo se ha documentado en *Heterandria formosa*,

Poecilia reticulata, *Xiphophorus maculatus* y *Poeciliopsis gracilis* (Turner, 1947; Contantz, 1989, Potter y Kramer, 2000; Kobayashi y Iwamatsu, 2002; Uribe *et al*., 2004; Uribe y Grier, 2011). Únicamente en *Heterandria formosa* y *Poecilia reticulata* se han observado estructuras que almacenan el esperma temporalmente (denominadas '*solid plugs*' y '*sperm storage micropockets*', respectivamente) y que se forman debido al cierre de los pliegues ováricos que contienen a los espermatozoides (Fraser y Renton, 1940; Kobayashi y Iwamatsu, 2002).

Debido a que la superfetación implica una producción constante de embriones en diferentes estadios de desarrollo (Turner, 1937, 1940; Scrimshaw, 1994), se espera que las especies con superfetación requieran fertilizar constantemente a sus ovocitos. Por lo que, en esta investigación se propuso como hipótesis que en las hembras de especies con superfetación, existen estructuras que almacenen espermatozoides y que les permiten estar disponibles para fertilizar constantemente a diferentes ovocitos. Hasta la fecha, no existen trabajos que demuestren una relación entre la retención de esperma y la superfetación en peces de la familia Poeciliidae. Por lo tanto, el segundo tema de investigación de la presente tesis se enfocará en demostrar la presencia de estructuras de almacenamiento de esperma y su posible relación con la presencia de superfetación.

Superfetación y tamaño del embrión

Finalmente, la asociación entre la matrotrofía y la superfetación ha llevado a la hipótesis de que la evolución de uno de estos rasgos ha facilitado la evolución del otro

(Pollux *et al.*, 2009, 2014; Trexler y DeAngelis, 2010; Meredith *et al*., 2011). La aparente coevolución de estas estrategias reproductoras puede llevar a importantes consecuencias en términos del esfuerzo reproductor. Por lo tanto, el significado adaptativo de la coevolución entre superfetación y matrotrofía podría encontrarse en algunas características de historias de vida como el tamaño de la cría al nacer.

Con base a lo anterior se espera que una hembra sin superfetación que produce grandes camadas de embriones debe sufrir de limitaciones tanto energéticas como de espacio dentro de su ovario, por lo que el tamaño individual de cada embrión debe ser relativamente pequeño (compromiso entre número y tamaño de las crías; Roff, 2002). En contraste, una hembra con superfetación reparte su esfuerzo reproductor produciendo pequeñas camadas que nacen frecuentemente (Reznick y Miles, 1989; Pollux *et al.*, 2009; Zuñiga-Vega *et al*., 2010). Es decir, las hembras superfetadoras dentro de su ovario producen varias camadas en diferente estadio de desarrollo, en consecuencia con distinto tamaño, entonces deberían liberar un poco de espacio dentro de su ovario (en comparación con especies que carecen de superfetación). Por lo que la hipótesis de esta sección es que el espacio adicional que tienen las hembras superfetadoras en su ovario podrían usarlo para producir crías más grandes. Ya que la producción de crías más grandes favorece su adecuación en ciertos ambientes (Reznick y Endler, 1982; Reznick *et al*., 1990; Bashey, 2008; Riesch *et al*., 2010

Por otro lado, la matrotrofía implica que la hembra provee los nutrientes necesarios después de la fertilización y durante todo el desarrollo embrionario. Por lo que los embriones son pequeños en los primeros estadios de desarrollo debido a su escaso vitelo y

más grandes en los últimos estadios del desarrollo. Una combinación de la presencia de superfetación y alta matrotrofía podría promover el incremento del tamaño de las crías al nacer. Similar a los dos temas antes planteados, no existen estudios previos enfocados al estudio de los patrones de coevolución en las estrategias reproductoras de superfetación-matrotrofía, y sus consecuencias sobre las características de historias de vida como en el tamaño de las crías al nacer. El tercer tema de investigación de la presente tesis es examinar esta posible relación.

En resumen, existen varias interrogantes sobre las consecuencias morfológicas y reproductoras de la convergencia evolutiva de la superfetación. ¿Qué relación hay entre la superfetación y las características placentarias de peces poecílidos? ¿Existe una relación entre la intensidad matrotrófica y la complejidad de las placentas? ¿Existen estructuras morfológicas especializadas para la retención de esperma en estos peces vivíparos? ¿Ha habido un efecto de la coevolución de las estrategias reproductoras superfetación-matrotrofía sobre el tamaño del embrión al nacer? La búsqueda de respuestas a estas interrogantes es la base de la presente investigación. Por lo tanto, mi objetivo principal es conocer la relación que existe entre superfetación, placentación, matrotrofía, retención de esperma y tamaño del embrión en diferentes especies de la familia Poeciliidae. Los resultados de la presente tesis son una contribución importante al entendimiento de los mecanismos reproductores de los peces vivíparos de la familia Poeciliidae. Las posibles consecuencias o cambios en los atributos de la historia de vida de los peces vivíparos provocados por la superfetación no se habían estudiado hasta esta investigación. Por lo que abordé varios temas interesantes relacionados con la superfetación que van desde cambios

en el tamaño de la cría al nacer hasta cambios morfológicos ultraestructurales de la placenta folicular. Adicionalmente, utilicé métodos innovadores y actuales (i.e. métodos comparativos filogenéticos y estereología) para poder unir los enfoques ecológico, evolutivo y morfológico de los mecanismos de la reproducción en las especies estudiadas.

La familia Poeciliidae y sitios de estudio

Los poecílidos son peces de agua dulce y salobre que se distribuyen desde el este de Estados Unidos hasta el noroeste de Argentina (Miller et al., 2009). Esta familia está conformada por 22 géneros y 180 especies, en general son pequeños y llegan a medir únicamente de 31 a 70 mm (Miller et al., 2009). La familia Poeciliidae tiene fecundación interna y son vivíparos (excepto el género *Tomeurus;* Miller *et al.*, 2009). Los machos tienen una aleta anal modificada (de los rayos 3 al 5) llamada gonopodio que permite fertilizar a la hembra (Miller *et al.*, 2009). Por su parte, la hembra debido a la carencia del conducto de Müller tiene un ovario en donde ocurre tanto la fertilización como la gestación (Amoroso, 1968; Turner 1947; Wourms, 1981). El tipo de gestación de estos poecílidos es intrafolicular, es decir que los ovocitos se fecundan dentro de un folículo materno en el ovario y se mantienen ahí hasta justo antes de su nacimiento (Fig. 1A-B; Wourms *et al*., 1988). Además, los poecílidos tienen una placenta llamada tipo folicular que se encuentra formada por los tejidos maternos y embrionarios (Turner, 1940). Los tejidos embrionarios son la piel absorbente del embrión, saco pericárdico y el saco vitelino (Turner, 1940). Mientras que los tejidos maternos son el epitelio folicular y el tejido conjuntivo laxo que lo rodea (Turner, 1940;

Grove y Wourms, 1994; Fig. 1C). En la presente tesis me enfoqué en 12 especies de la familia Poeciliidae. Las especies superfetadoras son: *Heterandria formosa* (obtenida de un acuario privado de Estados Unidos), *Poeciliopsis infans, P. gracilis, P. presidionis, P. prolifica* and *P. viriosa*. Mientras que las especies no superfetadoras son: *Pseudoxiphophorus bimaculatus*, *Xiphophorus hellerii, Poecilia mexicana, Priapella intermedia* y *Gambusia panuco*. Estas especies fueron seleccionadas debido a que traté de muestrear la mayor cantidad de especies de poecílidos en un área grande de la República Mexicana (Fig. 2; tabla 1).

Preguntas de investigación y presentación de los capítulos que componen la tesis

Para lograr el objetivo principal, la presente tesis se enfoca en contestar las preguntas de investigación que mencioné anteriormente. Estas preguntas de investigación definen los objetivos particulares de cada capítulo.

Capítulo 1

> *¿En qué difieren las placentas de especies de peces que superfetan con respecto a las placentas de especies que no superfetan? ¿Altos niveles de matrotrofía están asociados con placentas más complejas?*

Hipótesis:

a) Debido a que la superfetación requiere una regulación diferencial por parte de las hembras de las tasas de desarrollo de embriones en distintos estadios (Turner, 1937, 1940; Scrimshaw, 1944), las hembras de especies que superfetan tendrán una placenta más compleja (en su ultraestructura) comparada con la placenta de especies que no superfetan.

b) Debido a que la matrotrofía implica la transferencia activa de nutrientes por parte de la madre hacia sus embriones a lo largo de todo el desarrollo (Wourms, 1981; Marsh-Matthews, 2011), las hembras de especies con altos niveles de matrotrofía tendrán una placenta más compleja para cumplir esta función en comparación con las hembras de especies con lecitotrofía o matrotrofía incipiente.

Predicción:

Las placentas de hembras de especies con superfetación y altos niveles de matrotrofía, mostrarán características ultraestructurales que reflejen una mayor transferencia activa de nutrientes a través del epitelio folicular, en comparación con especies sin superfetación y bajos niveles de matrotrofía. Particularmente se espera observar mayor área y mayor grosor del folículo materno, presencia de microvellosidades y mayor cantidad de vesículas en las células foliculares. Estas características le confieren a la placenta mayor capacidad de eficiencia en el transporte de nutrientes desde la madre hacia sus embriones (Grove y Wourms, 1994).

Descripción general de los métodos:

Colecté tres hembras gestantes de especies superfetadoras y no superfetadoras de la familia Poeciliidae. Inmediatamente, se fijaron las muestras utilizando el protocolo específico para microscopía electrónica (Dykstra, 1993). Los métodos de microscopia electrónica permitieron observar y describir detalladamente la ultraestructura de los componentes maternos de la placenta folicular de todas las especies. Para cada especie, cuantifiqué algunas ultraestructuras placentarias que indican el grado de complejidad de la placenta folicular (mayor complejidad es debido a mayor valor de las ultraestructuras placentarias; Grove y Wourms, 1994). Mediante métodos filogenéticos comparativos realicé una comparación morfológica de las placentas foliculares entre las especies que superfetan y las que no superfetan. Además, también examiné mediante métodos filogenéticos comparativos la posible relación entre el nivel de matrotrofía de cada especie y el grado de complejidad de sus placentas.

Capítulo 2

¿Existen diferencias entre especies que superfetan y no superfetan en la capacidad de almacenar esperma?

Hipótesis:

Las hembras superfetadoras requieren fertilizar frecuentemente a distintos grupos de embriones, entonces las hembras de las especies que superfetan deberían tener

mayor capacidad de almacenar esperma en comparación con hembras de especies que no superfetan.

Predicción:

Las hembras de especies que superfetan deben tener más y más grandes (mayor volumen) estructuras para almacenar esperma dentro del ovario en comparación con hembras de especies que no superfetan.

Descripción general de los métodos:

Colecté tres hembras gestantes de especies superfetadoras y no superfetadoras de la familia Poeciliidae. Inmediatamente, se fijaron las muestras utilizando un protocolo histológico (Presnell *et al*., 1997). A través de cortes seriados y de la técnica de tinción histológica con hematoxilina-eosina examiné la morfología general del ovario tanto de especies superfetadoras como de especies no superfetadoras. En los ovarios de algunas especies observé estructuras que tienen la función de almacenar espermatozoides (llamadas espermatecas). Por medio de métodos estereológicos cuantifiqué el número, volumen total y volumen promedio de las espermatecas para cada especie. Finalmente, utilicé métodos filogenéticos comparativos para analizar si existe una relación entre las características de las espermatecas (número, volumen total y volumen promedio) y la presencia o ausencia de superfetación.

Capítulo 3

¿La presencia conjunta de superfetación y matrotrofía promueven la producción de crías más grandes en especies de la familia Poeciliidae?

Hipótesis:

La evolución conjunta de la superfetación y la matrotrofía en la familia Poeciliidae ha facilitado la evolución de crías más grandes.

Predicciones:

a) Las hembras de especies que presentan superfetación y altos niveles de matrotrofía tienen crías al nacer más grandes en comparación con hembras de especies que carecen de superfetación y que exhiben lecitotrofía o matrotrofía incipiente.

b) El tamaño de las crías al nacer ha evolucionado de manera correlacionada con el grado de superfetación y matrotrofía.

Descripción general de los métodos:

Se realizó una búsqueda bibliográfica del tamaño (masa) en el último estadio de desarrollo de las crías de distintas especies de la familia Poeciliidae. Además, se disecaron otros especímenes provenientes de la Colección Nacional de Peces (Instituto de Biología, UNAM) para medir directamente el tamaño promedio del último estadio de desarrollo para otras especies adicionales. A través de métodos filogenéticos comparativos, analicé si existe una relación entre el tamaño al último estadio de desarrollo y el nivel de

superfetación y matrotrofía. También estimé la heredabilidad filogenética de estas tres características para conocer si evolucionaron aproximadamente a la misma tasa, lo que indicaría cambios similares y probablemente paralelos en la superfetación, la matrotrofía y el tamaño al nacer, a lo largo de la historia evolutiva de esta familia de peces vivíparos.

LITERATURA CITADA

Amoroso EC. 1968. Evolution of viviparity. Proceedings of the Royal Society of Medicine Journal 61: 1188-1200.

Bashey F. 2008. Competition as a selective mechanism for larger offspring size in guppies. Oikos 117: 104-113.

Birkhead TR, Møller AP. 1993. Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals. Biological Journal of the Linnean Society 50: 295-311.

Blackburn DG. 1992. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. American Zoologist 32: 313-321.

Blackburn DG. 1999. Placenta and placental analogs in reptiles and amphibians. Encyclopedia of Reproduction 3: 840-847.

Carter AM, Mess A. 2007. Evolution of the placenta in eutherian mammals. Placenta 28: 259-262.

Constanz J. 1989. Reproductive biology of the poeciliid fishes. In: Meffe GK, Snelson FF, editors. Ecology and Evolution of Live Bearing Fishes (Poeciliidae). New Jersey: Prentice Hall. pp 33-50.

Cooley LR, Foighil DÓ. 2000. Phylogenetic analysis of the Sphaeriidae (Mollusca: Bivalvia) based on partial mitochondrial 16S rDNA gene sequences. Invertebrate Biology 119**:** 299-308.

Corner LA, Stuart LJ, Kelly DJ, Marples NM. 2015. Reproductive biology including evidence for superfetation in the european Badger *Meles meles* (Carnivora: Mustelidae). PloS one: 10e0138093.

Darling JDS, Noble ML, Shaw E. 1980. Reproductive strategies in the surfperches .1. Multiple insemination in natural-populations of the shiner perch, *Cymatogaster aggregata*. Evolution 34: 271-277.

Dykstra MJ. 1993. A manual of applied techniques for biological electron microscopy (No. 89). Springer Science & Business Media.

Fraser EA, Renton RM. 1940. Observation on the breeding and development of the viviparous fish. *Heterandria formosa*. Quarterly Journal of Microscopical Science 81: 479-520.

Grove BD, Wourms JP. 1991. The follicular placenta of the viviparous fish, *Heterandria formosa*. I: ultrastructure and development of the embryonic absorptive surface. Journal of Morphology 209: 265-284.

Gunn JS, Thresher RE. 1991. Viviparity and the reproductive ecology of clinid fishes (Clinidae) from temperate Australian waters. Environmental Biology of Fishes 31: 323-44.

Hetmanski T, Wolk E. 2005. The effect of environmental factors and nesting conditions on clutch overlap in the Feral Pigeon *Columba livia f. urbana* (Gm.). Polish Journal of Ecology 53: 523-534.

Hrbek T, Seckinger J, Meyer A. 2007. A phylogenetic and biogeographic perspective on the evolution of poeciliid fishes. Molecular Phylogenetics and Evolution 43: 986-998.

Kennedy H. 1978. Systematics and pollination of the "closed-flowered" species of *Calathea* (Marantaceae). University of California Publications in Botany 71:1-90.

Kobayashi H, Iwamatsu T. 2002. Fine structure of the storage micropocket of spermatozoa in the ovary of the guppy *Poecilia reticulata*. Zoological Science 19:545-555.

Koya Y, Munehara H, Takano K. 2002. Sperm storage and motility in the ovary of the marine sculpin Alcichthys alcilcornis (teleostei: Scorpaeniformes), with internal gametic association. Journal of Experimental Zoology 292: 145-155.

Kwan L, Fris M, Rodd FH, Rowe L, Tuhela L, Panhuis TM. 2015. An examination of the variation in maternal placentae across the genus *Poeciliopsis* (Poeciliidae). Journal of Morphology 276: 707-720.

Marsh-Matthews E. 2011. Matrotropy. In: Evans, JP; Pilastro, A.; Schlupp, I., editors. Ecology and evolution of poeciliid fishes. Chicago: The University of Chicago Press; 2011. p. 28-37.

Méndez-de la Cruz FR, Villagrán-Santa Cruz M, Andrews RM. 1998. Evolution of viviparity in the lizard genus *Sceloporus*. Herpetologica 54: 521-532.

Meredith RW, Pires MN, Reznick DN, Springer MS. 2011. Molecular phylogenetic relationships and the coevolution of placentotrophy and superfetation in (*Poecilia*) (Poeciliidae: Cyprinodontiformes). Molecular Phylogenetics and Evolution 59: 148-157.

Miller RRM, Norris WL, Soto SMS. 2009. Peces dulceacuícolas de México (No. EE/597.092972 M5).

Moll W. 1985. Physiological aspects of placental ontogeny and phylogeny. Placenta 6: 141-154.

Mossman HW. 1937. Comparative morphogenesis of the fetal membranes and accessory uterine structures. Carnegie Institute Contributions of Embryology 26: 129-246.

Mossman HW. 1987. Vertebrate fetal membranes. New Brunswick, N. J. Rutgers New Jersey, University press.

Olivera-Tlahuel C, Ossip-Klein AG, Espinosa-Pérez HS, Zúñiga-Vega JJ. 2015. Have superfetation and matrotrophy facilitated the evolution of larger offspring in poeciliid fishes? Biological Journal of the Linnean Society 116: 787-804.

Orr TJ, Zuk M. 2012. Sperm storage.Current Biology 22: R8-R10

Pape O, Winer N, Paumier A, Philippe HJ, Flatrès B, Boog G. 2008. Superfetation: case report and review of the literature. Journal de gynécologie, obstétrique et biologie de la reproduction 37: 791.

Perry JS. 1981. The mammalian fetal membranes. Journal of Reproduction and Fertility 62: 321-335.

Pires MN, Arendt J, Reznick DN. 2010. The evolution of placentas and superfetation in the fish genus *Poecilia* (Cyprinodontiformes: Poeciliidae: subgenera Micropoecilia and Acanthophacelus). Biological journal of the Linnean Society 99: 784-796.

Pires MN, McBride KE, Reznick DN. 2007. Interpopulation variation in life-history traits of *Poeciliopsis prolifica*: implications for the study of placental evolution. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 307: 113-125.

Pollux BJA, Meredith RW, Springer MS., Garland T, Reznick DN. 2014. The evolution of the placenta drives a shift in sexual selection in livebearing fish. Nature, 513: 233-236.

Pollux BJA, Pires MN, Banet AI, Reznick DN. 2009. Evolution of placentas in the fish family Poeciliidae: an empirical study of macroevolution. Annual Review of Ecology, Evolution, and Systematics 40:271-289.

Potter H, Kramer CR. 2000. Ultrastructural observations on sperm storage in the ovary of the platyfish, *Xiphophorus maculatus* (Teleostei: Poeciliidae): the role of the duct epithelium. Journal of Morphology 245: 110-129.

Pratt Jr HL. 1993. The storage of spermatozoa in the oviducal glands of western north Atlantic sharks. Environmental Biology of Fishes 38: 139-149.

Presnell JK, Schreibman MP, Humason GL. 1997. Humason's animal tissue techniques. San Francisco: Johns Hopkins University Press.

Reznick DN, Bryga HA, Endler JA. 1990. Experimentally induced life-history evolution in a natural population. Nature 346: 357-359.

Reznick DN, Endler JA. 1982. The impact of predation on life history evolution in Trinidadian guppies (Poecilia reticulata). Evolution 1982:160-177.

Reznick DN, Meredith R, Collette BB. 2007. Independent evolution of complex life history adaptations in two families of fishes, live-bearing halfbeaks (Zenarchopteridae, Beloniformes) and Poeciliidae (Cyprinodontiformes). Evolution 61: 2570-83.

Reznick DN, Miles DB. 1989. Review of life history patterns in poeciliid fishes. In: Meffe GK, Snelson FF Jr., editors. Ecology and evolution of livebearing fishes (Poeciliidae). Prentice Hall, New Jersey, p. 125-148.

Riesch R, Plath M, de León FJG, Schlupp I. 2010. Convergent life-history shifts: toxic environments result in big babies in two clades of poeciliids. Naturwissenschaften 97: 133-141.

Roellig K, Menzies BR, Hildebrandt TB, Goeritz F. 2011. The concept of superfetation: a critical review on a 'myth' in mammalian reproduction. Biological Reviews 86: 77-95.

Roff, D. 2002. Life history evolution. University of California, Riverside. First Edition,pp. 456. Editorial Sinauer Associates. Sunderland, Massachusetts. U.S.A.

Rosen DE, Bailey RM. 1963. The poeciliid fishes (Cyprinodontiformes): their structure, zoogeography, and systematics. Bulletin of the American Museum of Natural History 126: 1-176.

Scrimshaw NS. 1944. Superfetation in poeciliid fishes. Copeia 1944: 180-183.

Sever DM. 2002. Female sperm storage in amphibians. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 292: 165-79.

Stearns SC. 1992. The evolution of life histories. Vol. 249. Oxford: Oxford University Press.

Swain R, Jones SM. 2000. Facultative placentotrophy: half-way house or strategic solution? Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 127: 441-51.

Thibault RE, Schultz RJ. 1978. Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). Evolution 32: 320-333.

Travis J, Farr JA, Henrich S, Cheong RT. 1987. Testing theories of clutch overlap with the reproductive ecology of *Heterandria formosa*. Ecology 68: 611-623.

Trexler JC, DeAngelis DL. 2010. Modeling the evolution of complex reproductive adaptations in poeciliid fishes: matrotrophy and superfetation. In: Uribe, MC.

Grier, HJ., editors. Viviparous fishes II. Homestead: New Life Publications; 2010. p. 231-240.

Tuppen GD, Fairs C, De Chazal RC, Konje JC. 1999. Spontaneous superfetation diagnosed in the first trimester with successful outcome. Ultrasound in Obstetrics & Gynecology 14: 219-221.

Turner CL. 1937. Reproductive cycles and superfetation in poeciliid fishes. Biological Bulletin 72:145-164.

Turner CL. 1940. Pseudoamnion, pseudochorion, and follicular pseudoplacenta in poeciliid fishes. Journal of Morphology 67: 59-89.

Turner CL. 1947. Viviparity in teleost fishes. The Scientific Monthly 65: 508-518.

Uribe MC, Grier HJ. 2011. Oogenesis of microlecithal oocytes in the viviparous teleost *Heterandria formosa*. Journal of Morphology 272:241-257.

Uribe MC., De la Rosa-Cruz G, Guerrero-Estévez SM, García Alarcón A, Aguilar-Morales ME. 2004. Estructura del ovario de teleósteos vivíparos. Gestación intraovárica: Intraluminal en Ilyodon whitei (Goodeidae), e intrafolicular en *Poeciliopsis gracilis* (Poeciliidae). In: Lozano Vilano ML, Contreras Balderas AJ, editores. Homenaje al Dr Andrés Reséndez Medina. México: Dir. Pub. UANL. pp 31–45.

Vila S, Sàbat M, Hernandez MR, Munoz M. 2007. Intraovarian sperm storage in *Helicolenus dactylopterus dactylopterus*: fertilization, crypt formation and maintenance of stored sperm. The Raffles Bulletin of Zoology 14: 21-27.

Vogel P. 2005. The current molecular phylogeny of Eutherian mammals challenges previous interpretations of placental evolution. Placenta 26: 591-596.

Wourms JP, Grove BD, Lombardi J. 1988. The maternal-embryonic relationship in viviparous fishes. In: Fish Physiology: The Physiology of Developing Fish. Vol. 11, p. 1-134.

Wourms JP. 1981. Viviparity: the maternal-fetal relationship in fishes. American Zoologist 21: 473-515.

Yamaguchi N, Dugdale HL, Macdonald DW. 2006. Female Receptiveity, Embryonic Diapause, and Superfetation in the European Badger (*Meles Meles*: Implications for the Reproductive Tactics of Males and Females. The Quarterly Review of Biology 81: 33-48.

Yamaguchi N, Sarno RJ, Johnson WE, O'Brien SJ, Macdonald DW. 2004. Multiple paternity and reproductive tactics of free-ranging American minks, *Mustela vison*. Journal of Mammalogy 85: 432-439.

Zúñiga-Vega JJ, Macías-Garcia C, Johnson JB. 2010. Hypotheses to explain the evolution of superfetation in viviparous fishes. In: Uribe MC, Grier HJ, eds. Viviparous Fishes II. New life publications, 13-30.

Zúñiga-Vega JJ, Reznick DN., Johnson JB. 2007. Habitat predicts reproductive superfetation and body shape in the livebearing fish *Poeciliopsis turrubarensis*. Oikos 116: 995-1005.

LEYENDAS DE FIGURA

Fig. 1. Gestación intrafolicular de la familia Poeciliidae (*Poeciliopsis gracilis*). **A.** Se muestra el ovario tipo sacular con dos embriones en desarrollo. Las flechas indican el epitelio folicular que rodea a los embriones. (L) lumen del ovario, (V) vitelo embrionario, (E) embrión. **B.** Amplificación de un embrión en desarrollo. **C.** Amplificación del folículo materno que forma parte de la placenta folicular. Se muestra el folículo materno dividido en dos secciones, en la capa o epitelio folicular (CF) y el tejido conjuntivo (TC). Las líneas indican el grosor de cada sección.

Fig. 2. Mapa de los sitios de estudio de las especies en estudio de la familia Poeciliidae.

Tabla 1. Resumen de los datos y sitios de estudio de las 12 especies de la familia Poeciliidae.

ESPECIES	Coordenadas geográficas	Estado	Superfetación
Gambusia panuco	N 20° 41’ W 103° 57’	San Luis Potosí	NO
Poecilia butleri	N 20° 9’ W 103° 2’	Jalisco	NO
Poecilia mexicana	N 17° 26’ W 95° 26’	Oaxaca	NO
Priapella intermedia	N 17° 9’ W 95° 10’	Oaxaca	NO
Pseudoxiphophorus bimaculatus	N 17° 8’ W 95° 7’	Oaxaca	NO
Xiphophorus hellerii	N 20° 34’ W 104° 9’	Jalisco	NO
Heterandria formosa	Acuario privado	Estados Unidos	SI
Poecilioposis infans	N 20° 34’ W 104° 9’	Jalisco	SI
Poeciliopsis gracilis	N 21° 59’ W 99° 15’	San Luis Potosí	SI
Poeciliopsis presidionis	N 22° 29’ W 105° 21’	Nayarit	SI
Poeciliopsis prolifica	N 23° 03’ W 105° 50’	Sinaloa	SI
Poeciliopsis viriosa	N 21° 02’ W 104° 22’	Sinaloa	SI

Fig. 1.

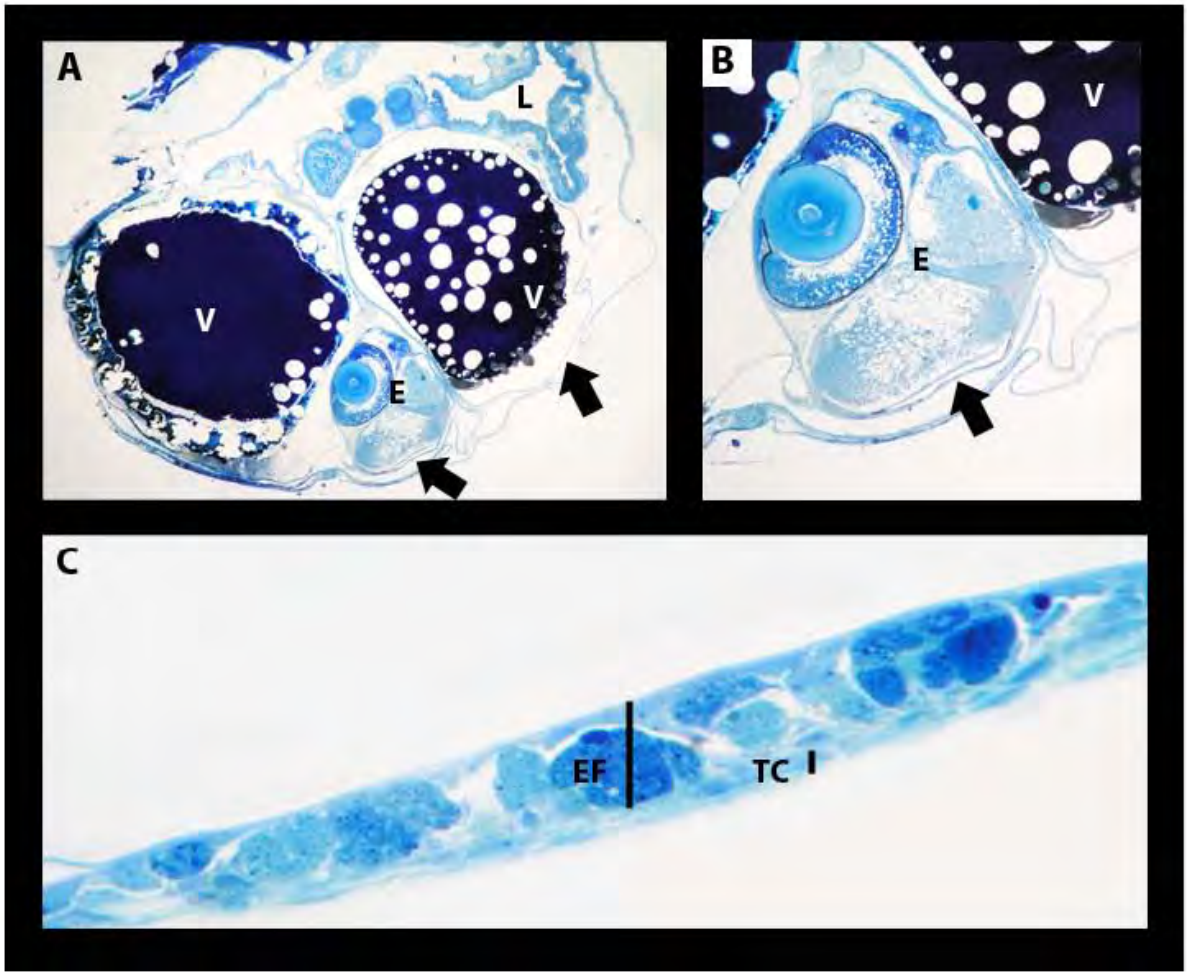
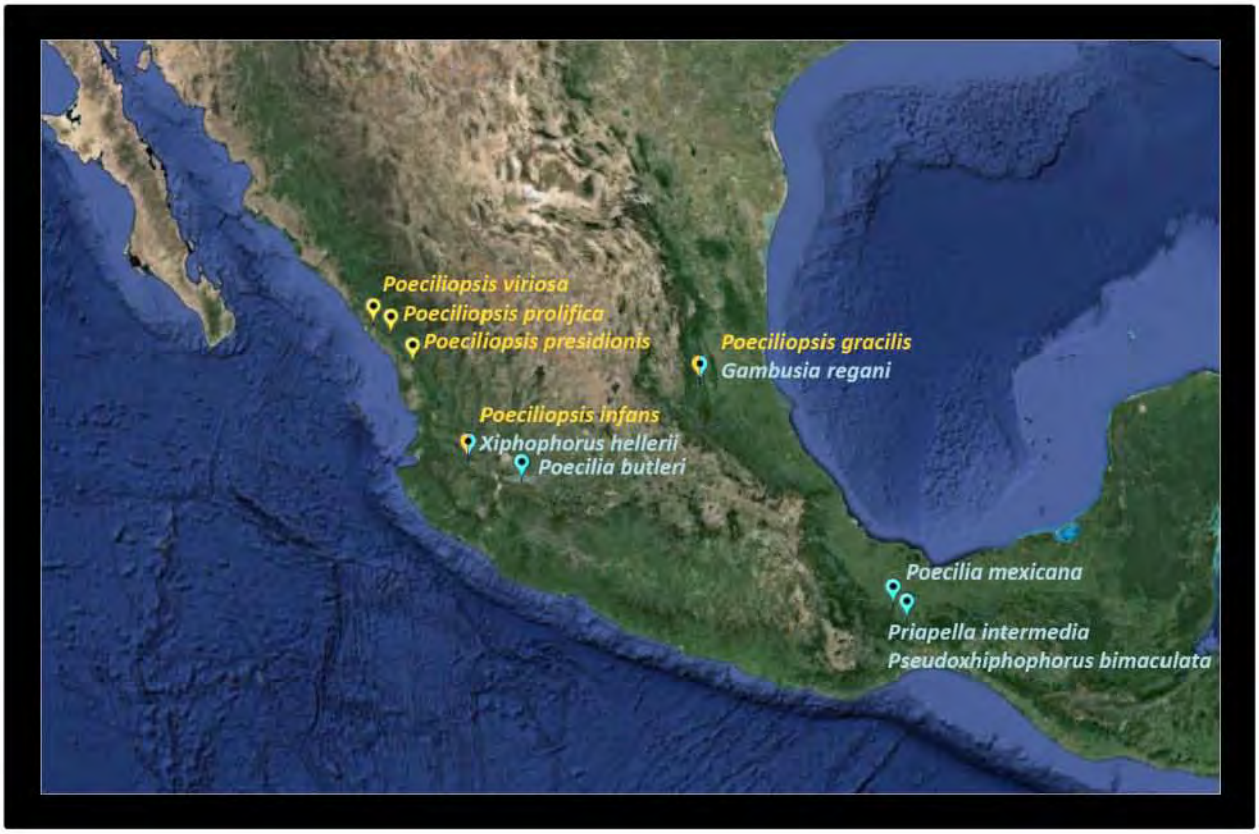


Fig. 2.


Poeciliopsis viriosa
Poeciliopsis prolifica
Poeciliopsis presidionis
Poeciliopsis gracilis
Gambusia regani
Poeciliopsis infans
Xiphophorus hellerii
Poecilia butleri
Poecilia mexicana
Priapella intermedia
Pseudoxhiphophorus bimaculata

CAPÍTULO I

Placental structures and their association with matrotrophy and superfetation in poeciliid fishes

Claudia Olivera-Tlahuel, Norma A. Moreno-Mendoza, Maricela Villagrán-Santa Cruz y J. Jaime Zúñiga-Vega

Prepared for submission to Zoomophology

Prepared for submission to Zoomophology

Placental structures and their association with matrotrophy and superfetation in poeciliid fishes

Claudia Olivera-Tlahuel[1], Norma A. Moreno-Mendoza[2], Maricela Villagrán-Santa Cruz[3] y J. Jaime Zúñiga-Vega[1]

Header: Degrees of placentation in poeciliids

Keywords: Matrotrophy; Poeciliidae; placentation; follicular placenta; superfetation

[1]Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de México. Ciudad Universitaria 04510, Ciudad de México, México

[2]Instituto de investigaciones Biomédicas, Universidad Nacional Autónoma de México. Ciudad Universitaria 04510, Ciudad de México, México

[3]Departamento de Biología Comparada, Facultad de Ciencias, Universidad Nacional Autónoma de México. Ciudad Universitaria 04510, Ciudad de México, México

*Correspondence to: J. Jaime Zúñiga-Vega, Facultad de Ciencias, Universidad Nacional Autónoma de México. Ciudad Universitaria 04510, Ciudad de México, México. E-mail: jzuniga@ciencias.unam.mx

ABSTRACT

Fish of the family Poeciliidae have a follicular placenta, the ultrastructure of which is little known. In this article, we describe ultrastructure of the maternal follicular placenta of 11 species of poeciliids using electron microscopy. In addition, using stereological methods and photomicrographs of placentas under electron microscopy, we examined and quantified six ultrastructure characteristics that reflect the degree of complexity in follicular placenta of each species. These characteristics included the number of vesicles, vesicles area, number of microvilli, microvilli length, thickness of maternal follicle, and follicular area. Additionally, using phylogenetic comparative methods, we evaluated the relationship between the degree of matrotrophy and placental characteristics. We also analyzed the possible relationship between the existence or lack of superfetation and different placental characteristics. We found that all species had a similar morphological structure; an inner follicular layer of epithelial nature as well as an outer follicular layer of connective nature. In particular, *Poeciliopsis presidionis* presents *hyper-placentation*, or more developed placental characteristics (thickness of maternal follicle and follicular area) for the exchange of nutrients between the mother and embryos. We found a positive relationship between the degree of matrotrophy and some placental characteristics, such as follicular area, number of microvilli, and number and size of vesicles. Similarly, follicular area and number and length of microvilli tended to be larger in species with superfetation than those without superfetation. As such, we conclude that high degrees of matrotrophy and superfetation are associated with placental characteristics that increase the efficiency of nutrient transfer between mother and embryos.

INTRODUCTION

During the evolutionary transition from oviparity to viviparity, the female reproductive system had to develop complex structures for the transfer of nutrients to the embryo (Blackburn, 1992). As such, distinct placental types have evolved over time (Pollux et al. 2009; Pollux et al. 2014). The placenta is the close apposition or fusion of maternal and fetal tissues for physiological exchange (Mossman, 1937). These tissues maximize the transfer of nutrients to the embryo. For examples, in lizards and snakes, some species have simple placentas, while others species (such as in the genera *Mabuya* and *Virginia*) have complex placentas which allow the rapid transfer of nutrients (Blackburn and Steward, 2011). It is therefore important to understand how different degrees of placentation are related to other morphological, physiological, and reproductive characteristics.

In fishes, viviparity implied that eggs had internal fertilization and were retained within female reproductive system (Amoroso, 1968). The fishes lack Müllerian duct, in consequence in the ovary occurs both fertilization and gestation (physiologically equivalent to the mammalian uterus; Amoroso, 1968; Turner 1947; Wourms 1981). Then, different types of placentas for the transfer of substances (nutrients, gases and waste) were developed for the maintenance of the embryo within the ovary (Amoroso, 1968; Blackburn, 2005, 2015). In particular, in the viviparous fish of the family Poeciliidae, the placenta is follicular and was first described by Turner (1940). The main components of the follicular placenta are the pericardial sac, the yolk sac, and the maternal follicle. The yolk sac and pericardial sac are embryonic tissues which are intimately related to the

maternal follicular cells. This close relationship between maternal and embryonic tissues allows the optimal exchange of nutrients and oxygen for the development of the embryo. Additionally, Turner (1940) observed that the follicular cells had microvilli whose shape varied among the species he analyzed (i.e. *Poeciliopsis sp.* and *Aulophallus elongatus*; Turner, 1940). Microvilli are prolongations of the membrane of the follicular cells which increase the surface area in contact with the embryo. Therefore, the increase in microvilli surface apparently increases the efficiency of the exchange of substances between mother and embryo. Likewise, Turner (1940) thus first inferred that the microvilli of the follicular placenta could differ among species in accordance with the amount of nutrients that the embryos receive directly from the mother during development.

Later, Grove and Wourms (1994) described the ultrastructure (characteristics observed under an electron microscope) of the components of the follicular placenta of *Heterandria formosa*. They demonstrated that the follicular cells had microvilli on their apical surface (the zone closest to the embryonic tissue), a complex system of vesicles, extensive endoplasmic reticulum and Golgi apparatus with 4 or 5 cisternae. As such, they concluded that all of these structures are necessary for the exchange of nutrients of the mother to the embryo. Then, in *Lebistes reticulatus* (*Poecilia reticulata*) it was observed that the maternal follicular placenta consists of two layers: an inner layer and an outer layer (Jollie and Jollie, 1964). The inner layer is conformed of epithelial follicular cells with numerous vesicles, which confers a great activity of nutrients transport. In other words, the vesicles permit the exchange and efficient movement of nutrients of the mother to the embryo through the follicular placenta. In contrast, the outer follicular layer is

conformed of lax connective tissue with high vascularization, fibroblasts, collagen fibers (Jollie and Jollie, 1964). Therefore, outer follicular layer has a protective and nutritive function, because the capillaries are very close with follicular cells, suggesting an intense transport of oxygen and nutrients to the embryo.

In addition to the variation in degree of placentation that can be found between species in this family of fish, the amount of nutrient transfer through the placenta that embryos experience over the course of their development varies widely among poeciliid species (Pollux et al. 2009, 2014). There are lecithotrophic species, in which nutrient transfer from mother to embryo occurs before the embryo is fertilized (Wourms, 1981; Marsh-Matthews, 2011). In these lecithotrophic species, the energy reserve devoted to embryonic development is stored in a nutritious substance known as yolk that is deposited into the mature oocyte. In contrast, some species are matrotrophic, such that most of the energy devoted to embryonic development is provided after fertilization and is transferred directly from mother to embryo through the placenta during embryonic development (Wourms, 1981; Marsh-Matthews, 2011). Therefore, embryos that are highly matrotrophic (extensive matrotrophy) have little yolk and are characterized by a large increase in weight during their development, since the mother is actively transferring nutrients to them (Pires et al. 2010). Species with moderate matrotrophy are those in which the mother deposits some yolk in the mature oocyte as well as transferring some nutrients directly during embryonic development. In the Poeciliidae family, there is therefore a continuum among species from completely lecithotrophic to extensively matrotrophic (Pollux et al. 2014).

Recently, Kwan et al. (2015) demonstrated that among eight species of poeciliids, the thickness of maternal follicle and vascularization and folding of the follicular epithelium increased with increasing degrees of matrotrophy. To date, this is the only study to examine the relationship between degree of matrotrophy and placental complexity, even though many studies have assumed that higher degrees of matrotrophy require more complex placentas (Reznick et al. 2002; Pollux et al. 2014; Ostrovsky et al. 2015). More comparative studies with larger numbers of species and which account for phylogenetic relationships are still needed. As such, one of the main objectives of this study was to analyze whether there is a relationship between degree of matrotrophy and several ultrastructural characteristics of the maternal follicular placenta. The hypothesis is that females that transfer more nutrients to their embryos during development (i.e. more matrotrophic females) require a more complex placenta (Turner, 1940; Jollie and Jollie, 1964; Grove and Wourms, 1994; Kwan et al. 2015). Some of the characteristics that we suggest could indicate a more highly developed placenta are the presence of numerous and long microvilli of follicular cells, higher number and area of vesicles of follicular cells, higher maternal follicle thickness, and higher follicular area.

On the other hand, superfetation is the capacity for females to simultaneously carry within the reproductive tract litters of embryos at different developmental stages (Turner, 1937, 1940; Scrimshaw, 1994). In poeciliids, the species of some genera (e.g., *Poeciliopsis*, *Heterandria* and *Neoheterandria*) exhibit superfetation, while those of other genera do not (e.g., *Alfaro*, *Brachyrhaphis* and *Xiphophorus*; Reznick and Miles, 1989; Zúñiga-Vega et al. 2010). The phylogenetic distribution of this

reproductive strategy suggests that superfetation has developed independently on multiple occasions (Olivera-Tlahuel et al. 2015). Because superfetation involves the differential development of distinct groups of embryos, we propose the hypothesis that the evolution of this reproductive strategy should be accompanied by the evolution of more specialized placentas which facilitate the differential regulation of nutrients to the distinct developmental stages. As such, the second objective of this study was to analyze whether there is a relationship between degree of placentation and presence or absence of superfetation.

We collected three visibly gestating females of superfetating and non-superfetating poeciliid species in different rivers in Mexico. The superfetating species were *Heterandria formosa, Poeciliopsis infans, P. gracilis, P. presidionis, P. prolifica* and *P. viriosa* (table 1), and the non-superfetating species were *Pseudoxiphophorus bimaculatus*, *Xiphophorus hellerii, Poecilia mexicana, Priapella intermedia* and *Gambusia panuco* (table 1). Each specimen was then anesthetized (using 3-amenobenzoic acid ethyl ester) and immediately sacrificed by an overdose of the same anesthetic. The ovaries were dissected from the collected females and immediately injected with Karnovsky fixative and were placed by immersion on the same fixative for later processing in the laboratory.

Electron microscopy

In the laboratory, the three ovary samples of each species were processed as follows. First, they were placed in 0.1 M sodium cocadylate buffer (0.1 M) for one night, then were transferred to 1% osmium tetroxide for one hour. Second, the ovaries were dehydrated in

gradually increasing concentrations of alcohol (70 %, 80 %, 90 %, 96 % and 100 %). Third, the processing was continued with acetonitrile for 20 minutes repeated at two different proportions (1:1 acetonitrile:Epon and 1:2 acetonitrile:Epon). Fourth, the samples were placed in increasing proportions of Epon until they were immersed in pure Epon. Finally, the samples were placed in plastic molds and allowed to polymerize in a 60°C oven for 24 hours.

The block obtained and included in Epon was cut into 2-micron thick slices, a semi-fine thickness. We used a Nova LKN BROMMA ultramicrotome to obtain serial sections of each of the ovaries. They were then stained with toluidine blue and using light microscopy, the follicular cells of ovarian follicles that contained an embryo in a relatively advanced developmental stage (between stages 8 and 9; Haynes, 1995) were located. Once follicular cells were located in each ovary, semi-fine sections were obtained. The samples were then moved to the Instituto de Fisiología Celular at the Universidad Nacional Autónoma de México (UNAM) and the Instituto de Pediatría to obtain fine (less than 1 micron thick) sections.

The fine sections were observed using the electron microscopes at the Instituto de Investigaciones Biomédicas and Instituto de Fisiología Celular at UNAM. Digital photomicrographs were taken of the follicular cells of the different species included in the study. Only 10 maternal follicles (one follicular cell and connective tissue) were randomly selected from each ovary for posterior analysis, such that 30 photographs of maternal follicles were obtained per species, since three ovaries were processed per species.

Standardization of developmental stage

During the course of embryonic development, follicular cells change in their components as well as their morphology (Jollie and Jollie, 1964; Guerrero-Estévez, 2005). At early stages, follicular cells are cuboidal and with low subjacent vascularization. On the contrary, at intermediate and at the beginning of late developmental stages, follicular cells are thinner and subjacent vascularization is high. At the most advanced stages of development, the follicular cells are extremely thin and their association with vascularization is limited. In order to avoid an erroneous comparative analysis, we standardized the stage of embryonic development by selecting only embryos at the beginning late developmental stages and close to the eye for the examination of maternal follicle morphology (between stages 8 and 9; Haynes, 1995). In this way, we assured that the differences among species reflected real morphological differences, and were not due simply to chance differences in the stage of embryonic development.

Stereology of the follicular epithelium

The placenta is composed of both maternal and fetal tissue. However, in this article we focus on maternal tissues (or maternal follicles). We describe the ultrastructure of the maternal follicle and we consider the differences of these substructures (number and size) as indicators of degree of complexity. For this, we used stereology, is a combination of simple and efficient methods for quantifying morphological structures using histological sections (Gundersen et al. 1988). However, stereology can also be applied to images obtained from electron microscopy. Using stereological methods it is possible to count,

measure, and calculate the volume of structures of interest. In this study we used the program ImageJ (Rasband, 1997-2016), which uses stereological tools to analyze photomicrographs taken using electron microscopy. In particular, we quantified the following characteristics of maternal follicle (one follicular cell and connective tissue): (1) number of vesicles, NV; (2) area of vesicles, AV; (3) number of microvilli, NM; (4) length of microvilli, LM; (5) thickness of maternal follicle, TF; (6) and area of maternal follicle, AF. We considered these six variables indicators of the degree of complexity of the placenta. In order to obtain a representative value for each of these variables for each species, we averaged the values of the three ovaries analyzed per species.

Matrotrophy index

The variations between species of the family Poeciliidae in the amount of nutrients transferred to embryos over the course of development (lecithotrophy to extensive matrotrophy) can be measured using the matrotrophy index (MI; Worms et al. 1988). This index represents the difference in dry mass between fertilization and the last stage of embryonic development. If the embryo weighs 30-35% less (due to metabolic costs) at the end of development than at oocyte fertilization, this indicates lecithotrophy (negative MI; Worms et al. 1988). While, if the embryo at the last stage of development is of a similar weight to that at oocyte fertilization, it has incipient matrotrophy (MI=0.6 to 0.7; Reznick et al. 2002). In other words, the energy necessary for development was obtained mostly from the yolk provided by the mother before oocyte fertilization. On the other hand, in a moderately matrotrophic species, the embryo's mass increases with progressive developmental stages ($0.8 < MI > 2$; Reznick et al. 2002), and the embryo obtains nearly all

its maternal provisioning during development through a placenta. The embryo therefore weighs more at the last stage of development than at oocyte fertilization. Extensive matrotrophy refers to species which completely lack yolk, such that all of the maternal provisioning occurs during embryonic development and weight at birth may be up to five times more than at oocyte fertilization (MI > 5 Reznick et al. 2002). There are extreme cases, such as in *Poecilia branneri* and *Poecilia bifura*, where the embryo at the last stage of development may weigh five times more than at fertilization (Pires et al. 2010). In each of the study species we obtained data on the matrotrophy index from previous studies (i.e. Pollux et al. 2014; Olivera-Tlahuel et al. 2015).

Estimation of the relationship between placental complexity, matrotrophy, and superfetation

Prior to comparative analyses, all variables, including matrotrophy index, were log-transformed to normalize the data. In addition, to explore whether maternal follicle size affected the magnitude of the morphological characters we studied, area of maternal follicle (AF) was included as an explanatory variable in linear regressions, in which each of the other five ultrastructural characteristics (NV, AV, NM, LM and TF) was used as the response variable. In the cases in which area of maternal follicle had a significant effect, the residuals of these linear regressions were used in subsequent comparative analyses.

We performed two separate sets of analyses. In the first set, we examined the relationship between matrotrophy index (MI) and our six morphological variables indicating placental complexity (NV, AV, NM, LM, TF and AF). In the second set, we looked

for differences between species with and without superfetation in the same six variables. In all cases we used two different evolutionary assumptions (i.e. Brownian motion, BM; Phylogenetic Generalized Least Squares, PGLS).

First, we assumed that the traits evolved by accumulating gradual changes and following a model of Brownian motion (BM), such that the phylogenetic relationships between species can explain a large part of the variation observed in the characteristics (Felsenstein, 1985). In the first set of analyses, we implemented this evolutionary model using Phylogenetic Generalized Least Squares (PGLS; Martins and Hansen, 1997), treating the matrotrophy index (MI) as a continuous independent variable and each of our six characteristics of interest as a response variable. In the second analysis, we also implemented a Brownian motion PGLS model to compare the six characteristics of interest between species with and without superfetation, treating the presence/absence of superfetation as a binary independent variable. PGLS is a regression technique which incorporates phylogenetic information into the error term of the model (Martins and Hansen, 1997).

In order to implement these comparative phylogenetic analyses, we used the family Poeciliidae phylogeny published by Pollux et al. (2014), trimmed to our species of interest. We created an ultrametric tree by transforming the original branch lengths to a scale based on relative time (Fig. 1). To do this transformation we used a semiparametric method based on penalized likelihood (Sanderson, 2002) implemented in the 'ape' package of the R program (Paradis et al. 2004; R Core Team, 2016).

Second, we assumed that traits evolved rapidly in the time and immediately adapted to the local environment with no trace of their phylogenetic history. Under this evolutionary assumption, phylogeny is unimportant, so we used Ordinary Least Squares regression (OLS). For the first set of data, we used this approach to look for a possible relationship between matrotrophy index (a continuous explanatory variable) and the six morphological characteristics of interest (dependent variables). For the second set of data, we used OLS to compare the different morphological characteristics between species with and without superfetation (binary explanatory variable; Garland et al. 2005; Lavin et al. 2008). We fit these PGLS and OLS regression models using the 'ape' and 'nlme' packages in R (Paradis et al. 2004; Pinheiro et al. 2016).

We used the Akaike Information Criterion (AIC) adjusted for small simple size (AICc; Burnham y Anderson, 2002) to compare the fit of the PGLS-BM and OLS models to the data for each of the morphological characteristics. The smallest AICc value indicates the model with the best fit, and a difference in AICc values between models greater than two units indicates a real difference in their capacity to explain the variation in the data.

RESULTS

Morphological description of maternal follicle

The follicular placenta in nearly all of the species was divided into two main layers, the inner and outer follicular layers. The inner follicular layer is the closest to the

277 embryonic placental tissues. This layer is structured by follicular epithelium with their
278 ovoid nucleus and organelles. Ultrastructurally is posible observe the density and size of
279 microvilli toward the embryonic part, and inside of follicular cells their nuclei, vesicles,
280 mitochondria and lipid droplets. We observed vesicles distributed randomly in the
281 follicular cells. Externally and in close contact with this inner follicular layer is found the
282 external follicular layer. In contrast, the outer follicular layer is composed mainly of blood
283 capillaries immersed in an extracellular matrix with collagen fibers. Presumably, the
284 function of this layer is to protect and nourish follicular cells and the embryo. In the
285 placentas of nearly all of the species we identified specific structures for nutrient
286 transport and cellular communication. In particular, in the follicular cells we found
287 prolongations of the membrane, or microvilli, oriented toward the embryo (Fig. 3A and
288 3C). In this same cells we also found a higher number of vesicles, mitochondria, and the
289 presence of cell junctions (i.e. desmosomes; 3A-C). All of these characteristics apparently
290 confer great nutrient exchange (Fig. 3B and 2C). In contrast, we found a large number of
291 blood capillaries in the external layer in close relation with follicular cells, which suggests a
292 high potential for gases and nutrients exchange (Fig. 3D).

293 We also observed the interaction between maternal and embryonic (pericardial
294 sac, yolk sac and tissue embryo) placental tissues (*Poeciliopsis prolifica,* MI=5.4; *Priapella*
295 *intermedia,* MI=0.6; Fig. 3E). However, both the follicular cells of the mother and the
296 embryonic tissue (pericardial sac) present microvilli, suggesting close physiological
297 communication. Specifically in *Poeciliopsis prolifica* and *Priapella intermedia,* we could
298 observe a large and uniform number of microvilli in the embryonic tissue (pericardial sac).

In fact, in these two species the microvilli are of different shape (Fig. 3E and 3F). In addition, in these species we observed a large number of blood capillaries that presumably transport oxygen and nutrients to embryo. This network of blood capillaries forms part of the embryonic pericardial sac.

In non-superfetating species (*Gambusia panuco,* MI=0.79*; Pseudoxiphophorus bimaculatus,* MI=0.65; *Poecilia mexicana,* MI=0.63; *Priapella intermedia,* MI=0.6; *Xiphophorus hellerii,* MI=0.61), like in superfetating species (*Heterandria formosa,* MI=35; *Poeciliopsis gracilis,* MI=0.84; *P. infans,* MI= 1.05; *P. prolifica*, MI=5.4; *P. viriosa,* MI=0.93), the follicular placenta consists of an inner and an outer follicular layer. In almost all species (except in two superfetating species: *Poeciliopsis presidionis* MI= 21.5 and *P. prolifica* MI=5.4), on the embryo side there is a layer without an apparent cellular structure called the follicular "ribbon" (described previously by Kwan et al. 2015). The follicular placentas of non-superfetating species were similar (Fig. 4A-E). In the maternal side, the follicular cells of inner follicular layer presented various types of cell junctions, nucleus, vesicles, mitochondria, and other organelles (Fig. 4A-E). In the outer follicular layer, we observed collagen fibers, fibroblasts, and blood capillaries. Only some of the species without superfetation presented microvilli of follicular cells (*Poecilia mexicana,* MI=0.63; *Priapella intermedia,* MI=0.6; *Xiphophorus hellerii,* MI=0.61). On average the non-superfetating species had 1.3 to 2 microvilli per cell, which is lower than in superfetating species (on average 7 microvilli per follicular cell). In contrast, non-superfetating species as *Gambusia panuco* (MI=0.79) and *Pseudoxiphophorus bimaculatus* (MI=0.65) did not present microvilli, which could suggest less nutrient transfer from

mother to embryos. In general non-superfetating species had a lower follicular cell thickness (6.46 µm) than superfetating species (9.87 µm; not statistically significant).

All of the superfetating species *Heterandria formosa,* MI=35; *Poeciliopsis gracilis,* MI=0.84; *P. infans,* MI= 1.05; *P. prolifica*, MI=5.4; *P. viriosa,* MI=0.93; *P. presidionis,* MI=21.5) had follicular placentas with similar morphological characteristics (Fig. 5-6). Like non-superfetating species, the follicular placenta of all superfetating species was composed of two layers (inner and outer follicular layers). In five of the six superfetating species, the inner follicular layer is formed by flat follicular epithelium with the nucleus, organelles, numerous vesicles, and microvilli (in the zone closest to the embryo; Fig. 5D-E). In the outer layer we found various arrangements of fibers and blood capillaries (Fig. 5A-E).In superfetating species the thickness of maternal follicle was greater than non-superfetating species, but only was a trend (average of 9.87 µm and 6.46 µm, respectively).

Unlike in all of the other (superfetating and non-superfetating) species, in *Poeciliopsis presidionis* (a superfetating specie; MI=21.5) the follicular placenta is arranged differently (Fig. 6). In this species, the epithelium of the inner follicular layer folds, forming peaks and valleys (Fig. 6A). We found that the epithelium has columnar cells and blood capillaries of outer follicular layer protrude towards the epithelium especially on the peaks (Fig. 6A). In contrast, the outer follicular layer maintains its lax connective tissue configuration and is composed of with high vascularization, fibroblasts, collagen fibers organized in several layers (Fig. 6A). In the inner follicular layer, the follicular cells have many vesicles, mitochondria and other organelles. In addition, the inner epithelium

343 presents fat droplets which stained strongly with uranyl (Fig. 6B-C). The thickness of
344 maternal follicle is clearly larger (average of 18.5 μm) than the other superfetating species
345 (average of 9.87 μm). Due to the large differences in the characteristics of the follicular
346 placenta in *P. presidionis* (MI=21.5) compared to the other species examined, we did not
347 include this species in the statistical comparisons between superfetating and non-
348 superfetating species.

349 **Relationship between degree of placentation, superfetation, and matrotrophy**

350 The electron microscopy and stereological methods we used allowed the clear
351 quantification of six follicular placenta characteristics (number of vesicles, NV; area of
352 vesicles, AV; number of microvilli, NM; length of microvilli, LM; thickness of maternal
353 follicle, TF; area of maternal follicle, AF; table 1). In the regressions in which we used AF as
354 an explanatory variable to examine the effect of maternal follicle on the other
355 morphological characteristics, NM and TF yielded significant results ($P < 0.05$ in both
356 cases), so the residuals from these regressions were used in subsequent analyses of these
357 two variables.

358 According to the models with the best fit, the number of vesicles (NV) and the area
359 of vesicles (AV) had a positive trend with the matrotrophy index (PGLS-BM model for NV:
360 slope = 0.05, $P = 0.098$; OLS model for AV: slope = 0.20, $P = 0.098$; table 2, Fig. 7). In the
361 particular case of the number of microvilli (NM), the best-fitting model (OLS) indicated
362 that the relationship between this variable and the matrotrophy index was positive but
363 not significant (table 2). However, assuming Brownian motion, this positive relationship

was significant (slope = 0.17, *P* = 0.052; Fig. 7). Under neither of the evolutionary assumptions was there a statistical relationship between LM or TF and matrotrophy index (table 2, Fig. 7). According to the OLS model (which had the best fit for this variable) the area of maternal follicle (AF) had a significant positive relationship with the matrotrophy index (slope = 0.16, *P* = 0.006, table 2; Fig. 7). This indicates that as a specie's matrotrophy index increases, so does its area of maternal follicle.

On the other hand, comparing the morphological ultrastructures of the placenta between superfetating (*Heterandria formosa, Poeciliopsis gracilis, P. infans, P.prolifica, P. viriosa*) and non-superfetating species (*Gambusia panuco, Pseudoxiphophorus bimaculatus, Poecilia mexicana, Priapella intermedia, Xiphophorus hellerii)*, the number of microvilli (NM) and area of maternal follicle (AF) were greater in species with superfetation (species with superfetation, NM= 7, AF= 159,464. 84 μm^2; species without superfetation NM= 1.3, AF= 66,833. 64 μm^2; Fig. 8) and in both cases the difference between superfetating and non-superfetating species was marginally significant in the best-supported models (NM: *P* = 0.074, AF: *P* = 0.059; table 3). In none of the other morphological variables and under neither of the evolutionary assumptions was there a significant difference between superfetating and non-superfetating species (table 3). However, in figure 8 we show that, in general, superfetating species had a trend toward positive values of most of the placental morphological characteristics we examined.

DISCUSSION

The follicular placenta of all species we analyzed, presented the same structures described previously by Jollie and Jollie (1964) in *Poecilia reticulata*. Specifically, we refer to the presence of an inner and an outer follicular layer. In the inner layer we identified numerous vesicles, microvilli on the apical surface (zone closest to embryonic tissue), cell junctions, and extensive endoplasmic reticulum. Grove and Wourms (1994) observed these same structures in the follicular placenta of *Heterendria formosa*. As such, we can conclude that the inner follicular layer in all species has a high degree of metabolic activity and is responsible for the transfer of nutrients from the mother to the embryo. For its part, the outer follicular layer is composed of lax connective tissue with high vascularization (blood capillaries), fibroblasts, and collagen fibers (Jollie and Jollie, 1964). As such, this outer layer appears to serve a protective function for the embryo and could facilitate gas exchange. An additional characteristic we noticed was the presence of a follicular ribbon or egg envelope (porous fertilization membrane), which was observed in some species of the genus *Heterandria* and *Poeciliopsis* (*Heterandria formosa, Poeciliopsis gracilis, P. latidens, P. occidentalis, P. turrubarensis* and *P. viriosa;* Grove and Wourms, 1994; Kwan et al. 2015). This structure is found between the inner follicular layer and the absorbent tissue of the embryo. In our study the follicular ribbon was observed in almost all species, except two species with superfetation and high matrotrophy index (*Poeciliopsis presidionis* MI= 21.5 and *P. prolifica* MI=5.4). This agrees with Kwan et al. (2015) that observed the follicular ribbon only in species with lecithotrophy and moderate

matrotroph (except in species with high matrotrophy index; *P. prolifica*, *P. turneri* y *P. retropinna*).

On the other hand, in *Poeciliopsis presidionis,* an extensively matrotrophic species (MI=21.5, Reznick et al. 2002), we observed hyper-placentation. This implies that the placenta of this specie has a totally different arrangement than that observed in the other ten species. This type of hyper-placentation consists of folds of the inner follicular layer and is composed of columnar-type cells. We found that the outer follicular layer follows the inner follicular layer fold (papillae-shaped) taking the capillaries close to the epithelium. In addition, the outer follicular layer presents connective tissue with several layers of collagen fibers. This type of placental morphology has been found in other species with extensive matrotrophy, such as *P. turneri* and *P. retropinna.* (Kwan et al. 2015). A similar arrangement to the type of hyper-placentation observed in these poecillid species has also been observed in the uterine epithelium of the omphalallantoic placenta in snakes (*Virginia striatula;* Blackburn and Steward, 2011). The association between a high matrotrophy index and follicular hyper-placentation indicates that the folding of the inner follicular layer (formed by columnar cells) maximizes the transfer of nutrients from the mother to the embryo due to a high secretory activity (Igarashi, 1961; Villagrán-Santa et al., 2005; Blackburn and Steward, 2011).

Several studies have used the matrotrophy index as an indicator of the degree of placentation (e.g. Reznick et al. 2002; Pollux et al. 2014; Ostrovsky et al. 2015). These studies have assumed that the species with a high matrotrophy index (which is simply the

quotient of the embryo's weight at birth divided by the egg's initial weight at fertilization) necessarily possess more specialized placentas. However, none of these studies used morphological evidence to validate this assumption. Recently, Kwan et al. (2015) were the first to demonstrate that, indeed, species with higher degrees of matrotrophy (i.e. higher values of the matrotrophy index) presented thicker and more structurally complex follicular placentas. In addition, they identified certain structures of the follicular placenta in these matrotrophic species, such as microvilli, which facilitate the exchange of nutrients between mother and embryo.

In this study, we present additional evidence that species with high degrees of matrotrophy have more complex placentas (higher values of the ultrastructural characteristics). In particular, we observed that area of maternal follicle, number of microvilli, number of vesicles, and size of these vesicles tend to increase with the matrotrophy index. These positive relationships indicate that species with elevated degrees of matrotrophy possess more specialized placentas *Heterandria formosa,* MI = 35; *Poeciliopsis prolifica,* MI = 5.4, which favor the active and continuous transfer of nutrients from the mother to the embryos during their development. In other words, more and larger vesicles permit the efficient exchange and movement of nutrients, such that the follicular cells present are more metabolically active (high micropinocytotic activity; Jollie and Jollie, 1964). In addition, the presence of microvilli indicates an increase in the surface area of contact between the follicular placenta and the embryo. So, the microvilli apparently increase the efficiency of the exchange of substances between the mother and embryo (Turner, 1940).

449 It is important to note that the species with the highest values of the matrotrophy
450 index (i.e. *Heterandria formosa,* MI = 35; *Poeciliopsis prolifica,* MI = 5.4) also had the
451 largest number and size of vesicles, highest number of microvilli, and highest follicular
452 area. This suggests that the specialization or complexity of the placentas in the species
453 studied only occurs at a matrotrophy index larger than five (species with extensive
454 matrotrophy, MI > 5; Reznick et al. 2002).

455 Finally, we should emphasize that the relationship between superfetation and
456 placental complexity had not been previously examined in the family Poeciliidae, and our
457 study is the first to demonstrate that superfetating species have placental ultrastructures
458 which reflect a higher degree of placental complexity (high exchange of substances) than
459 non-superfetating species. On average, superfetating species (*Heterandria formosa,*
460 *Poeciliopsis gracilis, P. infans, P. prolifica* and *P. viriosa)* had higher area of maternal
461 follicle and more microvilli than non-superfetating species (*Heterandria formosa,*
462 *Poeciliopsis gracilis, Poeciliopsis infans, Poeciliopsis prolifica, Poeciliopsis viriosa*). In
463 addition, superfetating species also showed a trend (though non-significant) toward
464 higher values of number and size of vesicles and length of microvilli compared to non-
465 superfetating species. These results support (despite the statistical power of the sample
466 size, 10 species) the hypothesis that species with superfetation should have a more
467 complex placenta given the need to differentially regulate the development of embryos at
468 different developmental stages (Zúñiga-Vega et al. 2010). This exploration of placental
469 complexity and its relationship with different levels of matrotrophy and superfetation still
470 needs to be carried out in a greater number of species.

ACKNOWLEDGEMENTS

This article was financed by the Consejo Nacional de Ciencia y Tecnología (CONACyT), project number 129675 and a doctoral fellowship (no. 368782/245650) granted to Claudia Olivera-Tlahuel. This article is a requirement for Claudia Olivera Tlahuel to obtain a doctorate in sciences in the Posgrado en Ciencias Biológicas at the Universidad Nacional Autónoma de México. The collection of specimens was authorized by the Comisión Nacional de Acuacultura y Pesca de México (permits SDPA/DGVS/03492, DGOPA-07010.210612.1749, and PPF/DGOPA-223/2013). We thank Pedro Medina Granados for help in obtaining fine sections and Israel Solano-Zavaleta for technical assistance.

REFERENCES

Amoroso EC. 1968. Evolution of viviparity. Proc. R. Soc. Med. J. 61: 1188-1200.

Blackburn D. G. 2015. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. J. Morph. 276: 961-990.

Blackburn DG, Stewart JR. 2011. Viviparity and placentation in snakes. Reproductive biology and phylogeny of snakes 9: 119-181.

Blackburn DG. 1992. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. Am. Zool. 32: 313-321.

Blackburn DG. 2005. Evolutionary origins of viviparity in fishes. In: Uribe MC, Grier HJ, eds. Viviparous Fishes I. New life publications, 287-301.

Burnham KP, Anderson DR. 2002. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. New York: Springer-Verlag.

Felsenstein J. 1985. Phylogenies and the comparative method. Am. Nat. 1985: 1-15.

Garland T, Bennett AF, Rezende EL. 2005. Phylogenetic approaches in comparative physiology. J Exp Biol 208:3015-3035.

Grove BD, Wourms JP. 1994. The follicular placenta of the viviparous fish, *Heterandria formosa*, In: ultrastructure and development of the embryonic absorptive surface. J. Morph. 209: 265-284.

Guerrero-Estévez SM. 2005. Estructura microscópica del ovario y la ovogénesis de dos especies de peces vivíparos: *Poeciliopsis gracilis* (poeciliidae) y *Chapalichthys encaustus* (Goodeidae). Tesis de maestría. Facultad de Ciencias, Universidad Nacional Autónoma de México.

Gundersen HJG., Bendtsen TF, KORBO L, Marcussen N, Møller A, Nielsen K., Nyengaard J. R, PAkkenberg B, Sørensen, FB, Vesterby A, West MJ. 1988. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. Apmis, 96: 379-394.

Gunn JS, Thresher, RE. 1991. Viviparity and the reproductive ecology of clinid fishes (Clinidae) from temperate Australian waters. Environ. Biol. Fish. 31; 323-344.

509 Haynes JL. 1995. Standardized classification of poeciliid development for life-history
510 studies. *Copeia*: 147-154.

511 Igarashi T. 1961. Histological and cytological changes in the ovary of a viviparous teleost,
512 *Neoditrema ransonneti* Steindachner during gestation. *北海道大學水産學部研究*
513 *彙報* Bulletin of the Faculty of Fisheries hokkaido University 12: 181-188.

514 Jollie WP, Jollie LG. 1964. The fine structure of the ovarian follicle of the ovoviviparous
515 poeciliid fish, *Lebistes reticulatus*. I. Maturation of follicular epithelium. J. Morph.
516 114: 479-501.

517 Kwan L, Fris M, Rodd FH, Rowe L, Tuhela L, Panhuis, TM. 2015. An examination of the
518 variation in maternal placentae across the genus *Poeciliopsis* (Poeciliidae). J.
519 Morph. 276: 707-720.

520 Lavin SR, Karasov WH, Ives AR, Middleton KM, Garland Jr T. 2008. Morphometrics of the
521 avian small intestine compared with that of nonflying mammals: a phylogenetic
522 approach. Physiol Biochem Zool 81: 526-550.

523 Marsh-Matthews E. 2011. Matrotropy. In: Evans JP, Pilastro A, Schlupp I, eds. Ecology and
524 evolution of poeciliid fishes. Chicago, IL: The University of Chicago Press, 28–37.

525 Martins EP, Hansen TF. 1997. Phylogenies and the comparative method: a general
526 approach to incorporating phylogenetic information into the analysis of
527 interspecific data. American Naturalist 149: 646–667.

528 Mossman HW. 1937. Comparative morphogenesis of the fetal membranes and accessory
529 uterine structures. Carnegie Inst. Contr. Embryol. 26: 129-246.

530 Olivera-Tlahuel C, Ossip-Klein AG, Espinosa-Pérez HS, Zúñiga-Vega JJ. 2015. Have
531 superfetation and matrotrophy facilitated the evolution of larger offspring in
532 poeciliid fishes? Biol. J. Linn. Soc. 116: 787-804.

533 Ostrovsky AN, Lidgard S, Gordon DP, Schwaha T, Genikhovich G, Ereskovsky AV. 2015.
534 Matrotrophy and placentation in invertebrates: a new paradigm. Biol. Rev. 91:
535 673-711.

536 Paradis E, Claude J, Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R
537 language. Bioinformatics 20:289–290.

538 Pinheiro J, Bates D, DebRoy S, Sarkar D and R Core Team. 2016. NLME: linear and
539 nonlinear mixed effects models. R package version 3.1-125. Available at
540 http://CRAN.R-project.org/package=nlme

541 Pires MN, Arendt J, Reznick DN. 2010. The evolution of placentas and superfetation in the
542 fish genus Poecilia (Cyprinodontiformes: Poeciliidae: subgenera Micropoeciliia and
543 Acanthophacelus). Biol. J. Linn. Soc. 99: 784–796.

544 Pollux BJA, Meredith RW, Springer MS, Reznick DN. 2014. The evolution of the placenta
545 drives a shift in sexual selection in livebearing fish. Nature 513:233–236.

546 Pollux BJA, Pires MN, Banet AI, Reznick DN. 2009. Evolution of placentas in the family
547 Poeciliidae: an empirical study of macroevolution. Ann. Rev. Ecol. Evol. Syst. 40:
548 271–289.

549 R Core Team. 2016. R: A language and environment for statistical computing. R
550 Foundation for Statistical Computing. Available at https://www.R-project.org/

551 Rasband WS. 1997-2016. ImageJ. U. S. National Institutes of Health, Bethesda, Maryland,
552 USA, https://imagej.nih.gov/ij/

553 Reznick DN, Mateos M, Springer MS. 2002. Independent origins and rapid evolution of the
554 placenta in the fish genus *Poeciliopsis*. Science 298 1018-1020.

555 Reznick DN, Meredith R, Collette BB. 2007. Independent evolution of complex life history
556 adaptations in two families of fishes, live-bearing halfbeaks (Zenarchopteridae,
557 Beloniformes) and Poeciliidae (Cyprinodontiformes). Evolution 61: 2570-83.

558 Reznick DN, Miles DB. 1989. Review of life history patterns in poeciliid fishes. In: Meffe GK,
559 Snelson FF Jr., editors. Ecology and evolution of livebearing fishes (Poeciliidae).

560 Sanderson MJ. 2002. Estimating absolute rates of molecular evolution and divergence
561 times: a penalized likelihood approach. Mol. Biol. Evol. 19:101–109.

562 Scrimshaw NS. 1944. Superfetation in poeciliid fishes. Copeia 1944: 180-183.

563 Turner CL. 1937. Reproductive cycles and superfetation in poeciliid fishes. Biol. Bull.
564 72:145-164.

565 Turner CL. 1940. Pseudoamnion, pseudochorion, and follicular pseudoplacenta in poeciliid
566 fishes. J. Morph. 67: 59-89.

567 Turner CL. 1947. Viviparity in teleost fishes. Sci. Mon. 65: 508-518.

568 Villagrán SM, Méndez FR, Stewart JR. 2005. Placentation in the Mexican lizard *Sceloporus*
569 *mucronatus* (Squamata: Phrynosomatidae). J. Morph. 264: 286-297.

570 Wourms JP, Grove BD, Lombardi J. 1988. The maternal-embryonic relationship in viviparous
571 fishes. Fish Physiol. 11: 1-134.

572 Wourms JP. 1981. Viviparity: the maternal fetal relationships in fishes. Am. Zool. 21: 473–
573 515.

574 Zúñiga-Vega JJ, Macías-Garcia C, Johnson J. B. 2010. Hypotheses to explain the evolution
575 of superfetation in viviparous fishes. In: Uribe MC, Grier HJ, eds. Viviparous Fishes
576 II. New life publications, 13-30.

577

578 **FIGURE LEGENDS**

579 **Fig. 1.** Phylogeny of the ten species used in the study. Black rectangles indicate presence
580 of superfetation. Modified from Pollux et al. (2014).

581 **Fig. 2.** Follicular placenta of *Poeciliopsis infans* (superfetating). Shown are the inner (IFL)
582 and outer (OFL) follicular layers. The arrow indicates a blood capillaries. Nucleus of

583 follicular cell (NF). The white lines represent the thickness of each layer. The scale bar
584 shows 2 µm.

585 **Fig. 3. A.** Sections of follicular placenta of *Poeciliopsis infans* (superfetating). Shown are
586 vesicles (V) and microvilli (MV) dispersed within the cells of the inner follicular layer. Inner
587 follicular layer (IFL), outer follicular layer (OFL). **B.** Section of the inner follicular layer of
588 *Poeciliopsis viriosa* (superfetating). Shown are vesicles (V), mitochondria (M) and cell
589 junctions (CJ) of the follicular cell. **C.** Section of the inner follicular layer of *Heterandria*
590 *formosa* (superfetating). Shown are vesicles (V) and microvilli (MV) in the apical side of
591 follicular cell. **D.** Section of the follicular placenta of *Poeciliopsis infans* (superfetating).
592 Blood capillary is shown (indicated by arrow) in outer follicular layer by close contact with
593 inner follicular layer. There are two types of nuclei, nucleus of follicular cell (NF) and
594 nucleus of connective tissue (NC). **E.** Interaction between maternal (IFL) and embryonic
595 tissues (E) in *Poeciliopsis prolifica* (superfetating). A large number of microvilli (MV) are
596 evident, nucleus of follicular cell and abundant endoplasmic reticulum (ER) are shown. The
597 embryo epithelium of pericardial sac shows several and thin microvilli. **F.** Vascularized
598 embryonic tissue (pericardial sac) of *Priapella intermedia* (non-superfetating). A large
599 number of microvilli are shown (MV). Arrows indicate blood capillaries. The scale bar
600 shows 2 µm in panels A, D, E, F and 200 nm in panel B.

601 **Fig. 4.** Different follicular placentas in species without superfetation **A.** *Gambusia panuco.*
602 **B.** *Pseudoxiphophorus bimaculatus.* **C.** *Poecilia mexicana.* **D.** *Priapella intermedia.* **E.**
603 *Xiphophorus hellerii*. Inner follicular layer (IFL), outer follicular layer (OFL), nucleus of

follicular cells (NF),), nucleus of follicular cells (NC), nucleus of endothelial cells (NE), vesicles (V), cell junctions (CJ) and follicular ribbon (R). The scale bar shows 2 µm.

Fig. 5. Different follicular placentas in species with superfetation **A.** *Heterandria formosa.* **B.** *Poeciliopsis gracilis.* **C.** *Poeciliopsis infans.* **D.** *Poeciliopsis prolifica.* **E.** *Poeciliopsis viriosa*. Inner follicular layer (IFL), outer follicular layer (OFL), nucleus of follicular cells (NF), nucleus of endothelial cells (NE), vesicles (V), cell junctions (CJ) and follicular ribbon (R). The scale bar shows 2 µm.

Fig. 6. Follicular hyper-placenta of *Poeciliopsis presidionis* (superfetating). The inner follicular layer (IFL) is folded and its cells are type columnar. The outer follicular layer (OFL) is composed of with high vascularization, fibroblasts, collagen fibers organized in several layers. Large quantities of lipid droplets (LD) are seen, strongly stained with uranyl. The follicular nuclei are columnar (FN) and the arrow indicates blood capillaries. Nucleus of follicular cells (NF). The scale bar indicates 2 µm.

Fig. 7. Relationships between matrotrophy and different morphological characteristics of the placentas of 10 species of poeciliid fish. The broken line indicates the relationship assuming Brownian motion (i.e. accounting for phylogeny using phylogenetic generalized least squares, PGLS-BM). The solid line shows the predicted relationship using ordinary least squares (OLS), which does not account for phylogeny.

Fig. 8. Average of the values of different morphological characters of the placentas for species without superfetation (gray bars) and with superfetation (black bars). Error bars show one standard error.

TABLE 1. Summary of data and results of the morphological characteristics of placentas of 10 species from the Poeciliidae family. MI indicates the matrotrophic index. SF indicates the presence or absence of superfetation. NV indicates the average number of vesicles per follicular cell. AV represents the average area of these vesicles. NM indicates the mean number of microvilli per follicular cell. LM indicates the average length of these microvilli. TF indicates the mean thickness of maternal follicle. AF represents the average area of maternal follicle.

SPECIES	Geographic coordinates	MI	SF	NV	AV (μm^2)	NM	LM (μm)	TF (μm)	AF (μm^2)
Gambusia panuco	N 20° 41' W 103° 57	0.79	NO	14.28	168.428	0	0	10.45	114228.75
Pseudoxiphophorus bimaculatus	N 17° 8' W 95° 7'	0.65	NO	21.07	38.237	0	0	5.64	62860.51
Heterandria formosa	Private aquarium	35	YES	48.83	535.205	13.60	0.85	16.92	352053.58
Poecilia mexicana	N 17° 26' W 95° 26'	0.63	NO	19.77	336.019	1.33	0.49	5.00	48473.19
Poeciliopsis gracilis	N 21° 59' W 99° 15'	0.84	YES	18.57	99.865	3.68	0.78	8.84	89901.33

Poeciliopsis infans	N 20° 34’ W 104° 9	1.05	YES	21.23	332.560	3.11	0.62	9.23	153007.38
Poeciliopsis prolifica	N 23° 03’ W 105° 50’	5.4	YES	22.07	527.738	8.10	0.86	6.87	99651.54
Poeciliopsis viriosa	N 21° 02’ W 104° 22’	0.93	YES	20.57	33.717	5.00	0.75	7.50	102710.40
Priapella intermedia	N 17° 9’ W 95° 10’	0.6	NO	36.43	53.694	1.50	1.22	4.86	38902.24
Xiphophorus hellerii	N 20° 34’ W 104° 9’	0.61	NO	20.67	312.286	1.33	1.71	6.39	69703.54

TABLE 2. Results of two regression models with different evolutionary assumptions examining the relationship between the matrotrophic index and different morphological characteristics of the placentas of 10 species from the Poeciliidae family. These two models used Phylogenetic Generalized Least Squares, assuming a Brownian motion evolutionary model (PGLS-BM) and an Ordinary Least Squares model, which assumes that characteristics adapt immediately to their environment. In all cases, the independent variable was the matrotrophic index and the dependent variables the average number of vesicles per follicular cell (NV), area of those vesicles (AV), average number of microvilli per follicular cell (NM) and the average length of these microvilli (LM), the average thickness of maternal follicle(TF) and the average area of maternal follicle (AF). The fit of each model was evaluated using the Akaike Information Criterion adjusted for small sample size (AICc). Also shown is the difference in AICc (⊿AICc) between each model and the model with the best fit. For each characteristic, the models are listed from best to worst fit.

Response variable	Regression model	AICc	ΔAICc	Slope	*P*
NV	PGLS-BM	3.62	0.00	0.052	0.098
	OLS	4.29	0.67	0.075	0.043
AV	OLS	23.52	0.00	0.195	0.097
	PGLS-BM	24.95	1.42	0.198	0.097
NM	OLS	16.91	0.00	0.082	0.266

	PGLS-BM	19.49	2.57	0.171	0.052
LM	OLS	4.13	0.00	0.227	0.503
	PGLS-BM	39.94	35.81	0.131	0.639
TF	PGLS-BM	-9.95	0.00	-0.009	0.452
	OLS	-9.86	0.09	-0.006	0.641
AF	OLS	9.74	0.00	0.164	0.006
	PGLS-BM	15.33	5.59	0.061	0.323

TABLE 3. Results of two regression models with different evolutionary assumptions examining the relationship between the presence or absence of superfetation and different morphological characteristics of the placentas of 10 species from the Poeciliidae family. These two models used Phylogenetic Generalized Least Squares, assuming a Brownian motion evolutionary model (PGLS-BM) and an Ordinary Least Squares model, which assumes that characteristics adapt immediately to their environment. In all cases, the independent variable was the matrotrophic index and the dependent variables the average number of vesicles per follicular cell (NV), area of those vesicles (AV), average number of microvilli per follicular cell (NM) and the average length of these microvilli (LM), the average thickness of maternal follicle (TF) and the average area of maternal follicle (AF). The fit of each model was evaluated using the Akaike Information Criterion adjusted for small sample size (AICc). Also shown is the difference in AICc (∆AICc) between each model and the model with the best fit. For each characteristic, the models are listed from best to worst fit. The regression slope shown denotes the difference between species with and without superfetation.

Response variable	Regression model	AICc	ΔAICc	Slope	*P*
NV	PGLS-BM	2.80	0.00	0.127	0.363
	OLS	6.48	3.68	0.058	0.579
AV	OLS	24.23	0.00	0.188	0.554

	PGLS-BM	24.85	0.62	0.224	0.679
NM	OLS	13.01	0.00	0.310	0.074
	PGLS-BM	20.12	7.11	0.286	0.483
LM	PGLS-BM	36.44	0.00	1.037	0.363
	OLS	38.46	2.02	1.082	0.183
TF	OLS	-11.96	0.00	-0.023	0.492
	PGLS-BM	-12.22	0.26	-0.009	0.870
AF	PGLS-BM	9.73	0.00	0.446	0.059
	OLS	11.20	1.47	0.344	0.034

Fig. 1

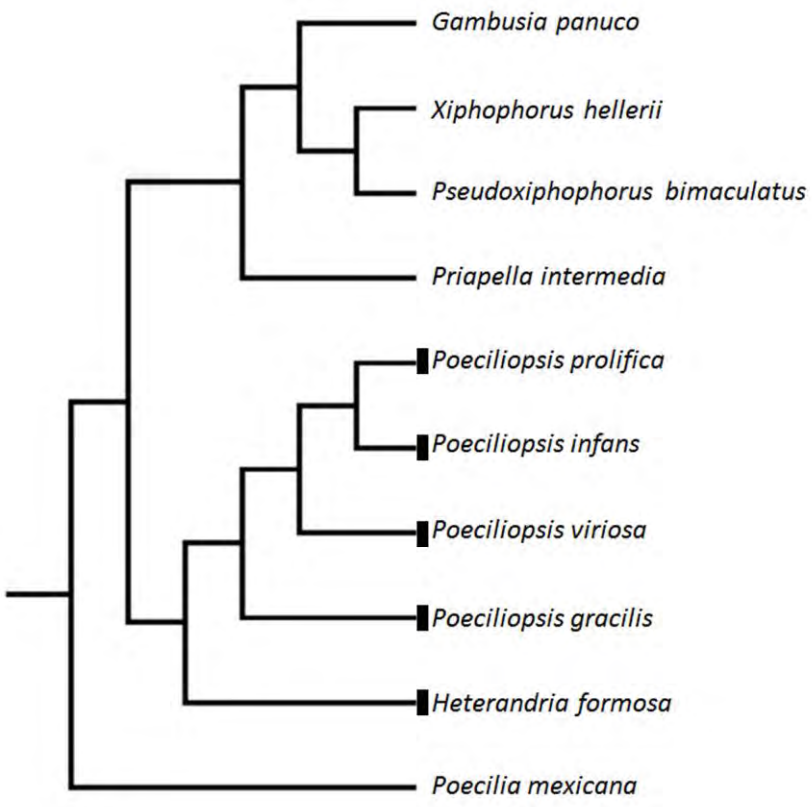

Gambusia panuco
Xiphophorus hellerii
Pseudoxiphophorus bimaculatus
Priapella intermedia
Poeciliopsis prolifica
Poeciliopsis infans
Poeciliopsis viriosa
Poeciliopsis gracilis
Heterandria formosa
Poecilia mexicana

Fig. 2

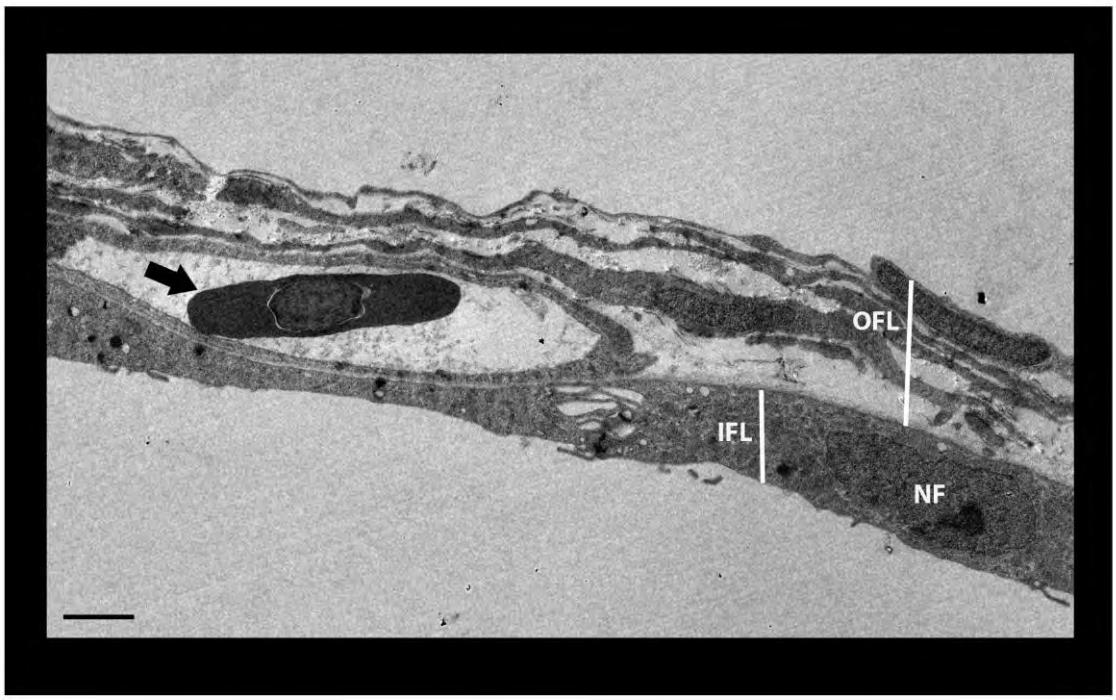


Fig. 3

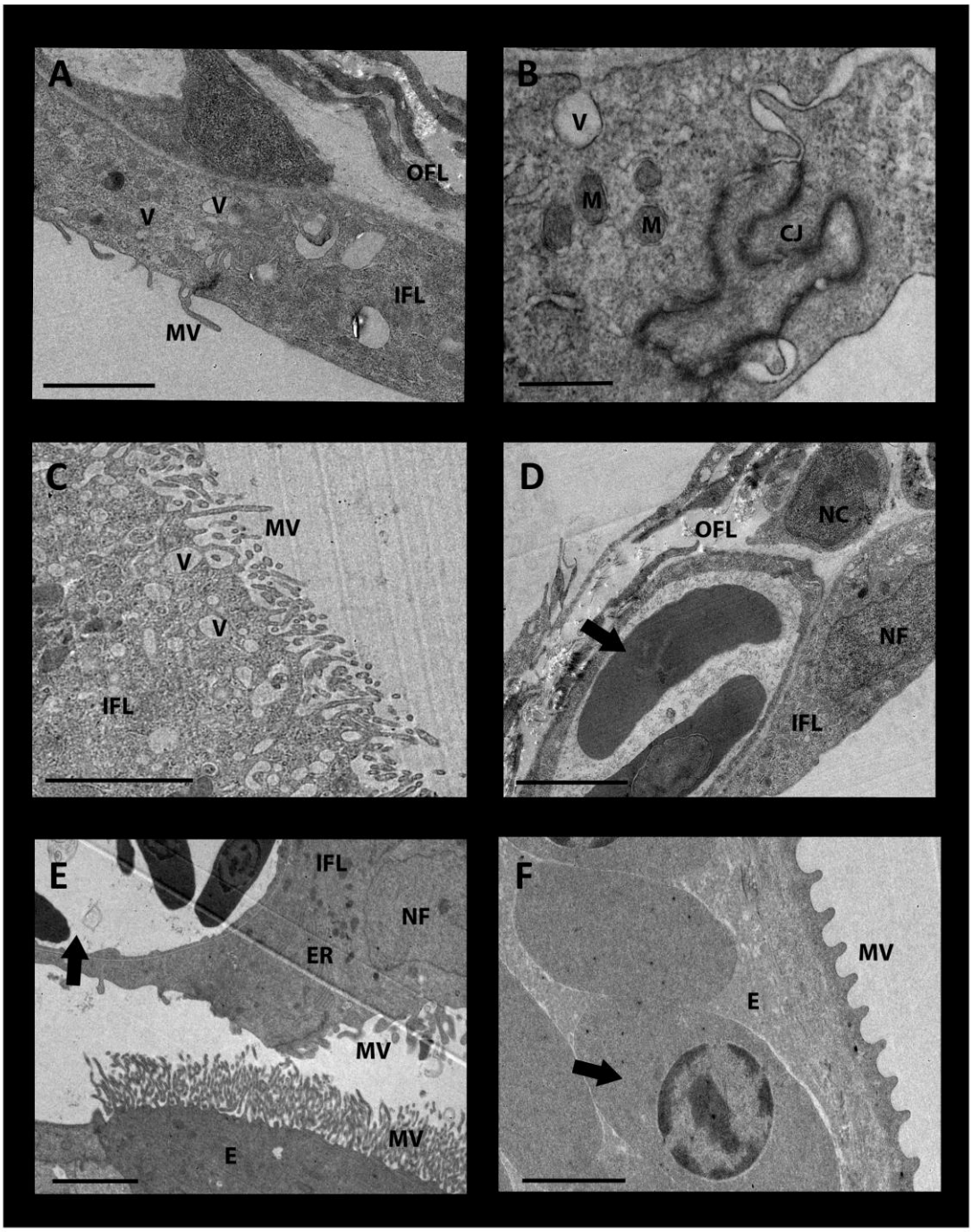

A
OFL
V
V
IFL
MV
B
V
M
M
CJ
C
MV
V
V
IFL
D
OFL
NC
NF
IFL
E
IFL
NF
ER
MV
MV
E
F
MV
E

Fig. 4

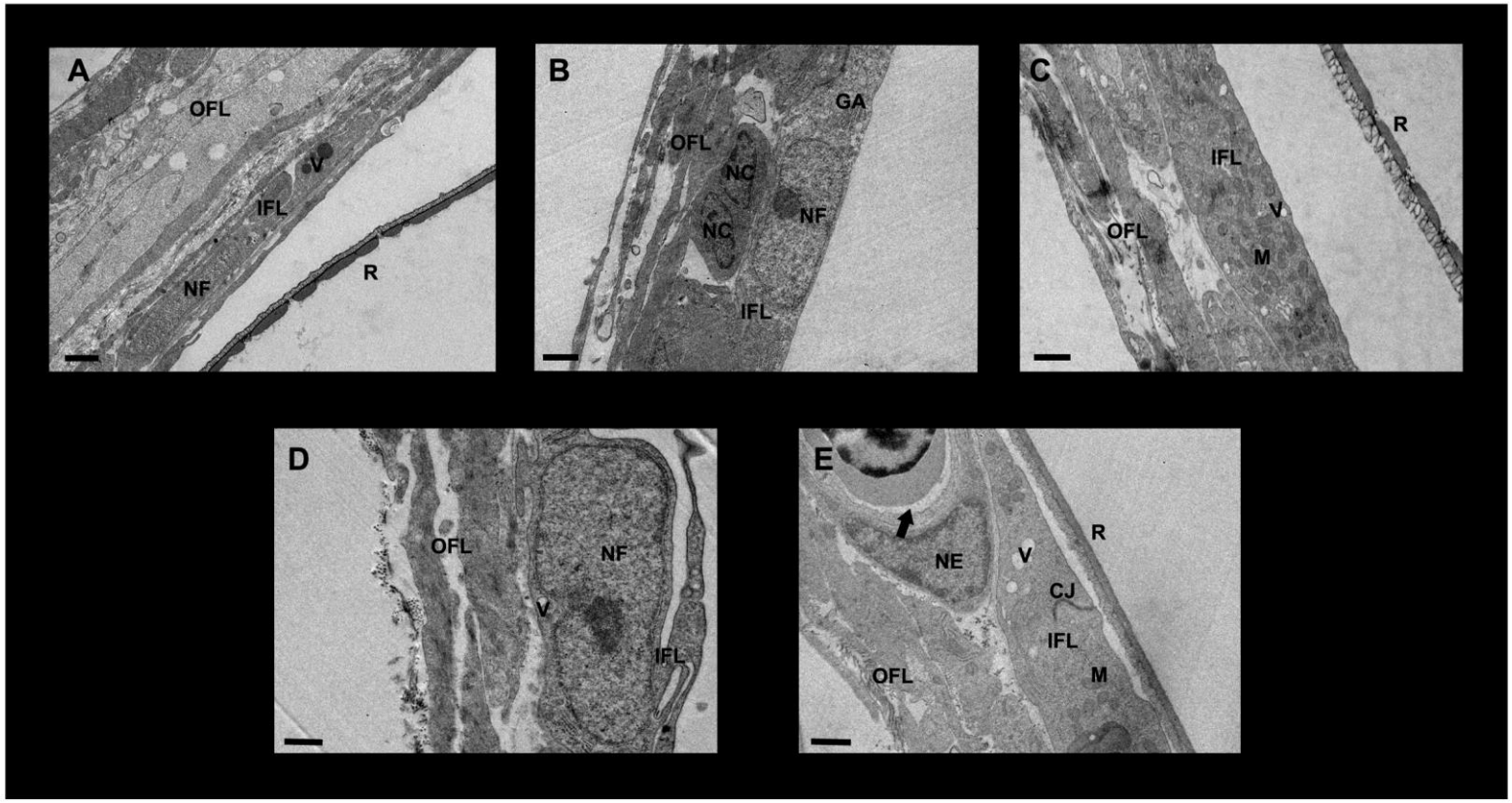

A
OFL
V
IFL
NF
R
B
GA
OFL
NC
NF
NC
IFL
C
R
IFL
V
OFL
M
D
OFL
NF
V
IFL
E
R
NE
V
CJ
IFL
OFL
M

Fig. 5

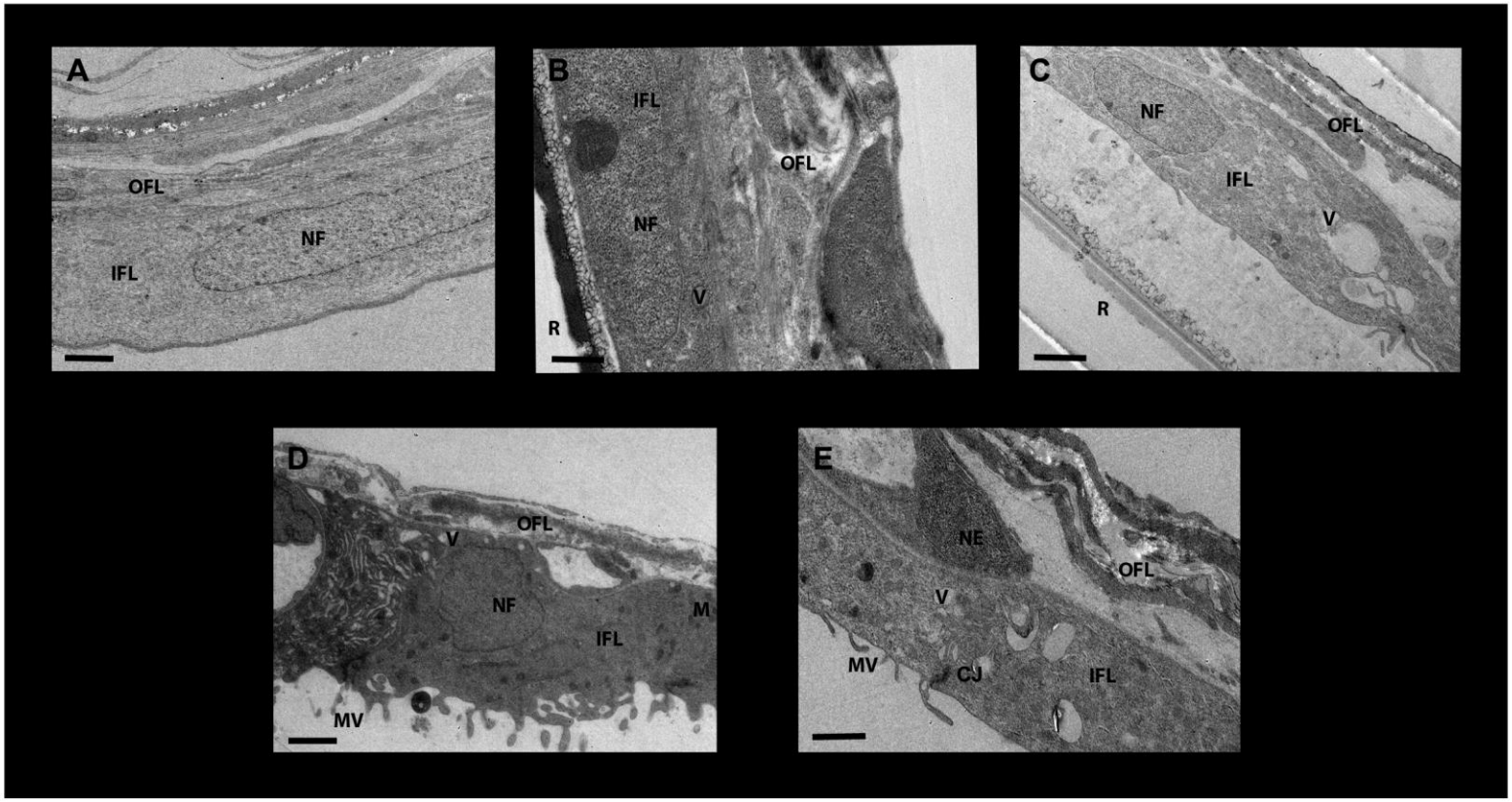

A
OFL
NF
IFL
B
IFL
OFL
NF
V
R
C
NF
OFL
IFL
V
R
D
V
OFL
NF
M
IFL
MV
E
NE
OFL
V
MV
CJ
IFL

Fig. 6

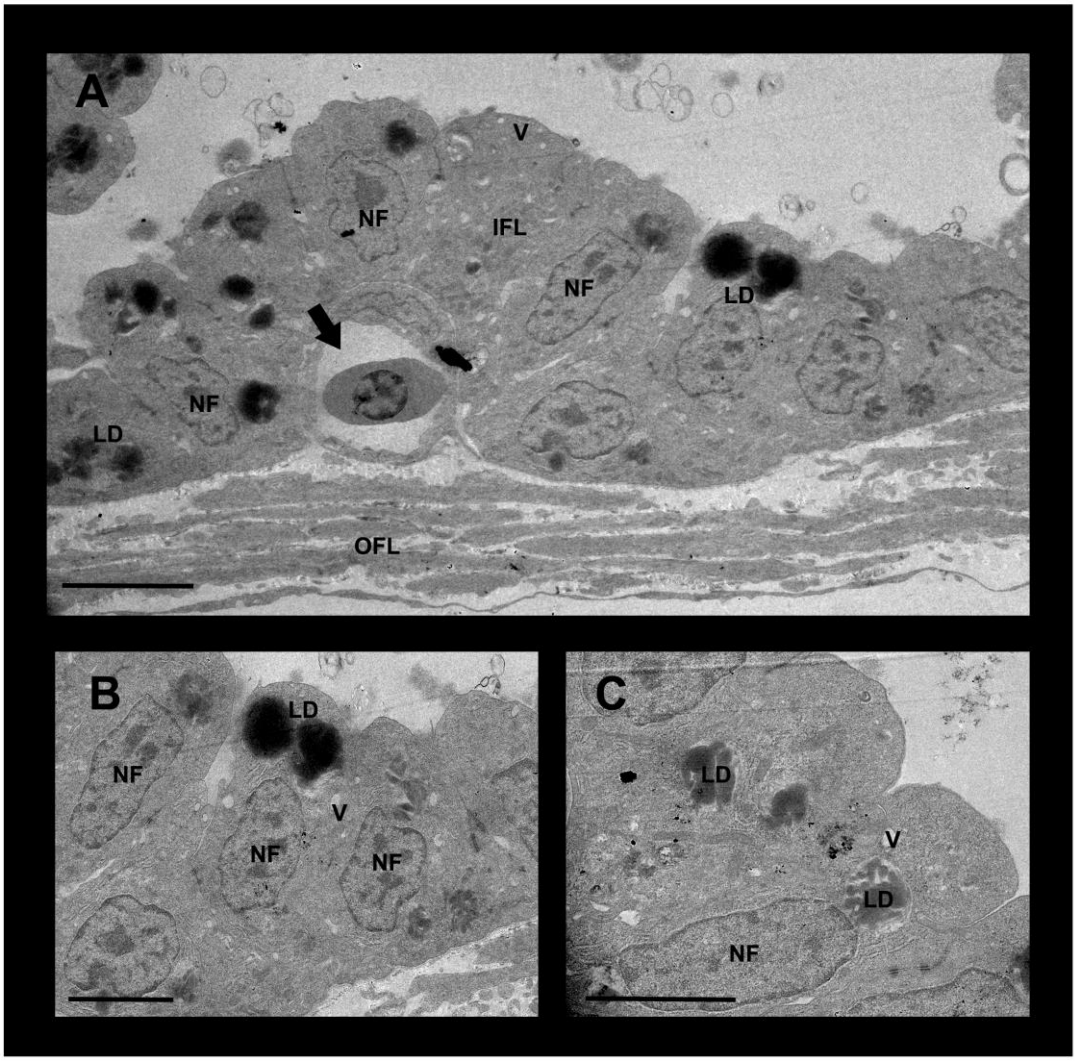


Fig. 7

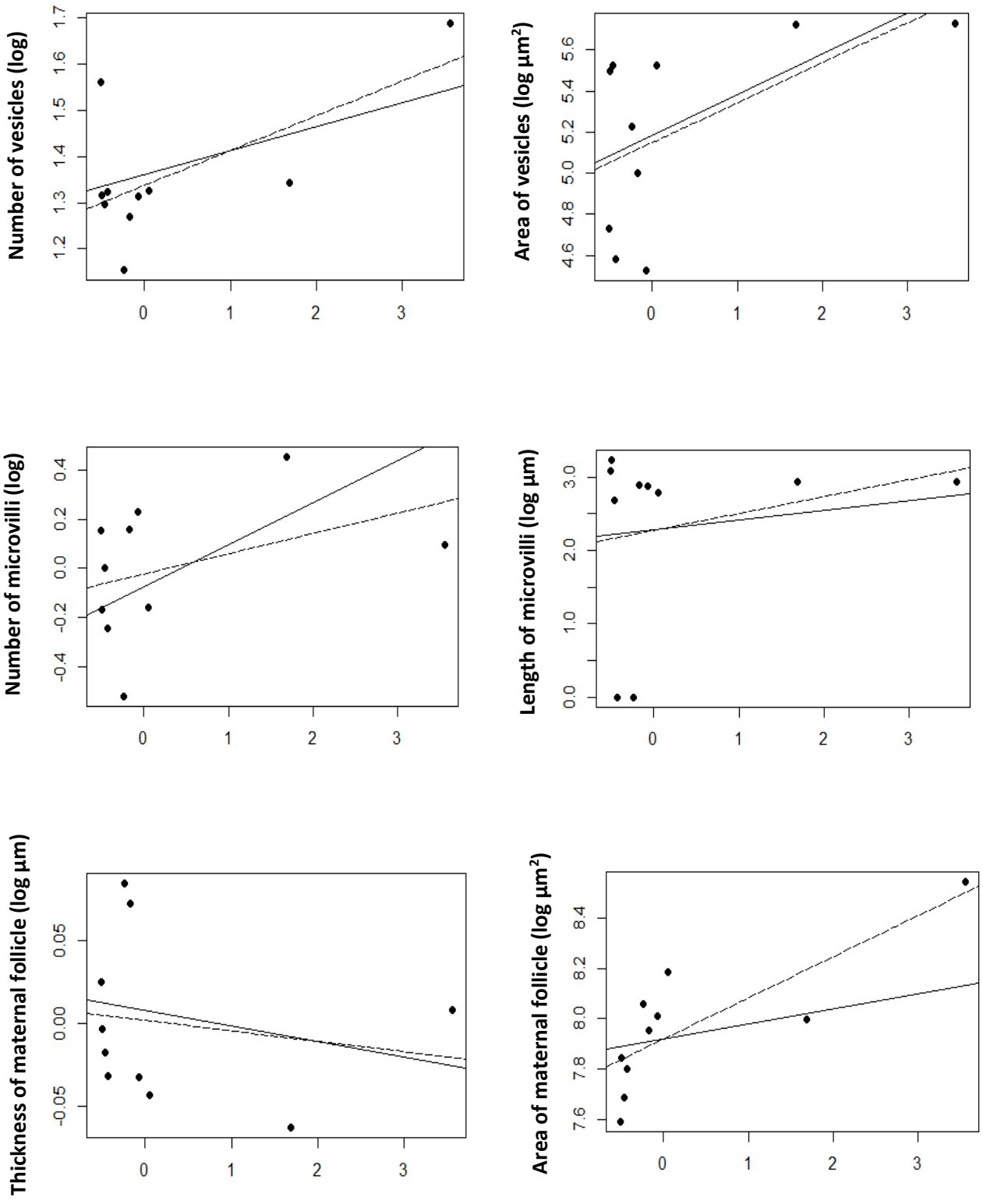
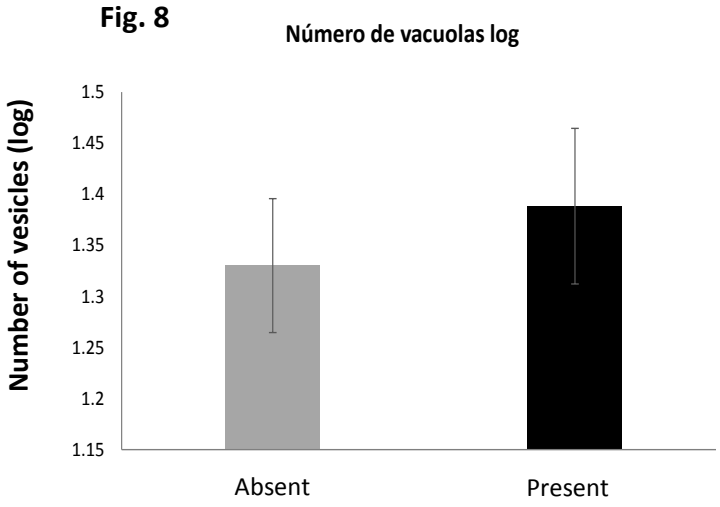
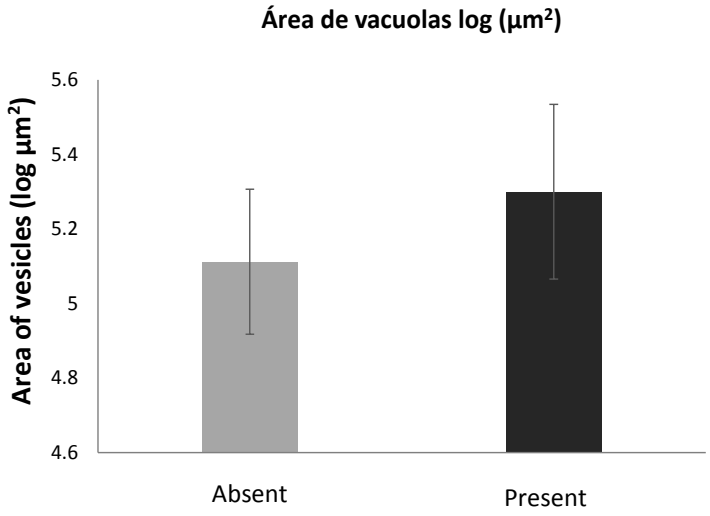
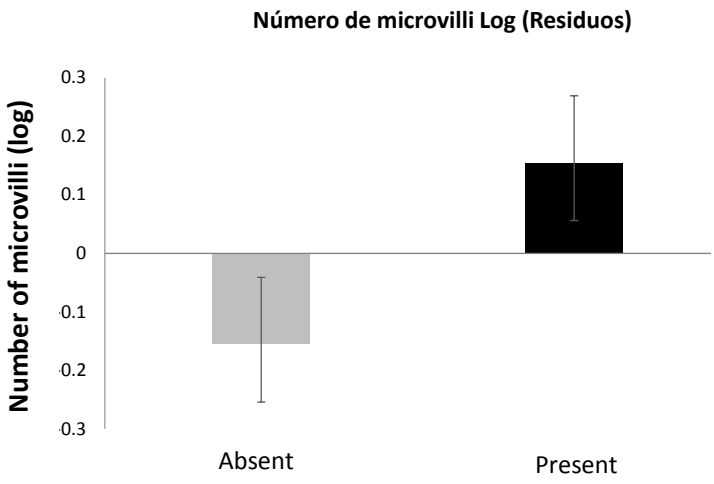
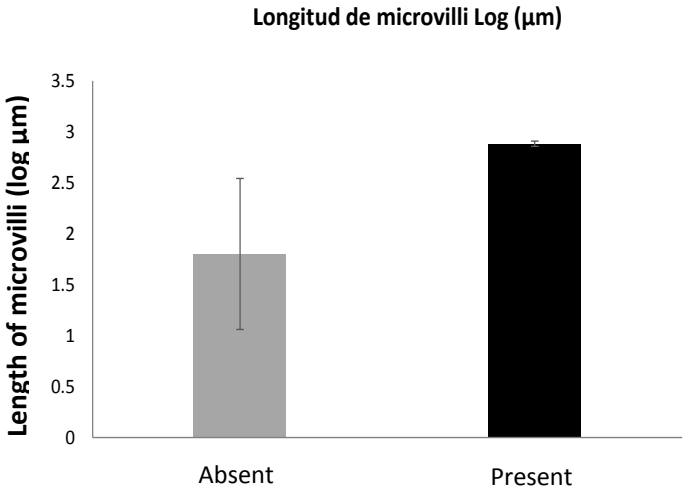

Number of vesicles (log)
Area of vesicles (log µm²)
Number of microvilli (log)
Length of microvilli (log µm)
Thickness of maternal follicle (log µm)
Area of maternal follicle (log µm²)
Matrotrophy index (log)

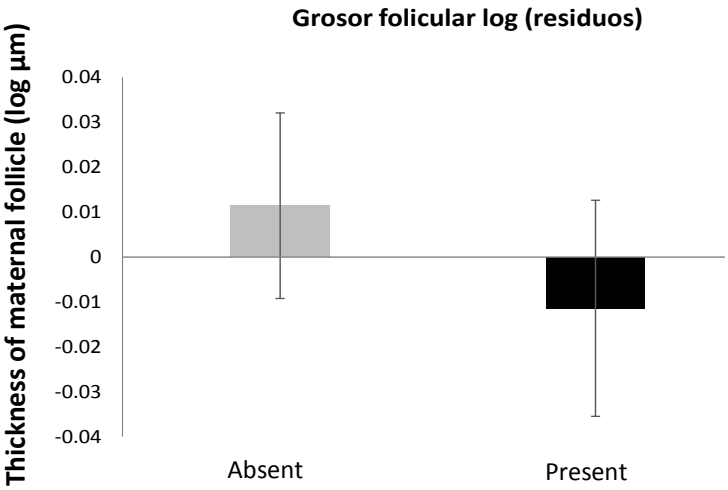
Fig. 8

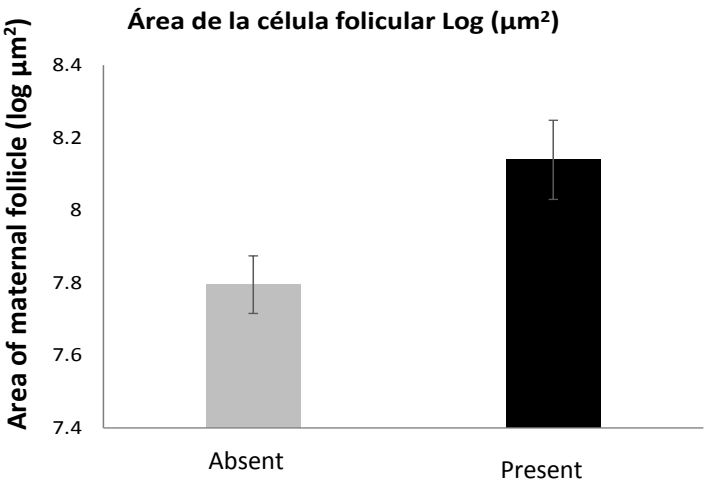












Superfetation

CAPÍTULO II

Morphological structures for potential sperm storage in poeciliid fishes. Does superfetation matter?

Claudia Olivera-Tlahuel, Maricela Villagrán-Santa Cruz, Norma A. Moreno-Mendoza, J. Jaime Zúñiga-Vega

Journal of Morphology, 2017: in press.

Received: 9 November 2016 | Revised: 26 January 2017 | Accepted: 17 March 2017

DOI: 10.1002/jmor.20684

RESEARCH ARTICLE

WILEY

Morphological structures for potential sperm storage in poeciliid fishes. Does superfetation matter?

Claudia Olivera-Tlahuel[1] | Maricela Villagrán-Santa Cruz[2] | Norma A. Moreno-Mendoza[3] | J. Jaime Zúñiga-Vega[1]

[1]Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria 04510, Ciudad de México, México

[2]Departamento de Biología Comparada, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria 04510, Ciudad de México, México

[3]Departamento de Biología Celular y Fisiología, Instituto de investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad Universitaria 04510, Ciudad de México, México

Correspondence
J. Jaime Zúñiga-Vega, Facultad de Ciencias, Universidad Nacional Autónoma de México. Ciudad Universitaria 04510, Ciudad de México, México.
Email: jzuniga@ciencias.unam.mx

Funding information
Funding for this project was provided by Consejo Nacional de Ciencia y Tecnología (CONACyT) and Secretaría de Educación Publica from México through the grant 129675. C.O.T received a doctorate scholarship from CONACyT (no. 368782/245650).

Abstract

Sperm storage within the female reproductive tract has been reported as a reproductive strategy in several species of vertebrates and invertebrates. However, the morphological structures that allow for sperm to be stored and kept viable for long periods are relatively unknown in osteichthyes. We use histological and stereological tools to identify and quantify sperm storage structures (spermathecae) in 12 species of viviparous Poeciliidae. We found spermathecae in nine species, six of which exhibit superfetation (the ability of females to simultaneously carry within the ovary two or more broods of embryos at different stages of development). These spermathecae are folds of ovarian tissue that close around spermatozoa. We compared the number and size (volume) of spermathecae between species with and without superfetation. Species that exhibit superfetation had a significantly higher number of spermathecae than species that do not exhibit this reproductive strategy. In addition, we found that the mean volume of spermathecae and total volume of spermathecae present in the ovary are marginally higher in species with superfetation. Our results contribute to the understanding of the morphological structures that allow for sperm storage in viviparous osteichthyes and suggest a positive relationship between superfetation and the capacity of females to store sperm.

KEYWORDS

Poeciliidae, sperm storage, spermathecae, superfetation, viviparity

1 | INTRODUCTION

In many animal species, females are able to store viable sperm within their reproductive tracts for long periods of time (Orr & Zuk, 2012). This phenomenon is present in some groups of insects (Simmons, 2001), reptilian sauropsids (Birkhead & Møller, 1993; Sever & Hamlett, 2002), amphibians (Sever, 2002), osteichthyes (Potter & Kramer, 2000), cartilaginous fishes (Pratt, 1993), and mammals (Birkhead & Møller, 1993). Among these diverse taxa, the retention time of sperm can vary from only a few hours to years after mating. For example, insects such as bee and termite queens can store sperm for nearly their entire lives (several years) (Orr & Zuk, 2012). Conversely, the majority of female mammals retain sperm for only a few hours (Orr & Zuk, 2012). There is also variation among taxa in the specific location within the female reproductive tract where spermatozoa are stored. For example, in reptilian sauropsids, sperm storage occurs in the oviduct (Villagrán-Santa Cruz, Mendoza-Cruz, Granados-González, Rheubert, & Hernández-Gallegos, 2017), whereas in bats spermatozoa are stored in the uterus (Birkhead & Møller, 1993).

In the osteichthyes, where internal fertilization has evolved independently several times (Gross & Shine, 1981; Mank, Promislow, & Avise, 2005), sperm retention has only been documented for Anablepidae, Cottidae, Embiotocidae, Sebastidae, and Poeciliidae (Darling, Noble, & Shaw, 1980; Fraser & Renton, 1940; Koya, Munehara, & Takano, 2002; Muñoz, Koya, & Casadevall, 2002; Turner, 1938; Vila, Sabat, Hernandez, & Munoz, 2007). In these groups of fish, sperm storage may occur in the ovary or in the gonoduct (Darling et al., 1980; Koya et al., 2002; Vila et al., 2007). For example, in *Helicolenus*

dactylopterus (Sebastidae) sperm retention occurs in storage chambers within the ovary that are lined by a cuboidal epithelium supported by muscle cells (Vila et al., 2007). The spermatozoa are concentrated inside these chambers, which are connected via a duct to the lumen of the ovary. In *Xiphophorus maculatus* (Poeciliidae), spermatozoa have been observed in association with the ovary and gonoduct epithelium (Potter & Kramer, 2000).

In poeciliid fishes, the ability to store sperm has been suggested because of the long time (several months) during which isolated females are able to produce young (Clark & Aronson, 1951; Greven, 2011; Hildemann & Wagner, 1954; Hubbs, 1964, 1997). However, the morphological structures where spermatozoa may be stored have only been examined in *Heterandria formosa*, *Poecilia formosa*, *Poecilia reticulata*, *X. maculatus*, and *Poeciliopsis gracilis* (Constantz, 1989, Kobayashi & Iwamatsu, 2002; Potter & Kramer, 2000; Turner, 1947; Uribe, De la Rosa-Cruz, Guerrero-Estévez, García Alarcón, & Aguilar-Morales, 2004; Uribe & Grier, 2011; Uribe, Grier, De la Rosa-Cruz, & Schartl, 2016). Temporary sperm storage structures (called "solid plugs" and "sperm storage micropockets") have been observed in *H. formosa* and *P. reticulata*; these structures are formed by the closure of ovarian folds that contain spermatozoa (Fraser & Renton, 1940; Kobayashi & Iwamatsu, 2002).

In this study, our first objective was to locate sperm storage structures, which we will refer to as "spermathecae", in viviparous species of Poeciliidae. The term "spermathecae" has been previously used to describe specialized sperm storage structures in other groups such as amphibians and insects (Dallai, 1975; Gupta & Smith, 1969; Pool & Hoage, 1973; Tombes & Roppel, 1972). For example, in salamanders, spermatozoa are stored in a pigmented structure (spermatheca) that opens into the oviducts (Pool & Hoage, 1973). Similarly, in beetles, spermathecae are abdominal invaginations near the reproductive tract which store viable sperm (Tombes & Roppel, 1972).

The study of morphological structures for potential sperm storage in poeciliid fishes may provide insight into some of the mechanisms and processes associated with superfetation, which is a particular mode of reproduction that occurs in some poeciliid species. Superfetation is the ability of females to simultaneously carry in the ovary multiple broods of embryos at different developmental stages (Turner, 1937, 1940; Zúñiga-Vega, Macías-Garcia, & Johnson, 2010). Apparently, superfetation has evolved independently several times within the Poeciliidae (Meredith, Pires, Reznick, & Springer, 2011; Pires, Banet, Pollux, & Reznick, 2011; Pollux, Pires, Banet, & Reznick, 2009). Some species have a high degree of superfetation; for example, *H. formosa* can carry up to eight simultaneous broods, each at a different developmental stage (Scrimshaw, 1944). Other species, such as *Poeciliopsis turrubarensis*, have a lower degree of superfetation, and can only carry two or three simultaneous broods (Zúñiga-Vega, Reznick, & Johnson, 2007). Many other species, such as *Gambusia vittata*, do not exhibit superfetation, and females only produce one group of embryos at a time (Pires et al., 2011).

Many studies have attempted to find adaptive explanations for superfetation (Frías-Alvarez & Zúñiga-Vega, 2016; Macías-Garcia & González-Zuarth, 2005; Olivera-Tlahuel, Ossip-Klein, Espinosa-Pérez, & Zúñiga-Vega, 2015; Pollux et al., 2009; Travis, Farr, Henrich, & Cheong, 1987; Zúñiga-Vega et al., 2007, 2010). However, no study to date has focused on describing the morphological and physiological mechanisms that may allow superfetation to occur. For this reason, the second objective of this study is to explore the possibility of a relationship between the capacity for sperm storage and the presence of superfetation in members of the family Poeciliidae.

This second objective is derived from the hypothesis that superfetating species need to frequently fertilize mature oocytes, which are constantly developing in order to form new broods (Macías-Garcia & González-Zuarth, 2005; Pollux, Meredith, Springer, & Reznick, 2014). In this sense, superfetation would depend, to some extent, on morphological and physiological mechanisms that would allow females constant access to spermatozoa. As such, we expected to observe more and larger spermathecae in species with superfetation compared to non-superfetating species, where we expected to observe fewer, smaller, or even no spermathecae, because females without superfetation only develop one brood of embryos at a time and would therefore not need such frequent access to spermatozoa.

In addition, the potential association between sperm storage within spermathecae and superfetation might as well be adaptive. Most superfetating species do not exhibit apparent signs of precopulatory female choice (Macías-Garcia & González-Zuarth, 2005; Pollux, Meredith, Springer, & Reznick, 2014). Males are smaller than females, lack ornaments, and do not exhibit courtship behaviors (Magurran, 2011). Thus, the concurrent presence of both superfetation and sperm storage may facilitate cryptic (post-copulatory) female choice because sperm competition may take place within spermathecae and spermatozoa of the highest quality could then be used to fertilize different broods (Evans & Pilastro, 2011). Previous studies in poeciliids have demonstrated that ovarian fluid mediates sperm competition by increasing the longevity of some particular spermatozoa (Gasparini & Evans, 2013; Gasparini & Pilastro, 2011). These physiological processes may take place within spermathecae.

2 | MATERIALS AND METHODS

2.1 | Study species and histological protocol

We collected gestating females of 12 viviparous species of Poeciliidae (Table 1). The majority of these females were collected from different rivers in Mexico (collection of individuals was conducted under ethical approval from the National Commission of Aquaculture and Fisheries from Mexico; permits DGOPA.07010.210612.1749 and PPF/DGOPA-233/2013). The only exceptions were females of *H. formosa*, which were acquired from a private aquarium in the United States. Six of our study species exhibit superfetation, whereas the other six do not (Table 1). Given that in Mexico most of the species with superfetation belong to the genus *Poeciliopsis* (Miller, Minckley, & Norris, 2005), our sample of superfetating species includes five from this genus.

We collected three visibly pregnant females of each species, which were anesthetized (using 3-amenobenzoic acid ethyl ester) and sacrificed (by means of an overdose of the same anesthetic) upon capture to extract ovaries. Immediately following their extraction, the ovaries

TABLE 1 Summary of data and results for spermathecae of 12 species of the family Poeciliidae

Species	Geographic coordinates	*N*	SF	Ovary volume (mm^3)	Number of spermathecae	Total volume of all spermathecae (mm^3)	Volume of each spermatheca (mm^3)	Relative volume of all spermathecae	Relative volume of each spermatheca
Gambusia panuco	N 20° 41'	3	NO	274.8	0.7	0.61	0.31	0.0022	0.0011
	W 103° 57'								
Poecilia butleri	N 20° 9'	3	NO	955.6	0.0	–	–	–	–
	W 103° 2'								
Poecilia mexicana	N 17° 26'	3	NO	637.0	4.7	2.67	0.63	0.0050	0.0010
	W 95° 26'								
Priapella intermedia	N 17° 9' W 95° 10'	3	NO	218.4	0.0	–	–	–	–
Pseudoxiphophorus bimaculatus	N 17° 8' W 95° 7'	3	NO	457.2	0.0	–	–	–	–
Xiphophorus hellerii	N 20° 34'	3	NO	441.6	2.0	4.04	0.67	0.0058	0.0010
	W 104° 9'								
Heterandria formosa	Private aquarium	3	YES	47.8	7.0	0.89	0.13	0.0196	0.0027
Poecilioposis infans	N 20° 34'	3	YES	222.0	17.7	24.74	1.59	0.1140	0.0074
	W 104° 9'								
Poeciliopsis gracilis	N 21° 59'	3	YES	297.1	15.3	43.25	2.63	0.1761	0.0129
	W 99° 15'								
Poeciliopsis presidionis	N 22° 29'	3	YES	240.4	4.7	1.61	0.35	0.0119	0.0034
	W 105° 21'								
Poeciliopsis prolifica	N 23° 03' W 105° 50'	3	YES	195.3	5.0	5.47	1.46	0.0408	0.0076
Poeciliopsis viriosa	N 21° 02'	3	YES	631.8	8.0	4.69	0.55	0.0118	0.0012
	W 104° 22'								

SF indicates presence or absence of superfetation. All values represent averages per species (across all individual ovaries). Relative volumes represent proportions with respect to total ovary volume. *N* = sample size.

were fixed in Bouin's solution for 30 min and then stored in 70% ethanol until processing. In the laboratory, each ovary was dehydrated prior to embedding in paraffin. Then, the entire ovaries were serially sectioned longitudinally in 6-μm section thickness using a rotary microtome (HistoStat 820). Finally, sections were stained using hematoxylin-eosin (Presnell, Schreibman, & Humason, 1997). We carried out this protocol for a total of 36 individual ovaries (three per each species).

2.2 | Identification and stereology of spermathecae

Each of the histological preparations (all serial sections of each ovary) was examined using a light microscope in order to detect as many spermathecae as possible. We defined a spermatheca as a closed structure (complete closure of the ovarian folds) that contains spermatozoa originating from the ovarian lumen after a mating event. The criterion for identifying a spermatheca was the presence of spermatozoa accumulated in a specific region completely surrounded by ovarian tissue (i.e., surrounded by a layer of epithelial cells, which was in turn surrounded by a layer of conjunctive tissue). We did not consider structures with small openings into the ovarian lumen to be spermathecae, but rather deep ovarian folds.

Each spermatheca was serially photographed (at 6-μm intervals) under 40x magnification. Photographs were then analyzed stereologically using the "Volumest" plug-in for the program ImageJ (Schneider, Rasband, & Eliceiri, 2012) to quantify the volume of each spermatheca. For each of the three ovaries that we examined per species, we quantified the total number of spermathecae, the total volume of all spermathecae, and the average volume per spermatheca. Then, for each of these variables we calculated the average across the three ovaries to obtain a representative value for each species.

2.3 | Estimating differences between species with and without superfetation

We searched for differences in the number, total volume, and average volume of spermathecae between species with and without

superfetation using two different evolutionary assumptions. First, we assumed that these traits evolved under Brownian motion (BM) and, hence, phylogenetic relatedness would account for most of the observed interspecific variation (Felsenstein, 1985). We implemented this evolutionary model in our comparison between species with and without superfetation by means of phylogenetic generalized least squares (PGLS) (Martins & Hansen, 1997), treating the presence/absence of superfetation as a binary (dummy) independent variable. PGLS is a regression technique that incorporates phylogenetic information into the error structure of the model. We used the phylogeny of the Poeciliidae published by Pollux et al. (2014), trimmed to our focal species. We created an ultrametric tree by transforming the original branch lengths to a scale based on relative time (Figure 1). To do so, we used a semiparametric smoothing method based on penalized likelihood (Sanderson, 2002) implemented in the R package "ape" (Paradis, Claude, & Strimmer, 2004; R Core Team, 2016). We used a smoothing parameter equal to 0.1, which is a conservative value (Revell & Reynolds, 2012). We used our resulting ultrametric tree to estimate how many times superfetation has evolved throughout the phylogenetic history of our study species by means of a likelihood-based ancestral reconstruction method implemented in the program MESQUITE 3.0 (Maddison & Maddison, 2009).

Second, we assumed that traits evolve fast and adapt immediately to their local environments, leaving behind no trace of their phylogenetic history. Under this evolutionary assumption, phylogeny is unimportant and, thus, we used ordinary least-squares regression (OLS) to compare number, total volume, and average volume of spermathecae between species with and without superfetation (Garland, Bennett, & Rezende, 2005; Lavin, Karasov, Ives, Middleton, & Garland, 2008). We fitted PGLS and OLS regression models using the R packages "ape" and "nlme" (Paradis et al., 2004; Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2016).

We compared the fit of these two evolutionary models using the Akaike information criterion (AIC) adjusted for small sample sizes (AICc) (Burnham & Anderson, 2002). The smallest AICc value indicates the best-fitting model. A difference between models in AICc values larger than 2 units indicates a real difference in their fit to the data. Originally, we considered using a third evolutionary model that would estimate the amount of phylogenetic signal in the relationship between superfetation and our response variables. This alternative model would indicate how important the phylogeny is in explaining the effect of superfetation, rather than assuming that phylogeny either explains most of the data (PGLS assuming BM) or is unimportant (OLS). However, such alternative model requires the estimation of one additional parameter (i.e., a measure of phylogenetic signal such as λ) (Freckleton, Harvey, & Pagel, 2002; Pagel, 1999) and our sample size (12 species) was too small to allow a precise estimation of this additional parameter.

Before analyses, we log-transformed the number of spermathecae. This variable was not statistically affected by the volume of the ovary (slope = –0.007, $p = 0.33$). In contrast, total and average volume of spermathecae were expressed as proportions of the total volume of the ovary to make these two variables fully comparable among species. These proportions were arcsine-transformed before analyses to meet assumptions of normality and homogeneity of variances (Zar, 2010). To test the effect of superfetation we used our full data set (12 species) for the number of spermathecae and a reduced data set (nine species) for total and average volume of spermathecae, because we did not find spermathecae in three species (Table 1).

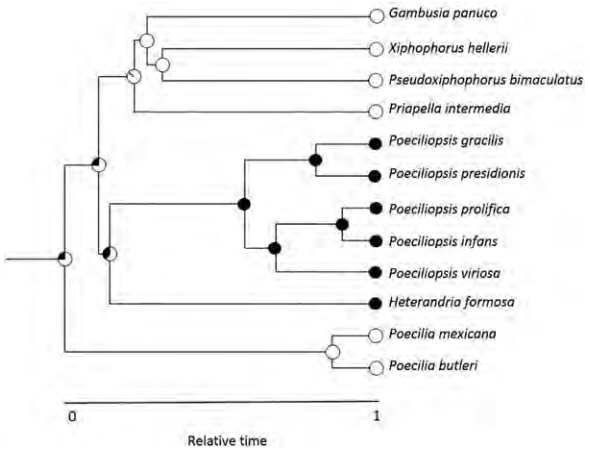


FIGURE 1 Ultrametric phylogenetic tree of the study species from the family Poeciliidae. Branch lengths are expressed in relative time. In the ancestral nodes, circles depict proportional likelihoods for the presence (black) and absence (white) of superfetation

3 | RESULTS

3.1 | Morphology of spermathecae

We found spermathecae in the ovaries of nine of the twelve species we examined (*Gambusia panuco*, *Xiphophorus hellerii*, *Poecilia mexicana*, *Poeciliopsis prolifica*, *Poeciliopsis infans*, *Poeciliopsis viriosa*, *Poeciliopsis presidionis*, *P. gracilis*, and *H. formosa*; Figures 2–4). In general, we observed that the spermathecae were not found in a specific area within the ovary, but rather were distributed more or less randomly, though always near the ovarian lumen. The spermathecae were frequently located next to blood vessels. For example, in *G. panuco*, *P. prolifica*, *P. infans*, and *P. viriosa* (Figures 2a–c and 3a–i), a large number of erythrocytes are visible near the spermathecae.

We confirmed that the spermathecae are composed of ovarian tissue and are generally oval or circular structures that completely surround spermatozoa to protect and store them within the ovary (Figures 2–4). In Figures 2–4, we present representative images of the proximal (a, d, g), middle (b, e, h), and distal sections of these structures (c, f, i). In all cases, the spermathecae were formed by thickened ovarian epithelium (brackets in Figures 2–4) that opens and closes to contain and protect spermatozoa. In Supporting Information, we provide additional serial sections of one spermatheca from each species to show their morphologies in greater detail (Supporting Information Figure S1).

The spermatozoa were oriented in a particular way within the spermathecae, with the heads in direct contact with the thickened

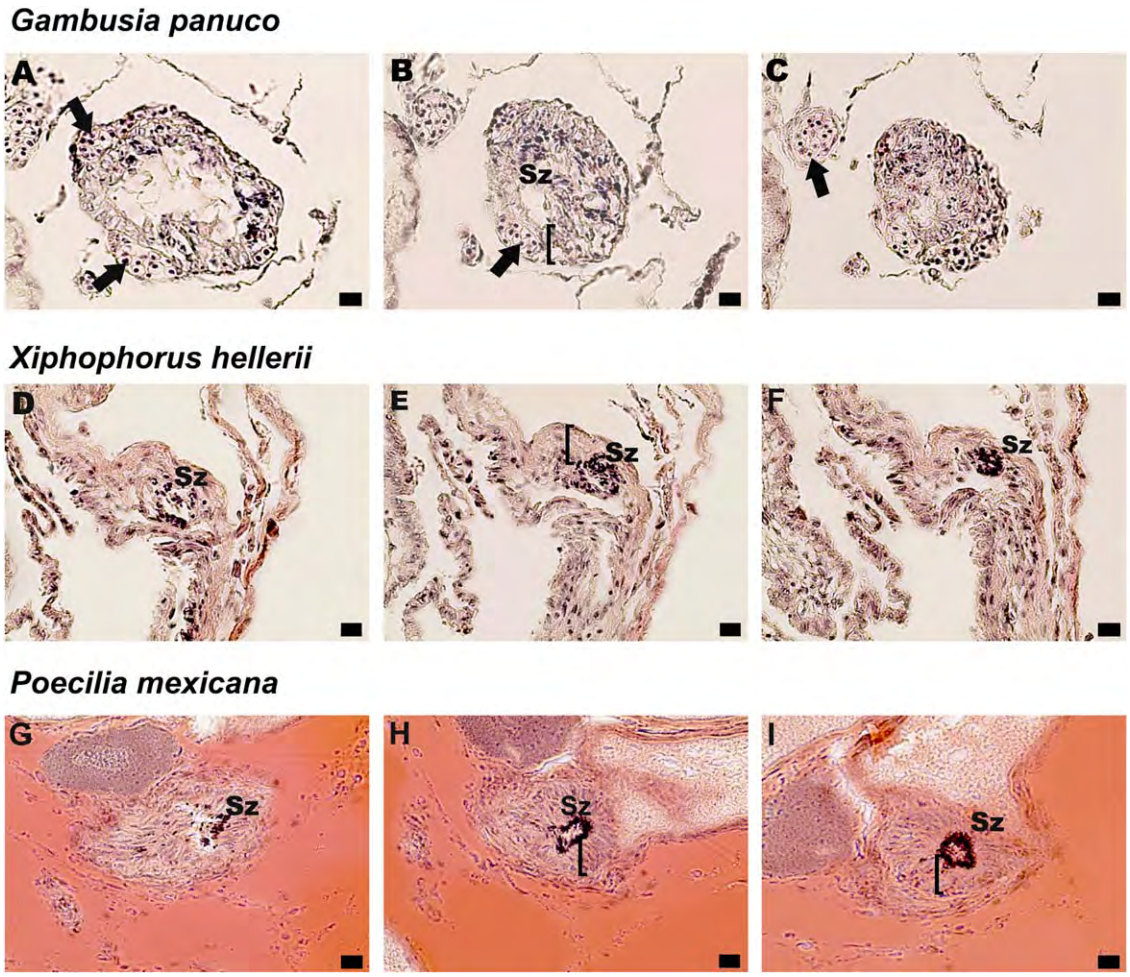


FIGURE 2 Sections of ovarian tissue stained using the hematoxylin-eosin technique that show the spermathecae observed in *G. panuco*, *X. hellerii*, and *P. mexicana* (species without superfetation). Heads of spermatozoa within the spemathecae stain blue (Sz). The different panels show representative images of the proximal (a, d, g), middle (b, e, h), and distal (c, f, i) sections of the spermathecae. Arrows point out erythrocytes. Brackets indicate thickened ovarian wall. Scale bar = 10 μm

ovarian tissue and the tails towards the lumen of the spermatheca (Figures 2h,i and 4g–i). However, we did observe some spermathecae in which spermatozoa were dispersed around the interior (e.g., *P. prolifica*, see Supporting Information, Figure S1).

3.2 | Quantification of the spermathecae and comparison between species with and without superfetation

All of the ovaries we examined from superfetating species had spermathecae. In contrast, the ovaries of three of the species that do not superfetate (*Priapella intermedia*, *Pseudoxiphophorus bimaculatus*, and *Poecilia butleri*) lacked spermathecae (Table 1). In addition, in two of the species that do not superfetate (*X. hellerii* and *G. panuco*), we found spermathecae in only one of the three ovaries we examined (6 and 2 spermathecae, respectively). We did not find apparent differences in the morphology of spermathecae between superfetating and non-superfetating species (Figures 2–4 and Supporting Information, Figure S1).

The maximum number of spermathecae that we observed per individual ovary was 22 in one female of *Poeciliopsis infans* (a species with superfetation). The absolute volume of each spermatheca varied widely, from 0.11 mm^3 in one female of *H. formosa* to 3.42 mm^3 in one female of *P. gracilis*. However, much of this variation in the size of spermathecae can be explained by variation in ovary size (*H. formosa* is a small species and *P. gracilis* is relatively large). After correcting for ovary size (expressing volume of spermathecae as proportions of ovary volume), the average relative volume of each spermatheca varied from 0.001 in *X. hellerii* and *P. mexicana* (both species without superfetation) to 0.0129 in *P. gracilis* (a species with superfetation). Table 1 shows average values per species for number, total volume, and average volume of spermathecae.

We identified three groups of species that clearly differ in the relative volume of all spermathecae (Table 1). First, species with the smallest proportion of total ovary volume (<.01) devoted to the production of spermathecae. This group is composed of the three species without superfetation in which we observed spermathecae (*G. panuco*, *P. mexicana*, and *X. hellerii*). The second group includes four superfetating

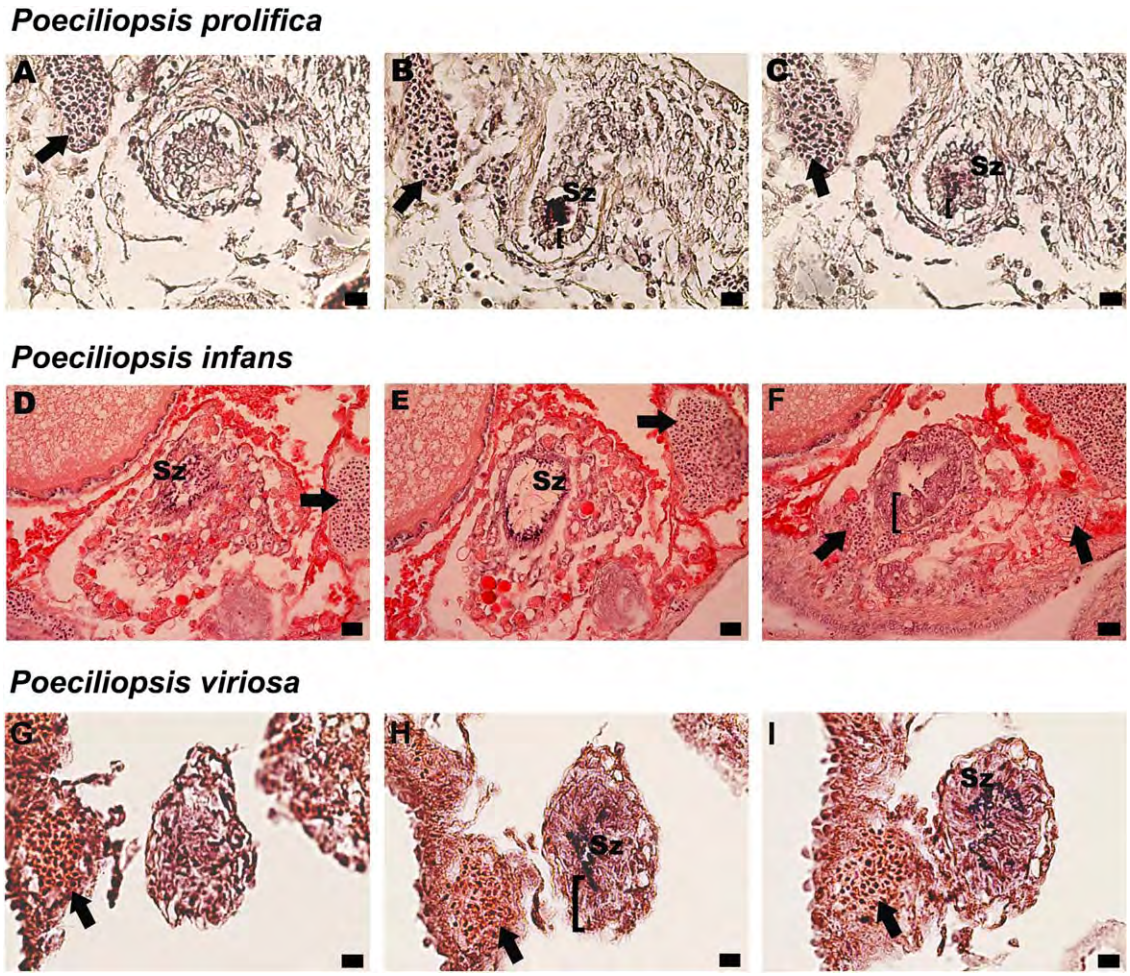


FIGURE 3 Sections of ovarian tissue stained using the hematoxylin-eosin technique that show the spermathecae observed in *P. prolifica*, *P. infans*, and *P. viriosa* (species with superfetation). Heads of spermatozoa within the spemathecae stain blue (Sz). The different panels show representative images of the proximal (a, d, g), middle (b, e, h), and distal (c, f, i) sections of the spermathecae. Arrows point out erythrocytes. Brackets indicate thickened ovarian wall. Scale bar = 10 μm

species with a total relative volume of spermathecae between 0.01 and 0.04 (*H. formosa*, *P. presidionis*, *P. prolifica*, and *P. viriosa*). The third group includes the two superfetating species with the largest proportions of ovary volume devoted to spermathecae (0.11 and 0.18 in *P. infans* and *P. gracilis*, respectively; Table 1).

We found some uncertainty with respect to how many times superfetation has evolved throughout the phylogenetic history of our study species. Both presence and absence of superfetation were almost equally likely in the common ancestor of *Poeciliopsis* and *Heterandria* (the two genera where superfetation is present; Figure 1). If this common ancestor exhibited superfetation, then this reproductive strategy only evolved once. In contrast, if this common ancestor lacked superfetation, then this mode of reproduction evolved twice independently: once on the branch leading to the common ancestor of all *Poeciliopsis* species and once on the branch leading to *H. formosa* (Figure 1).

In all three response variables, the model that assumed phylogenetic independence (OLS) outperformed the model that assumed a Brownian motion model of evolution ($\Delta AICc > 2$ in all three cases; Table 2). Therefore, phylogeny does not explain the effect of superfetation on number, total volume, and average volume of spermathecae. According to OLS regression, the total number of spermathecae was significantly higher in species with superfetation than in species without superfetation ($p = 0.001$; Table 2, Figure 5a). Also according to OLS regressions, total and average volume of spermathecae (after correcting for ovary size) were higher in species with superfetation than in species without superfetation, although these differences were only marginally significant ($p = 0.09$ and 0.06, respectively; Table 2 and Figure 5b,c).

4 | DISCUSSION

4.1 | Spermathecae in poeciliid fishes

The first work in poeciliid fishes to demonstrate temporary sperm storage in a structure formed by the closing of ovarian folds that contain spermatozoa (a structure they called a "solid plug") was carried out in *H. formosa* (Fraser & Renton, 1940). Later, Kobayashi and Iwamatsu (2002) described special sperm retention structures in *P. reticulata*,

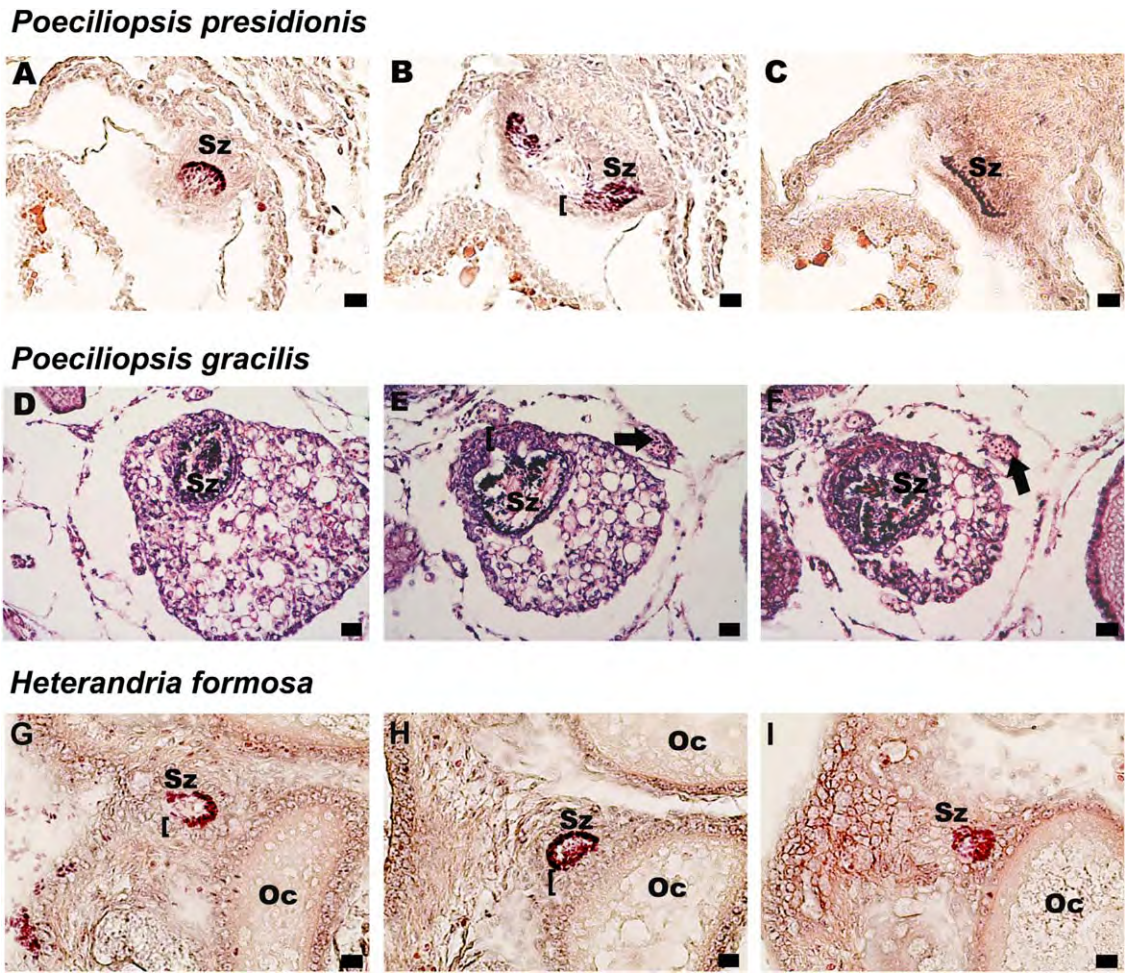


FIGURE 4 Sections of ovarian tissue stained using the hematoxylin-eosin technique that show the spermathecae observed in *P. presidionis*, *P. gracilis*, and *H. formosa* (species with superfetation). Heads of spermatozoa within the spemathecae stain blue (Sz). The different panels show representative images of the proximal (a, d, g), middle (b, e, h), and distal (c, f, i) sections of the spermathecae. Arrows point out erythrocytes. Brackets indicate thickened ovarian wall. Scale bar = 10 μm. Oocytes (Oc)

which they termed "sperm storage micropockets." In the present study, we describe similar structures in nine species of the Poeciliidae (*G. panuco*, *X. hellerii*, *P. mexicana*, *P. prolifica*, *P. infans*, *P. viriosa*, *P. presidionis*, *P. gracilis*, and *H. formosa*). However, we refer to these structures as "spermathecae" in order to be consistent with the term used for analogous structures in other taxonomic groups (Dallai, 1975; Pool & Hoage, 1973; Tombes & Roppel, 1972). Similar to "sperm storage micropockets" and "solid plugs", the spermathecae we observed are composed of ovarian folds, which close, completely surrounding the spermatozoa. However, spermatozoa may be temporarily stored as well in folds of the ovarian epithelium that do not close, but that instead connect directly to the ovarian lumen or to developing oocytes. These funnel-like folds have been called "dellen" (Greven, 2011; Mendoza, 1943). In fact, we observed spermatozoa within such deep ovarian folds (which we did not consider to be spermathecae) in most of the ovaries that we examined (Figure 6).

The spermathecae of all of the species we studied are composed of thickened ovarian epithelium. In general, the heads of the spermatozoa are oriented toward the ovarian epithelium and the tails toward the lumen of the spermatheca. This particular orientation of the spermatozoa could indicate that the internal ovarian tissue nourishes the spermatozoa to keep them viable. For example, in *X. maculatus*, the cells of the ovarian tissue that interact with spermatozoa exhibit abundant ribosomes, microvilli, appendices, and desmosomes, which are characteristics of cells that transport nutrients (Potter & Kramer, 2000). The blood vessels that we observed surrounding the spermathecae may also contribute nutrients to the spermatozoa stored within. Blood vessels transport oxygen and nutrients necessary for cells, so their proximity to spermathecae is additional evidence that the ovarian tissue of the female provides the necessary media to keep spermatozoa viable for a relatively prolonged period.

We still do not know how long spermathecae last within the ovary. However, there are studies that provide information about how long spermatozoa remain viable within the ovary, which is indirect evidence of the time between the formation and disappearance of spermathecae. For example, sperm may be stored for up to nine months in *H. formosa* and up to 10 months in *H. dactylopterus* (Fraser & Renton, 1940; Hubbs, 1964, 1997; Scrimshaw, 1944; Vila et al., 2007).

TABLE 2 Results from phylogenetic generalized least squares assuming a Brownian motion model of evolution (PGLS-BM) and ordinary least squares regression (OLS) fitted to the number, total volume, and average volume of spermathecae in ovaries of poeciliid fishes

Response variable	Regression model	AICC	ΔAICc	Regression slope	*p*
Number of spermathecae	OLS	31.48	0.00	1.70	0.001
	PGLS-BM	40.56	9.08	1.69	0.15
Total volume of spermathecae	OLS	3.27	0.00	0.16	0.09
	PGLS-BM	8.47	5.20	0.13	0.54
Average volume of spermathecae	OLS	−18.37	0.00	0.04	0.06
	PGLS-BM	−15.29	3.08	0.03	0.41

Total and average volume of spermathecae were treated as proportions of total ovary volume. In all cases, the explanatory variable was the presence or absence of superfetation. The fit of each model to the data was evaluated using the Akaike Information Criterion adjusted for small sample sizes (AICc), with the smallest value indicating the best-fitting model. Differences in AICc values between models (ΔAICc) are also shown. The regression slope represents the estimated difference between species with and without superfetation.

Other vertebrates exhibit structures analogous to the spermathecae of poeciliid fishes (Dallai, 1975; Holt & Lloyd, 2010; Palmer, Rostal, Grumbles, & Mulvey, 1998; Suarez, 2008). For example, in lizards, specialized tubules for sperm storage are found in the oviduct of females (Villagrán-Santa Cruz et al., 2017). In some amphibians, such as caecilians, particular cloacal structures function as sites of sperm storage (Kuehnel & Kupfer, 2012). In fish from other families, such as *H. dactylopterus* (Sebastidae), sperm retention occurs in storage chambers in the ovary (Vila et al., 2007).

4.2 | Does superfetation matter?

We have presented evidence that superfetation is positively related to the number and size of spermathecae. Independent of phylogenetic history, the superfetating species in our sample had more and larger spermathecae than non-superfetating species. As such, the former species devote a larger proportion of their ovarian volume to the formation of spermathecae than the latter. This difference between species with and without superfetation is substantial: the largest relative volumes of all spermathecae occurred in two species with superfetation and were on average over 30 times greater than the smallest relative volumes, which occurred in non-superfetating species. In addition, in three of our six non-superfetating species we found no spermathecae (Table 1). These results suggest a relationship between sperm storage within spermathecae and superfetation.

A potential caveat is our sample size. We examined three individual ovaries from each of the 12 species. Thus, the observed differences between species with and without superfetation should be interpreted with caution. In the case of number of spermathecae, the observed difference was significant at the conventional α level of 0.05. However, the differences in total and average volume of spermathecae could be considered significant only if we use a less conservative α level of 0.1, which may be appropriate for small sample sizes because the statistical power to detect differences is low. Regardless, in all three variables variation among individual ovaries (within each species) was lower than the variation that we observed among species (Supporting Information, Figure S2) and, therefore, the observed differences between species with and without superfetation are likely to remain with the inclusion of additional individuals.

In general, spermathecae are considered a morphological mechanism that allows a continuous supply of spermatozoa, facilitating reproduction when there is a low abundance of males or when reproductive cycles are asynchronous (Birkhead & Møller, 1993; Gist & Congdon, 1998). However, in populations of poeciliid fishes, males are abundant and females are receptive year-round (Frías-Alvarez, Garcia, Vázquez-Vega, & Zúñiga-Vega, 2014; Houde, 1997). Therefore, it is unlikely that in poeciliids these are the main functions of sperm storage within spermathecae. Based on our evidence of higher prevalence of spermathecae in superfetating species, we propose that sperm storage is an important mechanism for the development of superfetation, because it allows a constant supply of viable spermatozoa with which to fertilize frequently developing broods. However, this hypothesis associating superfetation with sperm storage assumes that the frequency of copulations is the same in species with and without superfetation, since superfetating species could acquire a constant supply of spermatozoa simply by copulating more frequently.

Another hypothesis that could explain the higher prevalence of spermathecae in species with superfetation suggests the presence of post-copulatory sexual selection. Males of some species of the family Poeciliidae exhibit elaborated ornaments and courtship behaviors, and females actively choose reproductive partners before copulation (Basolo, 1990; Bisazza, 1993). In contrast, males of other species lack these ornaments or courtship behaviors and their reproductive success is based on forced copulations (Magurran, 2011). Superfetation is more common in species where sexual coercion prevails and, as such, could be a reproductive strategy to compensate for the apparent lack of female mate choice (Macías-Garcia & González-Zuarth, 2005; Pollux et al., 2014). When females are unable to choose a high-quality mate

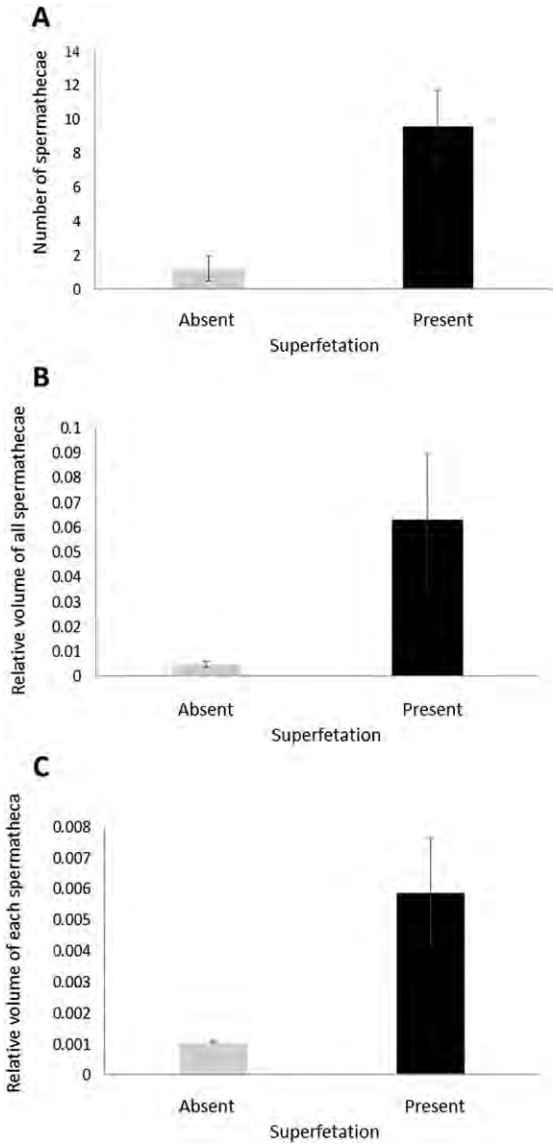


FIGURE 5 Total number (a), total volume (b), and average volume (c) of spermathecae in ovaries of poeciliid species with (black bars) and without (gray bars) superfetation. Volumes in b and c are expressed as proportions of total ovary volume. Error bars represent one standard error

before copulation, they could use mechanisms of post-copulatory sexual selection (i.e., cryptic female choice) to bias paternity in favor of some males and against others by means of physiological processes that occur within their ovaries (Birkhead & Pizzari, 2002; Evans & Pilastro, 2011). Sperm storage in spermathecae would have a fundamental role for this purpose in species with superfetation. Higher quality spermatozoa would have a higher likelihood of surviving within the spermathecae and would be stored for some period of time until being used by the females. In addition, sperm competition could occur within the spermathecae. Previous studies in poeciliids have documented sperm competition as well as an important influence of the female, through the ovarian fluid, on the motility and survival of spermatozoa (Gasparini & Evans, 2013; Gasparini & Pilastro, 2011; Gasparini, Simmons, Beveridge, & Evans, 2010).

Another possible advantage of sperm retention within spermathecae in species with superfetation is greater potential for storing sperm of different males, favoring the fertilization of oocytes by different genotypes and thus increasing their progeny's genetic variability (Macías-Garcia & González-Zuarth, 2005; Pollux et al., 2014). If females that superfetate usually lack mechanisms for pre-copulatory sexual selection, and therefore cannot assure the genetic quality of their young, they could at least increase their genetic variability. In this way, the ability to store sperm in spermathecae could facilitate multiple paternity, particularly in species with superfetation.

Finally, it is important to emphasize that we also found spermathecae (although fewer and smaller) in some species that do not superfetate. This indicates that cryptic female choice, sperm competition, and multiple paternity could also occur in non-superfetating species. In fact, multiple paternity has been reported in species with superfetation (e.g., *H. formosa*) (Soucy & Travis, 2003) as well as in species without superfetation (*Gambusia holbrooki*, *Poecilia latipinna*, and *P. reticulata*) (Kelly, Godin, & Wright, 1999; Travis, Trexler, & Mulvey, 1990; Zane, Nelson, Jones, & Avise, 1999). However, our evidence of higher prevalence of spermathecae in superfetating species suggests that these processes are more important and more frequent in the reproduction of species without apparent signs of pre-copulatory sexual selection, as is the case of the majority of species that exhibit superfetation (Macías-Garcia & González-Zuarth, 2005; Pollux et al., 2014).

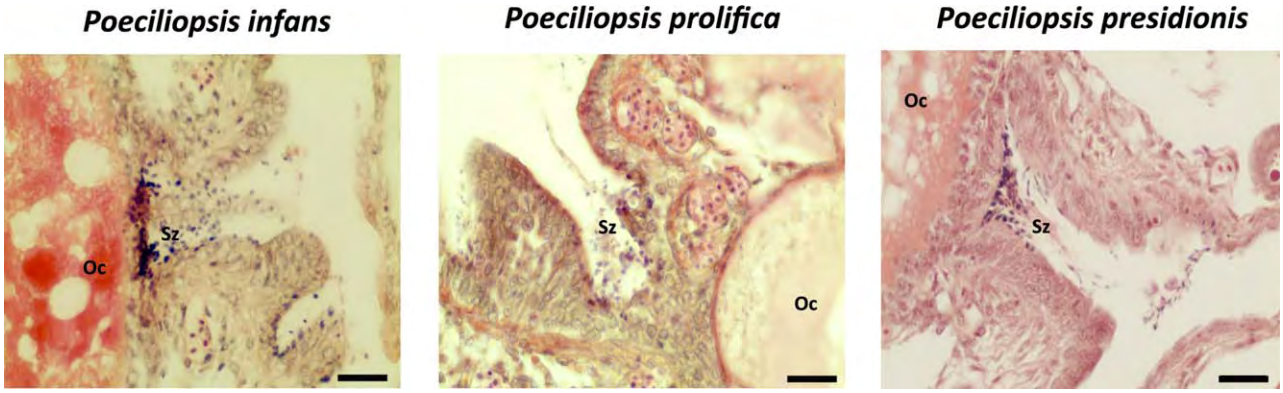


FIGURE 6 Sections of ovarian tissue stained using the hematoxylin-eosin technique that show spermatozoa (Sz) within folds of the ovarian epithelium observed in *P. infans*, *P. prolifica*, and *P. presidionis*. Heads of spermatozoa within the ovarian folds stain blue. Scale bar = 10 μm. Oocytes (Oc)

ACKNOWLEDGMENTS

Funding for this project was provided by Consejo Nacional de Ciencia y Tecnología (CONACyT) and Secretaría de Educación Pública from México through the grant 129675. C.O.T received a doctorate scholarship from CONACyT (no. 368782/245650). This paper is a requisite for C.O.T. to obtain the Ph.D. degree in the Posgrado en Ciencias Biológicas of Universidad Nacional Autónoma de México. Fieldwork and collection of specimens were authorized by Comisión Nacional de Acuacultura y Pesca from México (permits DGOPA.07010.210612.1749 and PPF/DGOPA-223/2013). We thank I. Solano-Zavaleta, E. Mendoza-Cruz, P. Mendoza-Hernández, M. Hernández-Apolinar, E. Ávila-Luna, I. A. Morales-Salas, M. E. Pérez-Cruz, B. Zúñiga-Ruiz, K. Morrison, and L. F. Vázquez-Vega for field and laboratory assistance.

AUTHOR CONTRIBUTIONS

C.O.T. and J.J.Z.V. designed the study and conducted fieldwork. C. O.T., M.V.S.C., and N.A.M.M. processed and analyzed all the samples. C.O.T. and J.J.Z.V. conducted all comparative analyses. All authors wrote the manuscript and gave final approval for publication.

REFERENCES

Basolo, A. L. (1990). Female preference for male sword length in the green swordtail, *Xiphophorus hellerii* (Pisces: Poeciliidae). *Animal Behaviour*, *40*, 332–338.

Birkhead, T. R., & Møller, A. P. (1993). Sexual selection and the temporal separation of reproductive events: Sperm storage data from reptiles, birds and mammals. *Biological Journal of the Linnean Society*, *50*, 295–311.

Birkhead, T. R., & Pizzari, T. (2002). Postcopulatory sexual selection. *Nature Reviews Genetics*, *3*, 262–273.

Bisazza, A. (1993). Male competition, female mate choice and sexual size dimorphism in poeciliid fishes. *Marine Behaviour and Physiology*, *23*, 257–286.

Burnham, K. P., & Anderson, D. R. (2002). *Model selection and multimodel inference: A practical information-theoretic approach*. New York: Springer-Verlag.

Clark, E, & Aronson, L. R. (1951). Sexual behavior in the guppy, *Lebistes reticulatus* (Peters). *Zoologica*, *36*, 49–66.

Constantz, J. (1989). Reproductive biology of the poeciliid fishes. In G. K. Meffe & F. F. Snelson (Eds.), *Ecology and evolution of live bearing fishes (Poeciliidae)* (pp. 33–50). Englewood Cliffs, NJ: Prentice Hall.

Dallai, R. (1975). Fine structure of the spermatheca of *Apis mellifera*. *Journal of Insect Physiology*, *21*, 89–109.

Darling, J. D. S., Noble, M. L., & Shaw, E. (1980). Reproductive strategies in the surfperches.1. Multiple insemination in natural-populations of the shiner perch, *Cymatogaster aggregata*. *Evolution*, *34*, 271–277.

Evans, J. P., & Pilastro, A. (2011). Postcopulatory sexual selection. In J. P. Evans, A. Pilastro, & I. Schlupp (Eds.), *Ecology and evolution of poeciliid fishes* (pp. 197–208). Chicago, IL: The University of Chicago Press.

Felsenstein, J. (1985). Phylogenies and the comparative method. *The American Naturalist*, *125*, 1–15.

Fraser, E. A., & Renton, R. M. (1940). Observation on the breeding and development of the viviparous fish, *Heterandria formosa*. *Quarterly Journal of Microscopical Science*, *81*, 479–520.

Freckleton, R. P., Harvey, P. H., & Pagel, M. (2002). Phylogenetic analysis and comparative data: A test and review of evidence. *The American Naturalist*, *160*, 712–726.

Frías-Alvarez, P., Garcia, C. M., Vázquez-Vega, L. F., & Zúñiga-Vega, J. J. (2014). Spatial and temporal variation in superfoetation and related life history traits of two viviparous fishes: *Poeciliopsis gracilis* and *P. infans*. *Naturwissenschaften*, *101*, 1085–1098.

Frías-Alvarez, P., & Zúñiga-Vega, J. J. (2016). Superfetation in livebearing fishes is not always the result of a morphological constraint. *Oecologia*, *181*, 645–658.

Garland, T., Bennett, A. F., & Rezende, E. L. (2005). Phylogenetic approaches in comparative physiology. *Journal of Experimental Biology*, *208*, 3015–3035.

Gasparini, C., & Evans, J. P. (2013). Ovarian fluid mediates the temporal decline in sperm viability in a fish with sperm storage. *PLoS One*, *8*, e64431.

Gasparini, C., & Pilastro, A. (2011). Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy. *Proceedings of the Royal Society of London B: Biological*, *278*, 2495–2501.

Gasparini, C., Simmons, L. W., Beveridge, M., & Evans, J. P. (2010). Sperm swimming velocity predicts competitive fertilization success in the green swordtail *Xiphophorus helleri*. *PLoS One*, *5*, e12146.

Gist, D. H., & Congdon, J. D. (1998). Oviductal sperm storage as a reproductive tactic of turtles. *Journal of Experimental Zoology*, *282*, 526–534.

Greven, H. (2011). Gonads, genitals, and reproductive biology. In J. P. Evans, A. Pilastro, & I. Schlupp (Eds.), *Ecology and evolution of poeciliid fishes* (pp. 3–17). Chicago, IL: The University of Chicago Press.

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

Gupta, B. L., & Smith, D. S. (1969). Fine structural organization of the spermatheca in the cockroach, *Periplaneta americana*. *Tissue Cell*, *1*, 295–324.

Hildemann, W. H., & Wagner, E. D. (1954). Intraspecific sperm competition in *Lebistes reticulatus*. *The American Naturalist*, *88*, 87–91.

Holt, W. V., & Lloyd, R. E. (2010). Sperm storage in the vertebrate female reproductive tract: How does it work so well? *Theriogenology*, *73*, 713–722.

Houde, A. E. (1997). *Sex, color, and mate choice in guppies*. New Jersey: Princeton University Press.

Hubbs, C. (1964). Interactions between a bisexual fish species and its gynogenetic sexual parasite. *Bulletin of the Texas Memorial Museum*, *8*, 1–72.

Hubbs, C. (1997). Sperm retention in poeciliids. *Bulletin of American Livebearer Association*, *146*, 9.

Kelly, C. D., Godin, J. G. J., & Wright, J. M. (1999). Geographic variation in multiple paternity within natural populations of the guppy (*Poecilia reticulata*). *Proceedings of the Royal Society of London B: Biological*, *266*, 2403–2408.

Kobayashi, H., & Iwamatsu, T. (2002). Fine structure of the storage micropocket of spermatozoa in the ovary of the guppy *Poecilia reticulata*. *Zoological Science*, *19*, 545–555.

Koya, Y., Munehara, H., & Takano, K. (2002). Sperm storage and motility in the ovary of the marine sculpin *Alcichthys alcilcornis* (teleostei: Scorpaeniformes), with internal gametic association. *Journal of Experimental Zoology*, *292*, 145–155.

Kuehnel, S., & Kupfer, A. (2012). Sperm storage in caecilian amphibians. *Frontiers in Zoology*, *9*, 12.

Lavin, S. R., Karasov, W. H., Ives, A. R., Middleton, K. M., & Garland, T., Jr. (2008). Morphometrics of the avian small intestine compared with that of nonflying mammals: A phylogenetic approach. *Physiological and Biochemical Zoology*, *81*, 526–550.

Macías-Garcia, C., & González-Zuarth, C. A. (2005). Reproductive behavior of viviparous fish and intersexual conflict. In M. C. Uribe & H. J. Grier HJ (Eds.), *Viviparous fishes* (pp. 289–302). Homestead, FL: New Life Publications..

Maddison, W. P., & Maddison, D. R. (2009). *MESQUITE: A modular system for evolutionary analysis*. Version 3.0. Retrieved from http://mesquiteproject.org

Magurran, A. E. (2011). Sexual coercion. In J. P. Evans, A. Pilastro, & I. Schlupp (Eds.), *Ecology and evolution of poeciliid fishes* (pp. 209-217). Chicago, IL: The University of Chicago Press.

Mank, J. E, Promislow, D. E. L., & Avise, J. C. (2005). Phylogenetic perspectives in the evolution of parental care in ray-finned fishes. *Evolution*, *59*, 1570-1578.

Martins, E. P., & Hansen, T. F. (1997). Phylogenies and the comparative method: A general approach to incorporating phylogenetic information into the analysis of interspecific data. *The American Naturalist*, *149*, 646-667.

Mendoza, G. (1943). The reproductive cycle of the viviparous teleost, *Neotoca bilineata*, a member of the family Goodeidae. IV. The germinal tissue. *The Biological Bulletin*, *84*, 87-97.

Meredith, R. W., Pires, M. N., Reznick, D. N., & Springer, M. S. (2011). Molecular phylogenetic relationships and the coevolution of placentotrophy and superfetation in *Poecilia* (Poeciliidae: Cyprinodontiformes). *Molecular Phylogenetics and Evolution*, *59*, 148-157.

Miller, R. R., Minckley, W. L., & Norris, S. M. (2005). *Freshwater fishes of Mexico*. Chicago, IL: The University of Chicago Press.

Muñoz, M., Koya, Y., & Casadevall, M. (2002). Histochemical analysis of sperm storage in *Helicolenus dactylopterus dactylopterus* (Teleostei: Scorpaenidae). *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *292*, 156-164.

Olivera-Tlahuel, C., Ossip -Klein, A. G., Espinosa-Pérez, H. S., & Zúñiga-Vega, J. J. (2015). Have superfetation and matrotrophy facilitated the evolution of larger offspring in poeciliid fishes? *Biological Journal of the Linnean Society*, *116*, 787-804.

Orr, T. J., & Zuk, M. (2012). Sperm storage. *Current Biology*, *22*, R8-R10.

Pagel, M. (1999). Inferring the historical patterns of biological evolution. *Nature*, *401*, 877-884.

Palmer, K. S., Rostal, D. C., Grumbles, J. S., & Mulvey, M. (1998). Long-term sperm storage in the desert tortoise (*Gopherus agassizii*). *Copeia*, *1998*, 702-705.

Paradis, E., Claude, J., & Strimmer, K. (2004). APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics*, *20*, 289-290.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team. (2016). NLME: Linear and nonlinear mixed effects models. R package version 3.1-125. Retrieved from http://CRAN.R-project.org/package=nlme

Pires, M. N., Banet, A. I., Pollux, B. J. A., & Reznick, D. N. (2011). Variation and evolution of reproductive strategies. In J. P. Evans, A. Pilastro, & I. Schlupp (Eds.), *Ecology and evolution of poeciliid fishes* (pp. 28–37). Chicago, IL: The University of Chicago Press.

Pollux, B. J.A., Meredith, R. W., Springer, M. S., & Reznick, D. N. (2014). The evolution of the placenta drives a shift in sexual selection in livebearing fish. *Nature*, *513*, 233–236.

Pollux, B. J. A., Pires, M. N., Banet, A. I., & Reznick, D. N. (2009). Evolution of placentas in the family Poeciliidae: An empirical study of macroevolution. *Annual Review of Ecology, Evolution, and Systematics*, *40*, 271–289.

Pool, T. B., & Hoage, T. R. (1973). The ultrastructure of secretion in the spermatheca of the salamander, *Manculus quadridigitatus* (Holbrook). *Tissue Cell*, *5*, 303–313.

Potter, H., & Kramer, C. R. (2000). Ultrastructural observations on sperm storage in the ovary of the platyfish, *Xiphophorus maculatus* (Teleostei: Poeciliidae): The role of the duct epithelium. *Journal of Morphology*, *245*, 110–129.

Pratt, H. L.,Jr. (1993). The storage of spermatozoa in the oviducal glands of western north Atlantic sharks. *Environmental Biology of Fishes*, *38*, 139–49.

Presnell, J. K., Schreibman, M. P., & Humason, G. L. (1997). *Humason's animal tissue techniques*. San Francisco: Johns Hopkins University Press.

R Core Team. (2016). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from https://www.R-project.org/

Revell, L. J., & Reynolds, R. G. (2012). A new Bayesian method for fitting evolutionary models to comparative data with intraspecific variation. *Evolution*, *66*, 2697–2707.

Sanderson, M. J. (2002). Estimating absolute rates of molecular evolution and divergence times: A penalized likelihood approach. *Molecular Biology and Evolution*, *19*, 101–109.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*, 671–675.

Scrimshaw, N. S. (1944). Embryonic growth in the viviparous poeciliid, *Heterandria formosa*. *The Biological Bulletin*, *87*, 37–51.

Sever, D. M. (2002). Female sperm storage in amphibians. *Journal of Experimental Zoology*, *292*, 165–179.

Sever, D. M., & Hamlett, W. C. (2002). Female sperm storage in reptiles. *Journal of Experimental Zoology*, *292*, 187–199.

Simmons, L. W. (2001). *Sperm competition and its evolutionary consequences in the insects*. Princeton: Princeton University Press.

Soucy, S., & Travis, J. (2003). Multiple paternity and population genetic structure in natural populations of the poeciliid fish, *Heterandria formosa*. *Journal of Evolutionary Biology*, *16*, 1328–1336.

Suarez, S. S. (2008). Regulation of sperm storage and movement in the mammalian oviduct. *The International Journal of Developmental Biology*, *52*, 455–462.

Tombes, A. S., & Roppel, R. M. (1972). Ultrastructure of the spermatheca of the granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). *International Journal of Insect Morphology*, *1*, 141–152.

Travis, J., Farr, J. A., Henrich, S., & Cheong, R. T. (1987). Testing theories of clutch overlap with the reproductive ecology of *Heterandria formosa*. *Ecology*, *68*, 611–623.

Travis, J., Trexler, J. C., & Mulvey, M. (1990). Multiple paternity and its correlates in female *Poecilia latipinna* (Poeciliidae). *Copeia*, *1990*, 722–729.

Turner, C. L. (1937). Reproductive cycles and superfetation in poeciliid fishes. *The Biological Bulletin*, *72*, 145–164.

Turner, C. L. (1938). Adaptations for viviparity in embryos and ovary of *Anableps anableps*. *Journal of Morphology*, *62*, 323–349.

Turner, C. L. (1940). Pseudoamnion, pseudochorion, and follicular pseudoplacenta in poeciliid fishes. *Journal of Morphology*, *67*, 59–89.

Turner, C. L. (1947). Viviparity in teleost fishes. *Scientific Monthly*, *65*, 508–518.

Uribe, M. C., De la Rosa-Cruz, G., Guerrero-Estévez, S. M., García Alarcón, A., & Aguilar-Morales, M. E. (2004). Estructura del ovario de

teleósteos vivíparos. Gestación intraovárica: Intraluminal en *Ilyodon whitei* (Goodeidae), e intrafolicular en *Poeciliopsis gracilis* (Poeciliidae). In M. L. Lozano Vilano & A. J. Contreras Balderas (Eds.), *Homenaje al Dr Andrés Reséndez Medina* (pp. 31–45). Mexico City: Dirección de Publicaciones Universidad Autónoma de Nuevo León.

Uribe, M. C., & Grier, H. J. (2011). Oogenesis of microlecithal oocytes in the viviparous teleost *Heterandria formosa*. *Journal of Morphology*, *272*, 241–257.

Uribe, M. C., Grier, H. J., De la Rosa-Cruz, G., & Schartl, M. (2016). The occurrence of spermatozoa in the ovary of the gynogenetic viviparous teleost *Poecilia formosa* (POECILIIDAE). *Journal of Morphology*, *277*, 341–350.

Vila, S., Sabat, M., Hernandez, M. R., & Munoz, M. (2007). Intraovarian sperm storage in *Helicolenus dactylopterus dactylopterus*: Fertilization, crypt formation and maintenance of stored sperm. *Raffles Bulletin of Zoology*, *14*, 21–7.

Villagrán-Santa Cruz, M., Mendoza-Cruz, E., Granados-González, G., Rheubert, J. L., & Hernández-Gallegos, O. (2017). Sperm storage in the viviparous lizard *Sceloporus bicanthalis* (Squamata: Phrynosomatidae), a species with continuous spermatogenesis. *Zoomorphology*, *136*, 85–93.

Zane, L., Nelson, W. S., Jones, A. G., & Avise, J. C. (1999). Microsatellite assessment of multiple paternity in natural populations of a live-bearing fish, *Gambusia holbrooki*. *Journal of Evolutionary Biology*, *12*, 61–69.

Zar, J. H. (2010). *Biostatistical analysis*. Upper Saddle River: Prentice Hall.

Zúñiga-Vega, J. J., Macías-Garcia, C., & Johnson, J. B. (2010). Hypotheses to explain the evolution of superfetation in viviparous fishes. In M. C. Uribe & H. J. Grier (Eds.), *Viviparous fishes II* (pp. 241–254). Homestead, FL: New Life Publications.

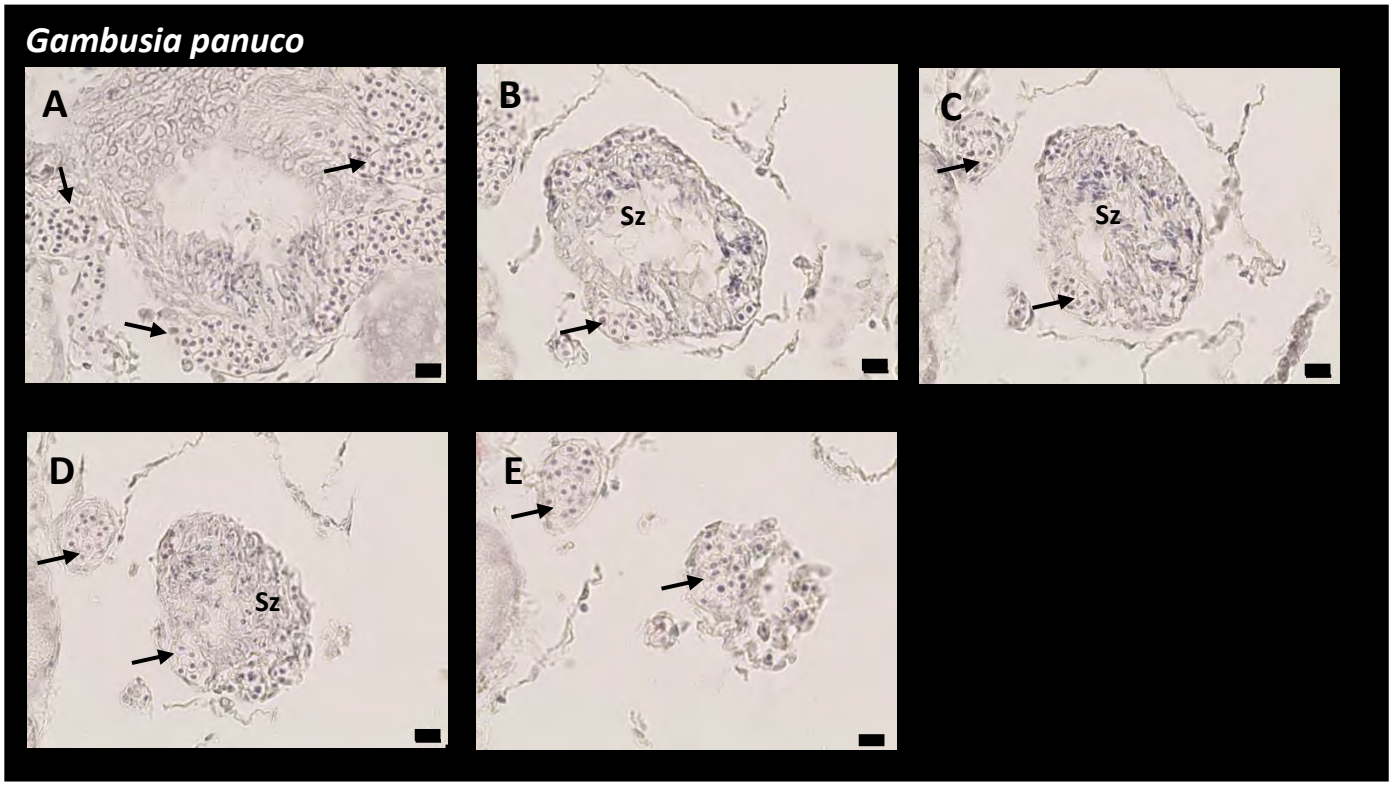
Zúñiga-Vega, J. J., Reznick, D. N., & Johnson, J. B. (2007). Habitat predicts reproductive superfetation and body shape in the livebearing fish *Poeciliopsis turrubarensis*. *Oikos*, *116*, 995–1005.

SUPPORTING INFORMATION

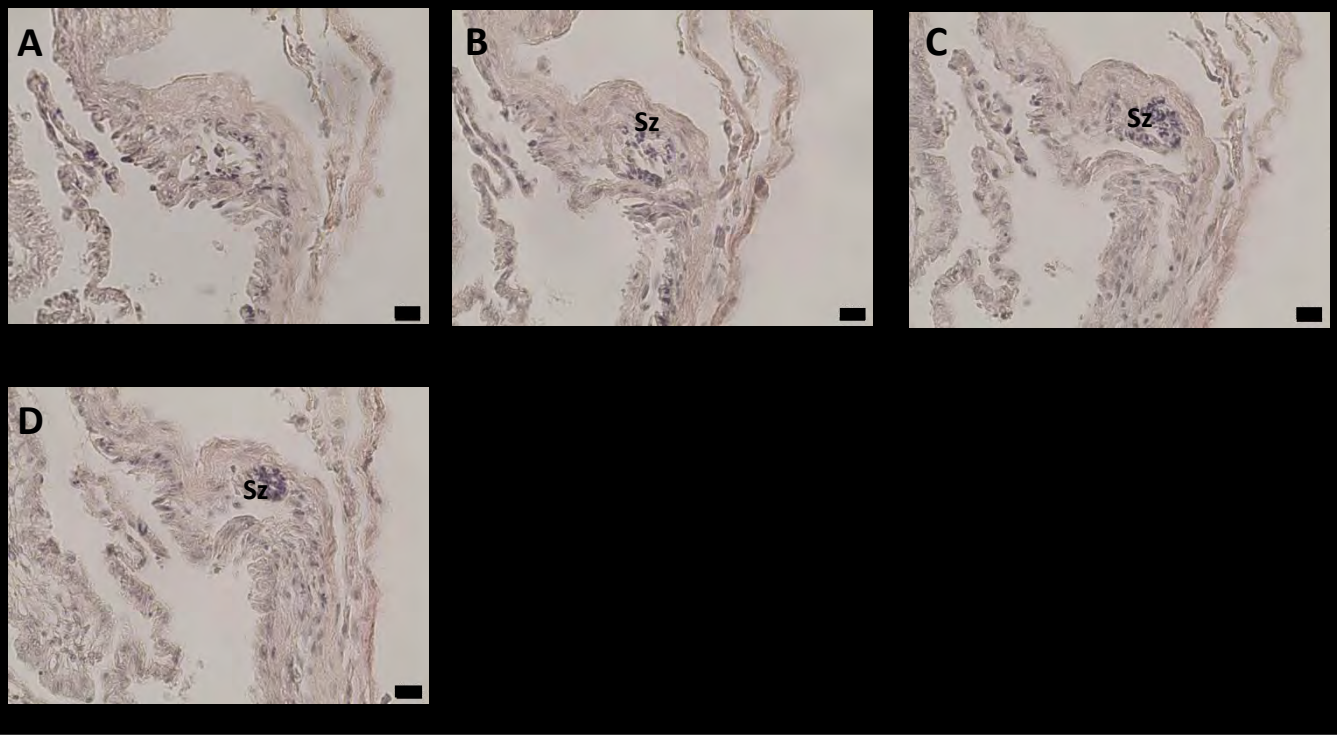
Additional Supporting Information may be found online in the supporting information tab for this article.

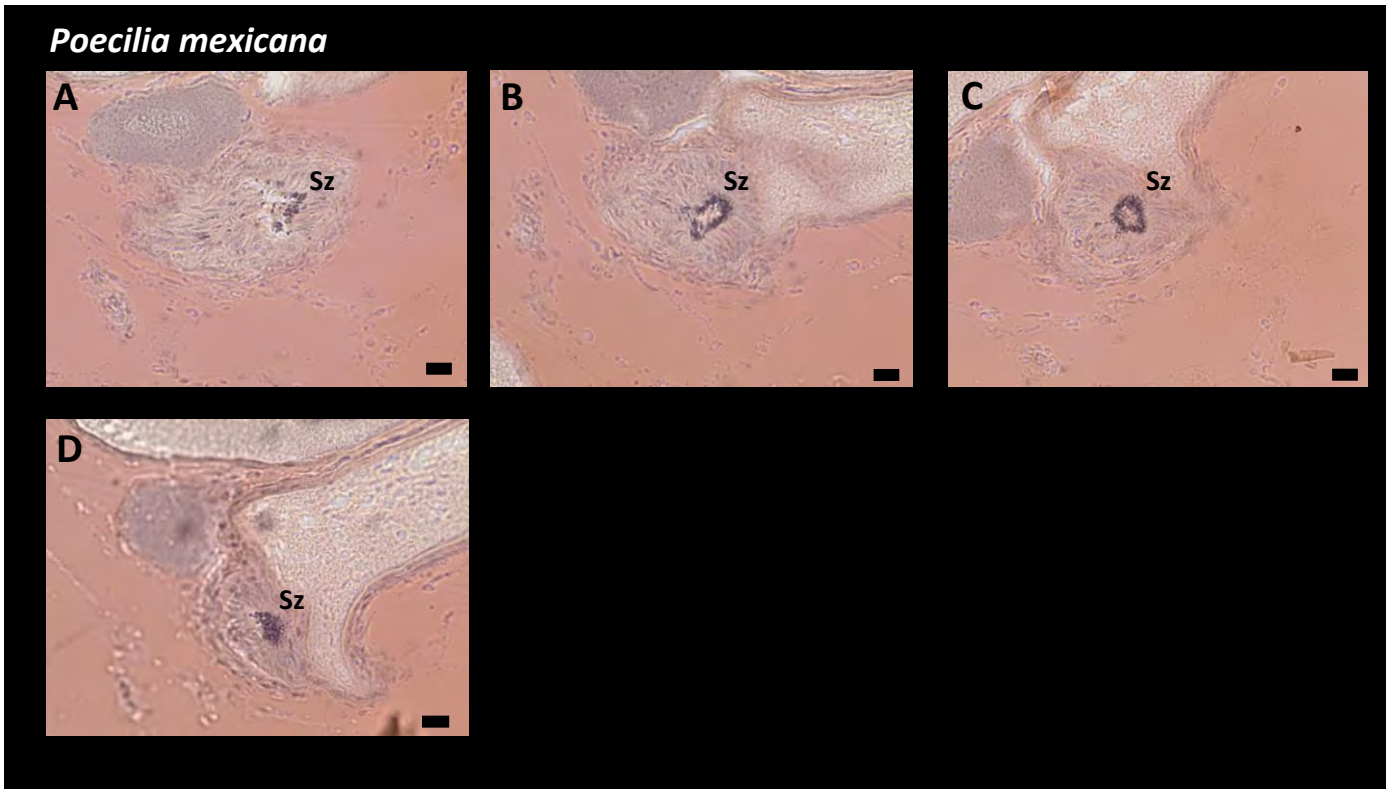
How to cite this article: Olivera-Tlahuel C, Villagrán-Santa Cruz M, Moreno-Mendoza NA, Zúñiga-Vega JJ. Morphological structures for potential sperm storage in poeciliid fishes. Does superfetation matter?. *Journal of Morphology*. 2017;00:1–12. https://doi.org/10.1002/jmor.20684

Fig. S1. Hematoxylin-eosin stained serial sections of spermathecae for each study species. For *Poeciliopsis gracilis* we show spermathecae from two different females. Heads of spermatozoa (Sz) within spermathecae stain blue. Arrows point out erythrocytes. Scale bar = 10 µm.

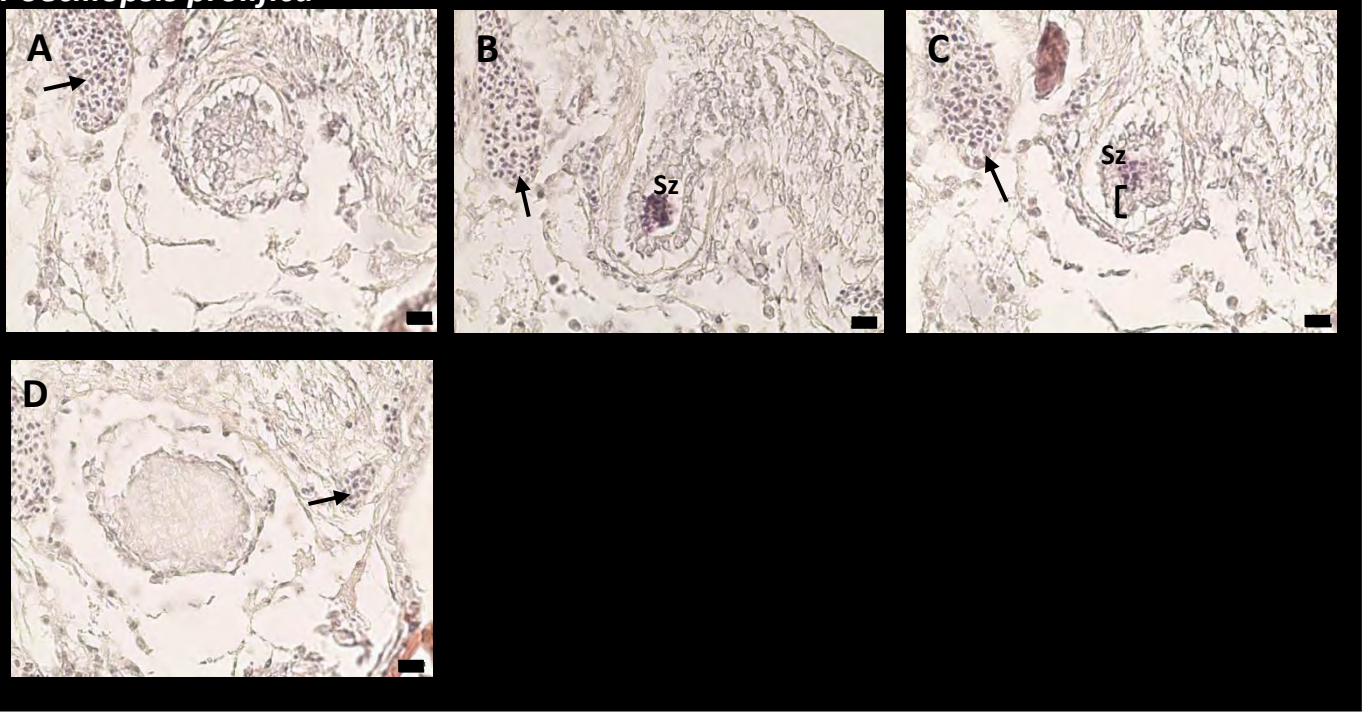


Xiphophorus hellerii

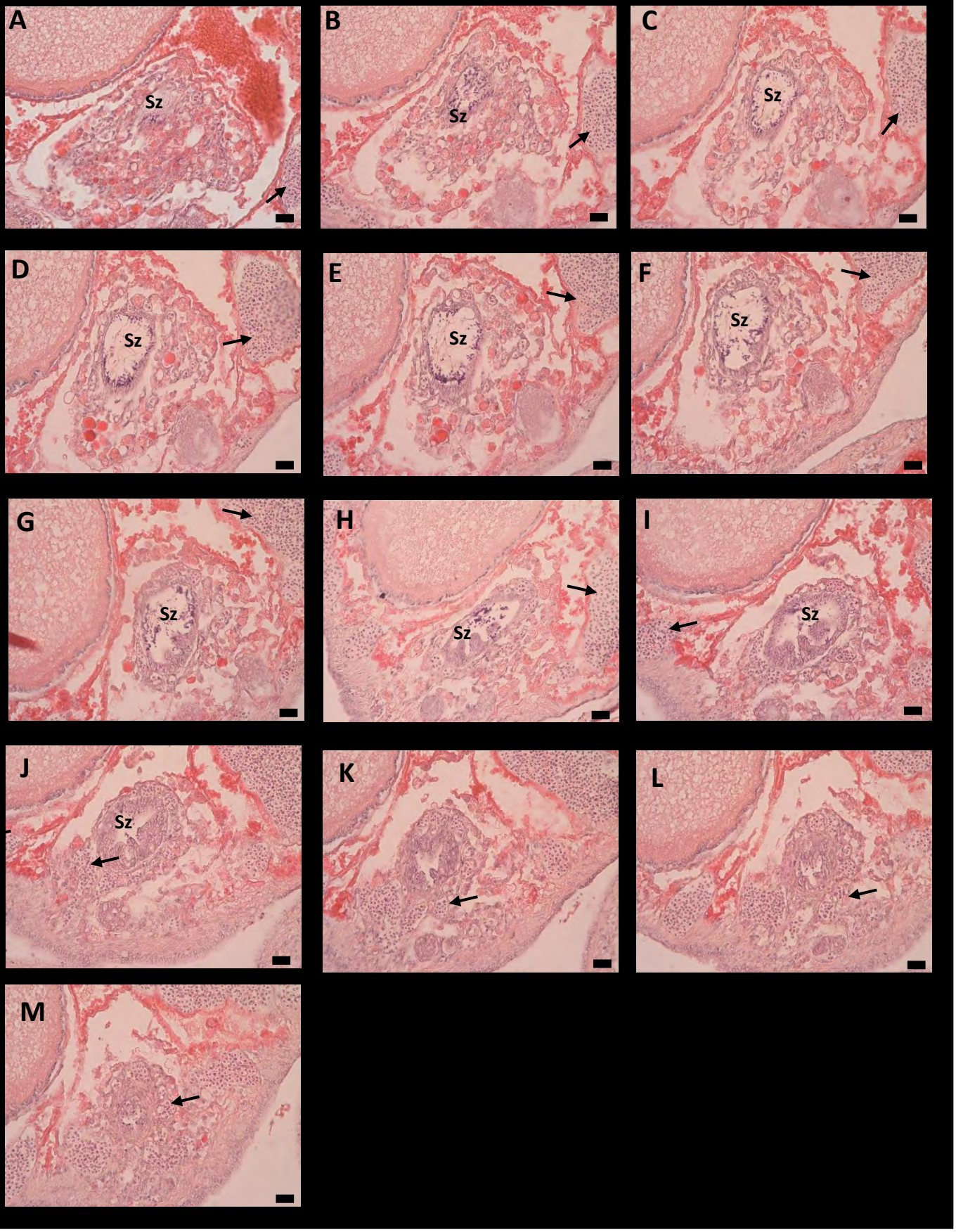



Poecilia mexicana
A
Sz
B
Sz
C
Sz
D
Sz

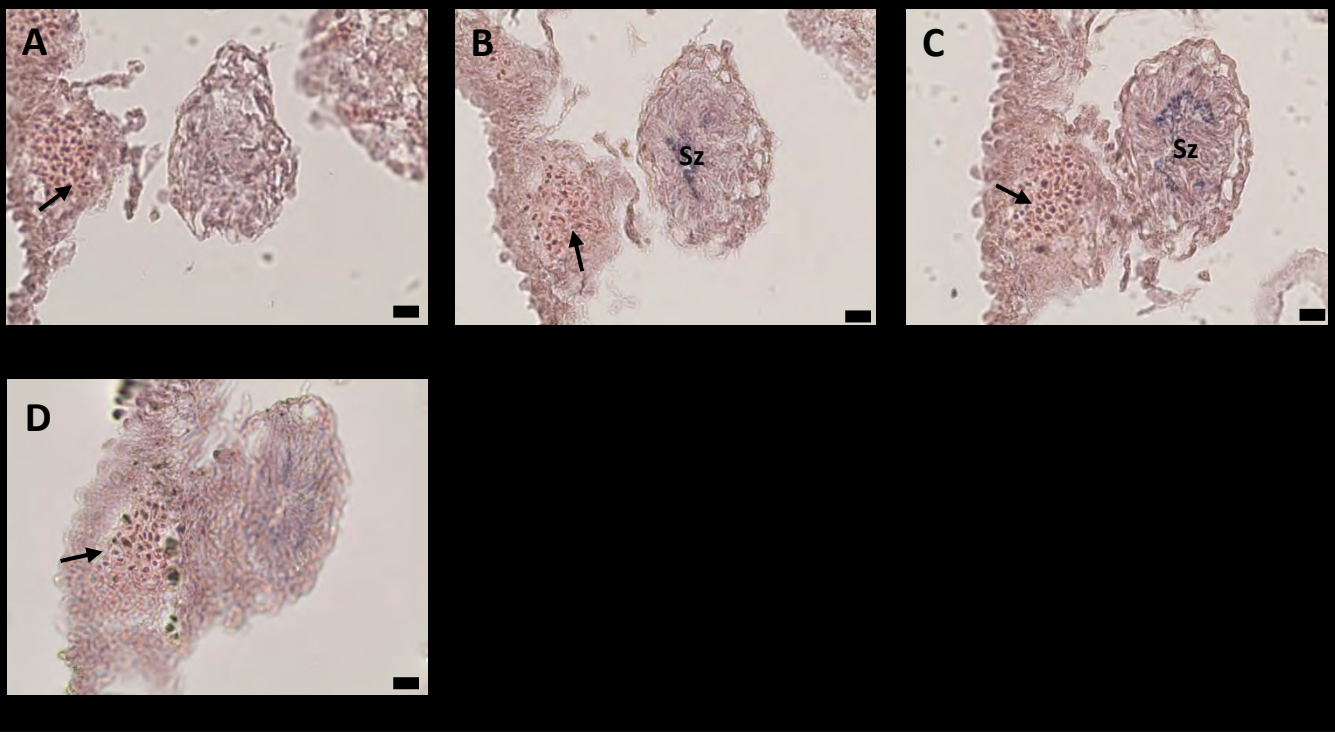
Poeciliopsis prolifica



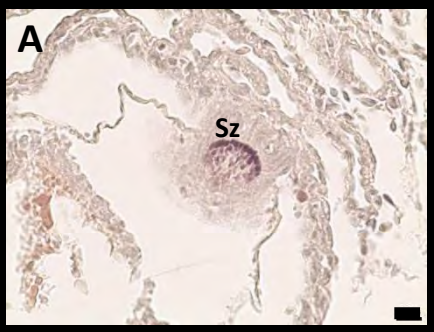
Poeciliopsis infans

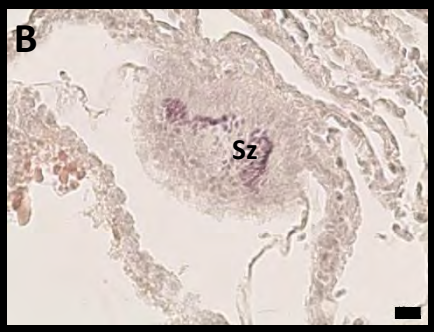


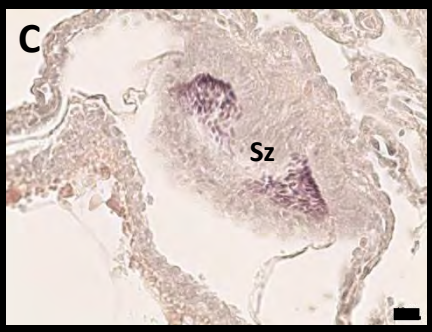
Poeciliopsis viriosa

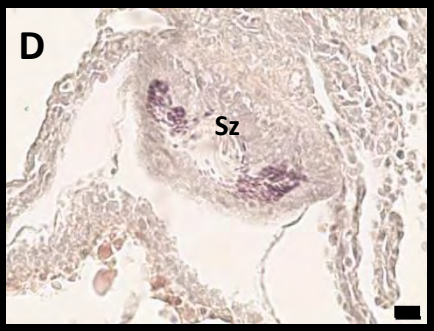


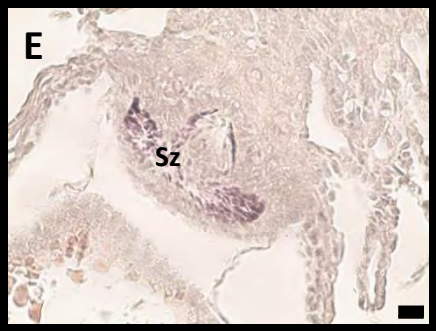
Poeciliopsis presidionis

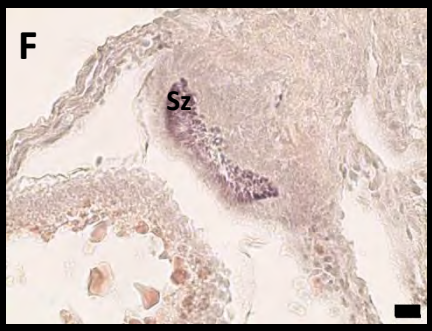


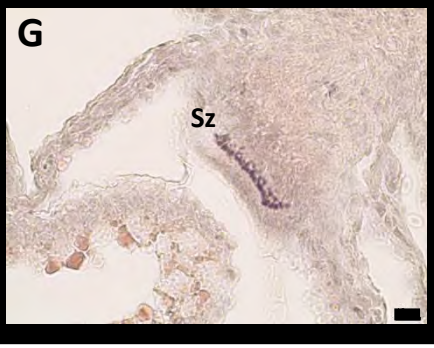


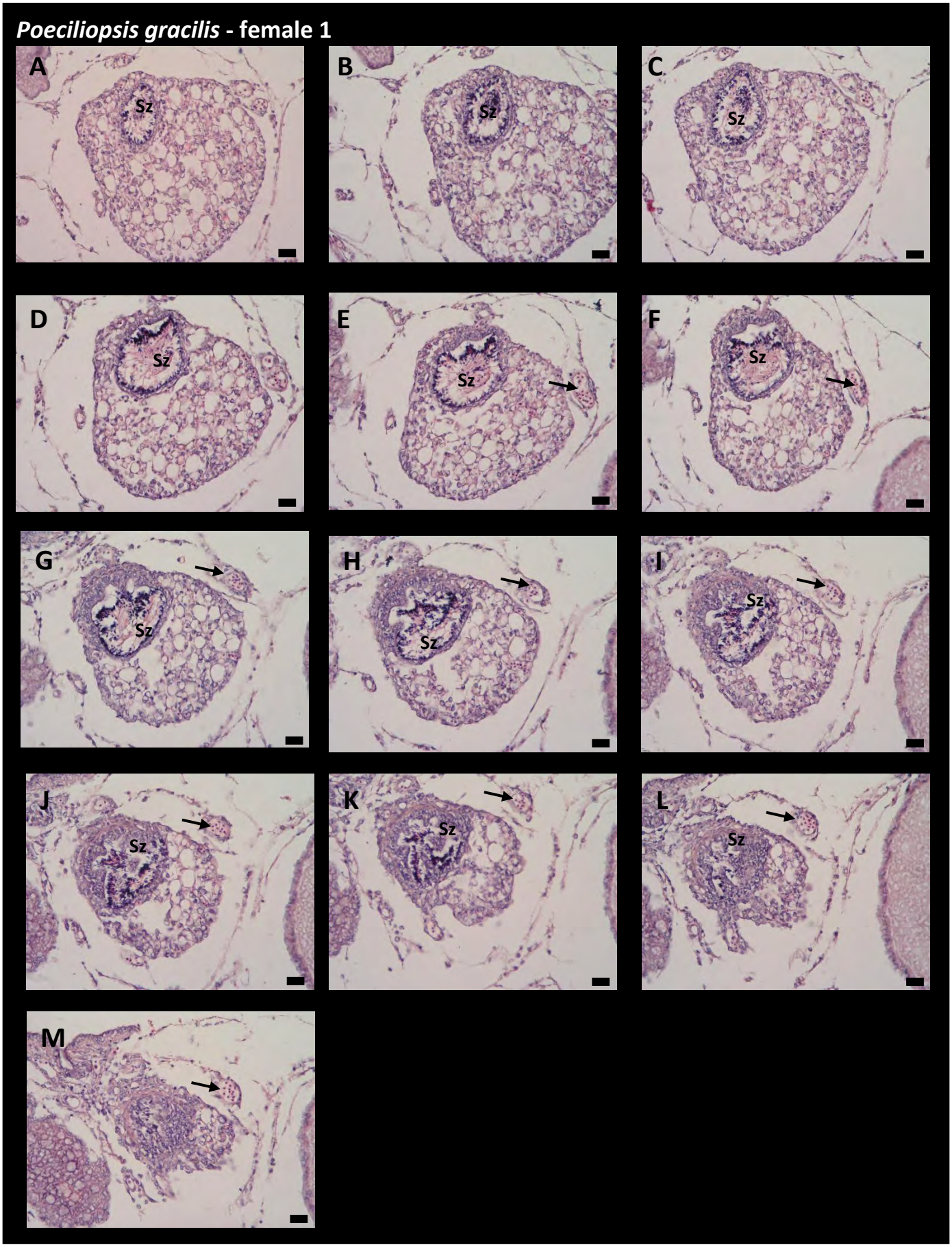




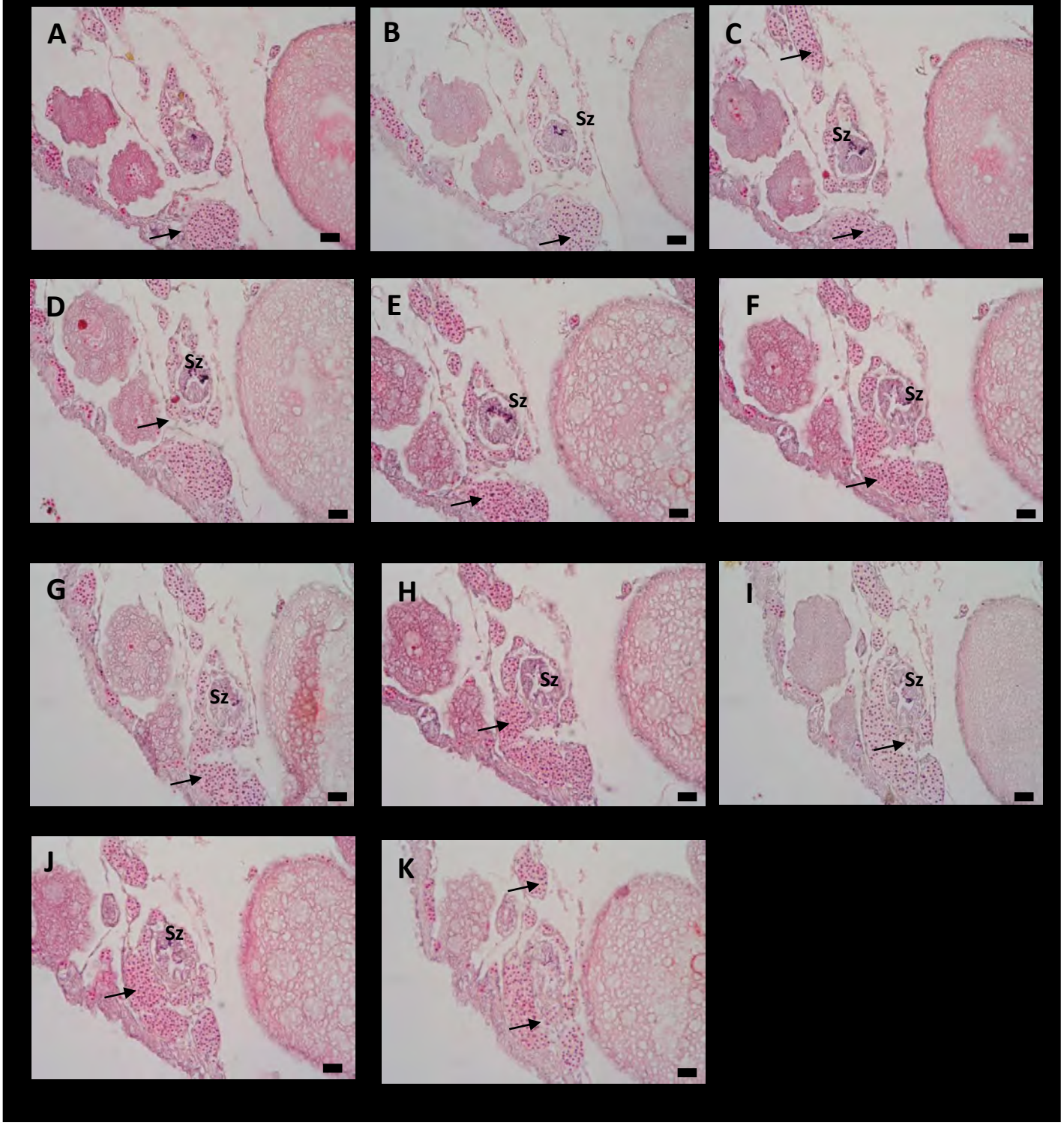







Poeciliopsis gracilis - female 1
A
Sz
B
Sz
C
Sz
D
Sz
E
Sz
F
Sz
G
Sz
H
Sz
I
Sz
J
Sz
K
Sz
L
Sz
M

Poeciliopsis gracilis – female 2



Heterandria formosa

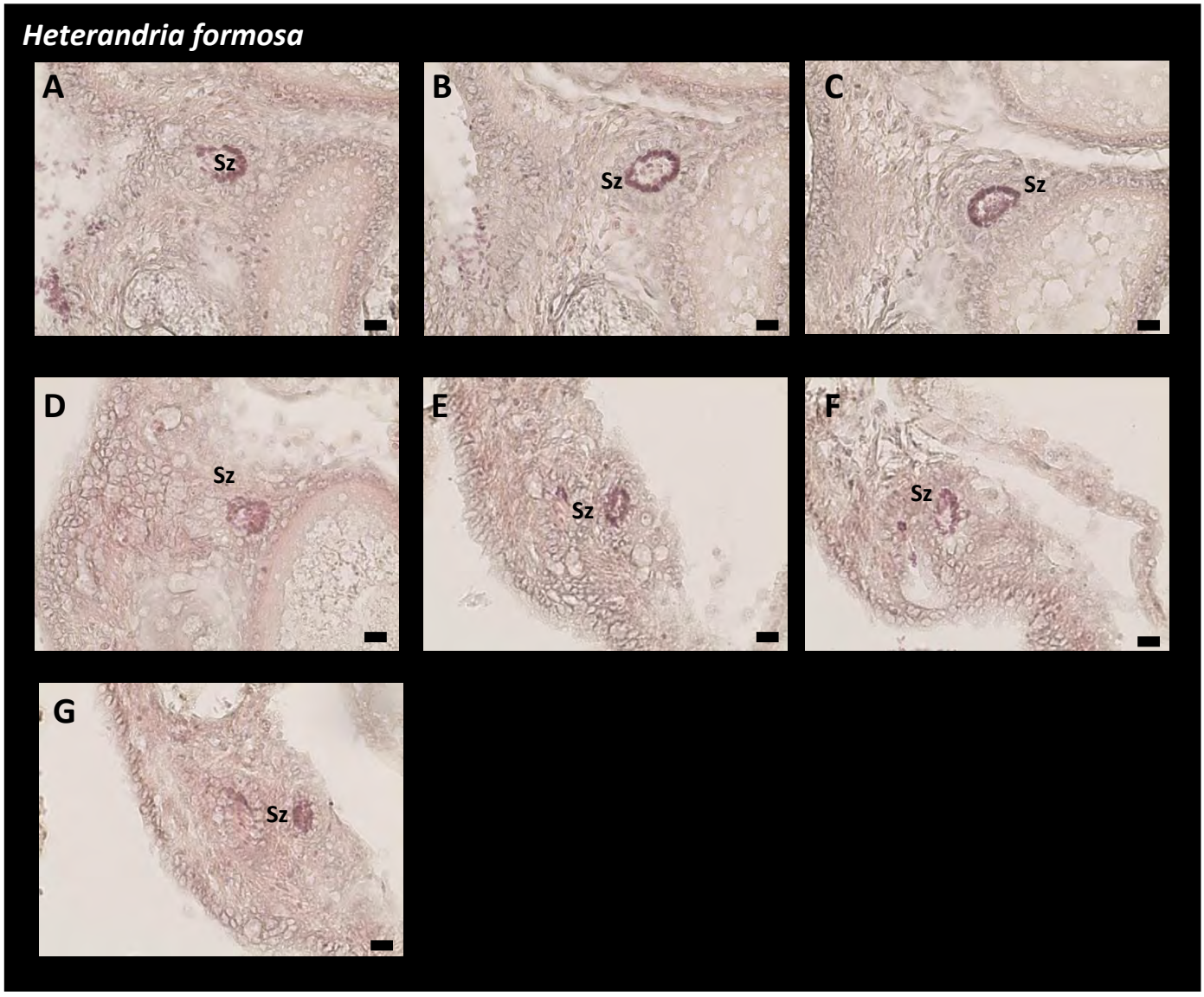
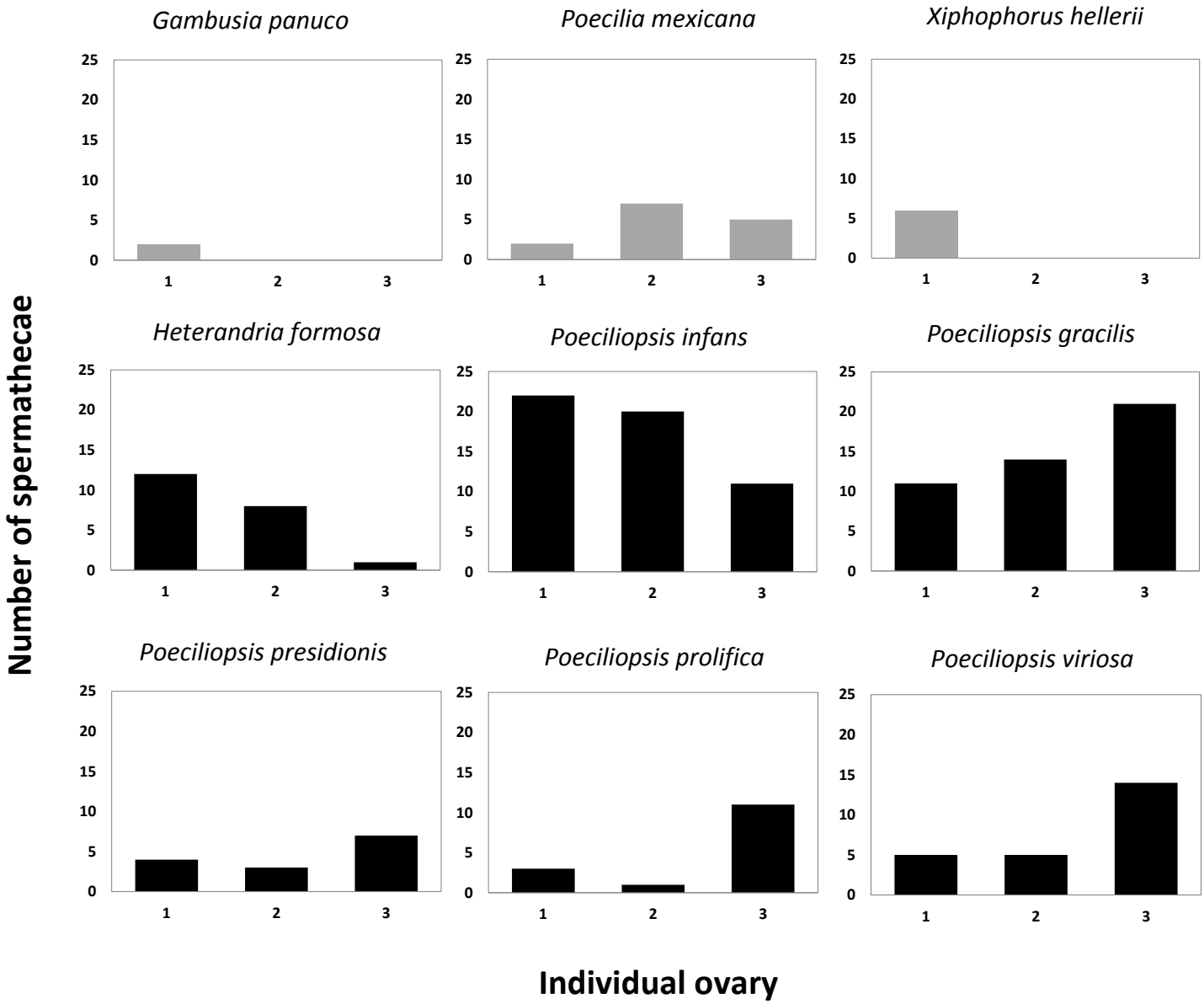
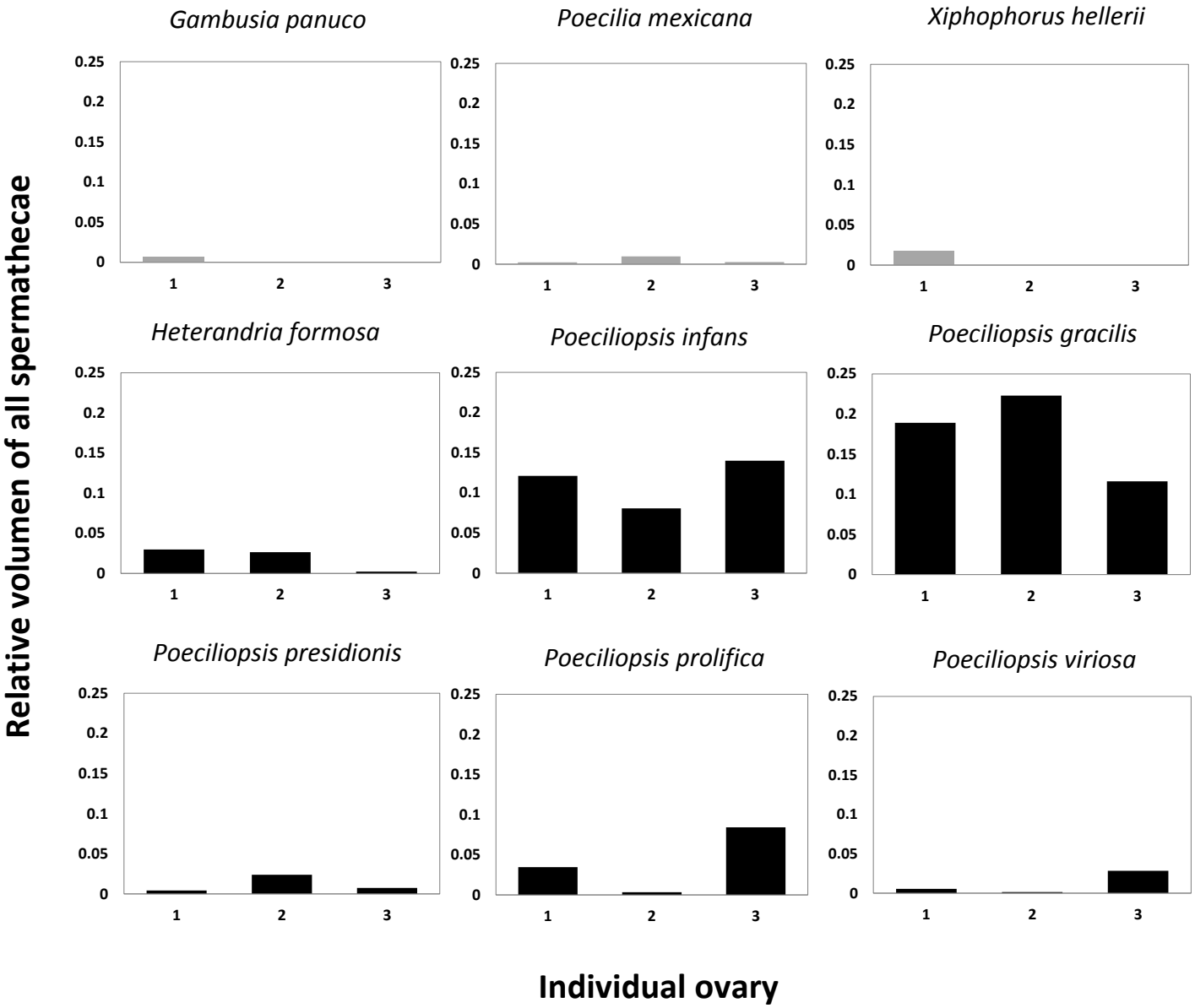
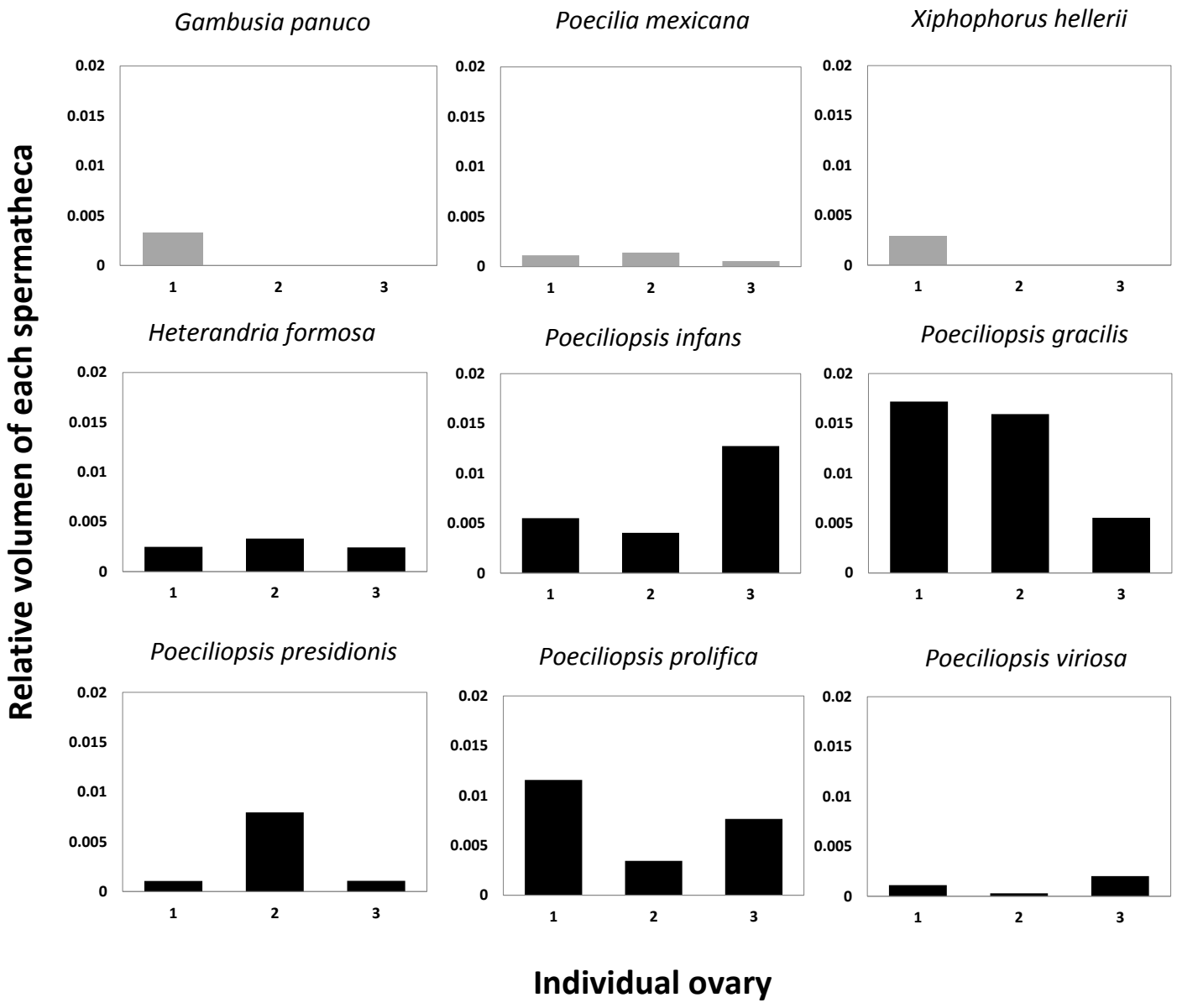


Fig. S2. Variation among individual ovaries in total number, total volume, and average volume of spermathecae for nine of the 12 species that we examined. Total and average volume of spermathecae are expressed as proportions of total ovary volume. Grey bars correspond to species without superfetation and black bars correspond to species with superfetation. We did not find spermathecae in the remaining three species (*Poecilia butleri, Priapella intermedia,* and *Pseudoxiphophorus bimaculatus*).




Gambusia panuco
Poecilia mexicana
Xiphophorus hellerii
Heterandria formosa
Poeciliopsis infans
Poeciliopsis gracilis
Poeciliopsis presidionis
Poeciliopsis prolifica
Poeciliopsis viriosa
Relative volumen of all spermathecae
0.25
0.2
0.15
0.1
0.05
0
1
2
3
Individual ovary


Gambusia panuco
Poecilia mexicana
Xiphophorus hellerii
Heterandria formosa
Poeciliopsis infans
Poeciliopsis gracilis
Poeciliopsis presidionis
Poeciliopsis prolifica
Poeciliopsis viriosa
Relative volumen of each spermatheca
Individual ovary
0.02
0.015
0.01
0.005
0
1
2
3

CAPÍTULO III

Have superfetation and matrotrophy facilitated the evolution of larger offspring in poeciliid fishes?

Claudia Olivera-Tlahuel, Alison G. Ossip-Klein, Héctor S. Espinosa-Pérez, and J. Jaime Zúñiga-Vega

Biological Journal of the Linnean Society, 2015, 116: 787-804.

The Linnean Society of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2015, **116**, 787–804. With 3 figures.

Have superfetation and matrotrophy facilitated the evolution of larger offspring in poeciliid fishes?

CLAUDIA OLIVERA-TLAHUEL[1], ALISON G. OSSIP-KLEIN[2], HÉCTOR S. ESPINOSA-PÉREZ[3] and J. JAIME ZÚÑIGA-VEGA[4]*

[1]*Posgrado en Ciencias Biológicas, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria, Distrito Federal, 04510, México*
[2]*Department of Biology, Indiana University, Bloomington, IN, 47405, USA*
[3]*Colección Nacional de Peces, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria, Distrito Federal, 04510, México*
[4]*Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria, Distrito Federal, 04510, México*

Received 22 May 2015; revised 10 July 2015; accepted for publication 13 July 2015

Superfetation is the ability of females to simultaneously carry multiple broods of embryos, with each brood at a different developmental stage. Matrotrophy is the post-fertilization maternal provisioning of nutrients to developing embryos throughout gestation. Several studies have demonstrated that, in viviparous fishes, superfetation and matrotrophy have evolved in a correlated way, such that species capable of bearing several simultaneous broods also exhibit advanced degrees of post-fertilization provisioning. The adaptive value of the concurrent presence of both reproductive modes may be associated with the production of larger newborns, which in turn may result in enhanced offspring fitness. In this study, we tested two hypotheses: (1) species with superfetation and moderate or extensive matrotrophy give birth to larger offspring compared with species without superfetation or matrotrophy; (2) species with higher degrees of superfetation and matrotrophy (i.e. more simultaneous broods and increased amounts of post-fertilization provisioning) give birth to larger offspring compared with species with relatively low degrees of superfetation and matrotrophy (i.e. fewer simultaneous broods and lesser amounts of post-fertilization provisioning). Using different phylogenetic comparative methods and data on 44 species of viviparous fishes of the family Poeciliidae, we found a lack of association between offspring size and the combination of superfetation and matrotrophy. Therefore, the concurrent presence of superfetation and moderate or extensive matrotrophy has not facilitated the evolution of larger offspring. In fact, these traits have evolved differently. Superfetation and matrotrophy have accumulated gradual changes that largely can be explained by Brownian motion, whereas offspring size has evolved fluidly, experiencing changes that probably resulted from selective responses to the local conditions. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **116**, 787–804.

ADDITIONAL KEYWORDS: lecithotrophy – offspring size – phylogenetic comparative methods – Poeciliidae – simultaneous broods – viviparous fishes.

INTRODUCTION

Superfetation is the ability of females to simultaneously carry multiple broods of embryos at different developmental stages (Turner, 1937, 1940; Scrimshaw, 1944). This mode of reproduction involves the occurrence of fertilization and development of a new brood before a preceding brood is born and can be clearly identified by the presence of groups of embryos within a single female that are in discretely different and well-separated stages of development (Pires *et al.*, 2011a; Roellig *et al.*, 2011). Superfetation is a relatively rare phenomenon that occurs in some viviparous species of three phylogenetically distant families of fishes: Clinidae (Gunn & Thresher, 1991), Zenarchopteridae (Reznick, Meredith & Collette, 2007a) and Poeciliidae (Turner, 1937, 1940; Scrimshaw, 1944; Schultz, 1961; Thibault & Schultz,

*Corresponding author. E-mail: jzuniga@ciencias.unam.mx

1978; Pollux *et al.*, 2009, 2014). Some species of the bivalve family Sphaeriidae exhibit brood masses that contain developmentally discrete subsets of embryos, with each subset encapsulated in a different brood sac. This phenomenon has been termed 'sequential brooding' but is equivalent to superfetation (Cooley & Foighil, 2000).

Within the fish family Poeciliidae, superfetation occurs in some genera such as *Poeciliopsis*, *Poecilia*, *Heterandria* and *Neoheterandria,* whereas it is absent in other genera (e.g. *Brachyrhaphis*, *Gambusia*, *Xhiphophorus* and *Alfaro*; Pires, Arendt & Reznick, 2010; Zúñiga-Vega, Macías-Garcia & Johnson, 2010). The phylogenetic distribution of superfetation within the family Poeciliidae suggests that it has evolved independently multiple times (Reznick & Miles, 1989; Pollux *et al.*, 2009; Pires *et al.*, 2010; Meredith *et al.*, 2011). In addition, the number of simultaneous broods present within females (i.e. degree of superfetation) varies widely among superfetating species. For example, *Heterandria formosa* exhibit a high degree of superfetation (females can carry up to eight different broods), whereas *Poecilia parae* exhibit a relatively low degree of superfetation (the maximum number of simultaneous broods is two; Travis *et al.*, 1987; Pires *et al.*, 2010). The multiple independent origins, along with the observed interspecific variation, suggest that superfetation might convey certain adaptive advantages that are not yet completely understood (Zúñiga-Vega *et al.*, 2010).

In addition to superfetation, the amount of nutrients that are transferred from mothers to embryos during development varies widely among viviparous species of the family Poeciliidae (Reznick, Mateos & Springer, 2002; Pollux *et al.*, 2009, 2014). Some species provide all the necessary nutrients for development before fertilization in the form of yolk without additional post-fertilization nutrient transfer. Hence, embryos lose mass during development due to metabolic costs (embryo mass decreases ~ 35% from fertilization to birth). This mode of maternal provisioning is called lecithotrophy (Wourms, 1981; Marsh-Matthews, 2011). In contrast, other species provide nutrients to developing embryos after fertilization through specialized morphological structures (i.e. placentas; Turner, 1940; Lombardi & Wourms, 1985; Mossman, 1991). This mode of maternal provisioning is called matrotrophy (Wourms, 1981; Marsh-Matthews, 2011). The relative amount of pre- and post-fertilization maternal provisioning varies widely among species (Pollux *et al.*, 2009, 2014). Incipient matrotrophy refers to species in which females provide only small amounts of nutrients to developing embryos after fertilization in addition to the yolk that was previously allocated to eggs. In such cases embryo mass decreases only slightly (10–15%) or remains constant throughout development. Moderate matrotrophy refers to species in which embryo mass increases moderately throughout development, reflecting a relatively larger provisioning from the mother after fertilization, although still relying to some extent on pre-fertilization yolk allocation. Extensive matrotrophy refers to species in which yolk is absent from mature ova, the mother throughout gestation actively provides all the nutrients that are necessary for development, and the mass at birth is at least five times larger than the egg mass at fertilization (Reznick *et al.*, 2002; Marsh-Matthews, 2011; Pires *et al.*, 2011a). In some species, the increase in embryo size throughout development reaches 60 000% (*Poecilia branneri* and *P. bifurca;* Pires *et al.*, 2010). There exists a continuum in the modes of maternal provisioning among poeciliid species, from strict lecithotrophy to extensive matrotrophy (Pollux *et al.*, 2009, 2014).

Some studies have demonstrated an evolutionary link between superfetation and matrotrophy (Reznick & Miles, 1989; Pollux *et al.*, 2009, 2014; Trexler & DeAngelis, 2010; Meredith *et al.*, 2011). Most species with superfetation also exhibit some degree of post-fertilization maternal provisioning, although a few exceptions exist (e.g. *Priapichthys festae*, *Neoheterandria tridentiger*, *N. elgans* and *Poeciliopsis monacha* exhibit superfetation and lecithotrophy; Reznick & Miles, 1989; Pollux *et al.*, 2009). This association has led to the hypothesis that the evolution of one of these two traits has facilitated the evolution of the other (Pollux *et al.*, 2009, 2014; Trexler & DeAngelis, 2010; Meredith *et al.*, 2011). The apparent coevolution of these reproductive strategies may have important consequences in terms of reproductive output. Hence, the adaptive significance of matrotrophy and superfetation could be associated with their patterns of covariation with life-history traits, such as number and size of offspring. For instance, species with superfetation tend to produce smaller broods and do so more often than non-superfetating species (Reznick & Miles, 1989; Pollux *et al.*, 2009). This mode of reproduction allows females to give birth continuously, with the interval between broods being as short as a few days. In contrast, species without superfetation, which for the most part exhibit lecithotrophy or only incipient matrotrophy, tend to produce larger broods and give birth less frequently, with many days between broods (Fraser & Renton, 1940; Thibault & Schultz, 1978; Cheong *et al.*, 1984; Leips *et al.*, 2009). The production of larger broods in non-superfetating species could result in smaller offspring because viviparous females have limited space within their reproductive tracts and, hence, they should

experience the widely recognized trade-off between number and size of offspring (Smith & Fretwell, 1974; Brockelman, 1975; Stearns, 1976).

In contrast, superfetating species that exhibit certain amounts of post-fertilization nutrient transfer might be able to increase the size of individual offspring. When matrotrophic females transfer an amount of nutrients during development that results in at least a slight increase in embryo mass from fertilization to birth (i.e. moderate matrotrophy), early-stage embryos are smaller than late-stage embryos. In combination with superfetation, where smaller broods imply fewer full-term embryos at any given time, this amount of post-fertilization nutrient transfer should promote less space requirements within the reproductive tract because these females bear a mix of early (small) and late (large) embryos compared with non-superfetating females that, at some point, would bear a high number of large full-term embryos. Therefore, the additional body volume that may be available to females exhibiting superfetation and at least moderate matrotrophy could be used to produce larger newborns. The combined effects of superfetation and matrotrophy on the potential reduction of space requirements within the reproductive tract could be even stronger in those species that exhibit extensive matrotrophy because at fertilization the ova is remarkably smaller than late-stage embryos. Thus, superfetation and extensive matrotrophy promote the presence of very small early-stage embryos, few large-late stage embryos and, potentially, low ovarian volume. This in turn could more easily allow for increases in the size of individual neonates.

Producing large newborns may increase their probability of surviving to maturity, at least under particular ecological conditions (Reznick, 1982; Reznick, Bryga & Endler, 1990; Bashey, 2008; Gordon *et al.*, 2009; Riesch *et al.*, 2010a). Therefore, if the coevolution of superfetation and matrotrophy has facilitated the evolution of larger offspring in poeciliid fishes, then the adaptive significance of the combined presence of these two reproductive modes could be associated with enhanced offspring fitness.

In this study we used modern phylogenetic comparative methods to analyse the evolutionary relationship between offspring size and the combined presence of superfetation and matrotrophy in 44 species of viviparous fishes of the family Poeciliidae. We focused on testing two main hypotheses. (1) Species with superfetation and at least moderate matrotrophy give birth to larger offspring compared with species without superfetation, species with superfetation and lecithotrophy, and species with superfetation and incipient matrotrophy (after accounting for differences among species in the size of adult females). (2) Species with higher degrees of superfetation and matrotrophy (i.e. more simultaneous broods and increased post-fertilization maternal provisioning) give birth to larger offspring compared with species with relatively low degrees of superfetation and matrotrophy (i.e. fewer simultaneous broods and less post-fertilization provisioning). In other words, offspring size should increase as the number of simultaneous broods and the amount of post-fertilization nutrient transfer increase. This hypothesis is based on the observation that superfetating species with more simultaneous broods and relatively high levels of matrotrophy tend to produce fewer newborns per brood compared with superfetating species with fewer simultaneous broods and lower levels of matrotrophy. For example, *Xenodexia ctenolepis* (dry weight at birth is over three times larger than unfertilized ova) and *Poecilia branneri* (dry weight at birth is over 86 times larger than unfertilized ova) produce on average 4.13 and 4.77 simultaneous broods, with 4.40 and 4.13 newborns per brood, respectively (Reznick *et al.*, 2007b; Pires *et al.*, 2010). In contrast, *Poeciliopsis monacha* (embryo dry weight decreases during development) and *P. turrubarensis* (embryo dry weight barely increases during development) produce on average 2 and 2.12 simultaneous broods, with 11.80 and 7.64 newborns per brood, respectively (Thibault & Schultz, 1978; Zúñiga-Vega, Reznick & Johnson, 2007). For those species with advanced degrees of superfetation and matrotrophy, more smaller broods probably imply very few large full-term embryos, more small embryos of earlier stages, less total space requirements within the reproductive tract and, therefore, the possibility of producing considerably large offspring.

MATERIAL AND METHODS

Data collection

We conducted an extensive literature search to extract data on average standard length (mm) of reproductive females, embryo dry mass (mg) at the last stage of development [i.e. stage 11 according to Haynes (1995) or the equivalent stage in other classification methods of embryonic development], degree of superfetation (average number of simultaneous broods at different developmental stages) and matrotrophy index (MI) for species of the family Poeciliidae (Table 1). We considered dry mass at the last stage of development as an appropriate proxy for size (mass) at birth (Haynes, 1995). MI is a standard measure of the amount of post-fertilization maternal provisioning and is calculated as the dry mass of the offspring at birth divided by the dry mass of the egg

Table 1. Female size (standard length), offspring size (mass), degree of superfetation (average number of simultaneous broods per female) and matrotrophy index (MI) for 44 species of the fish family Poeciliidae

Species	Female size (mm)	Offspring size (mg)	Offspring size (residuals)	Superfetation (no. of broods)	MI	PC1	Reference(s)
Brachyrhaphis rhabdophora	37.11	1.64	−0.458	1.00	0.77	−0.623	Johnson & Belk (2001), Pollux *et al.* (2014)
Brachyrhaphis episcopi	30.08	2.43	0.823	1.00	0.83	−0.621	Jennions & Telford (2002)
Brachyrhaphis holdridgei	22.61	0.6	−0.485	1.00	0.66	−0.628	Pollux *et al.* (2014), This study
Heterandria formosa	18.88	0.58	−0.244	3.09	35.00	2.439	Schrader & Travis (2012), Leips & Travis (1999), Pollux *et al.* (2014)
Poeciliopsis lucida	32.5	0.71	−1.066	3.00	2.00	0.968	Thibault & Schultz (1978), Pollux *et al.* (2014)
Poeciliopsis pleurospilus	45.01	1.04	−1.612	1.35	0.50	−0.367	This study
Poeciliopsis occidentalis	34.20	1.66	−0.235	1.64	1.12	−0.114	Pollux *et al.* (2014), This study
Poeciliopsis prolifica	24.63	0.59	−0.636	3.30	5.40	1.343	Pires, McBride & Reznick (2007), Pollux *et al.* (2014)
Poeciliopsis infans	28.75	0.82	−0.694	1.79	1.05	−0.003	Frías-Alvarez *et al.* (2014), Molina-Moctezuma (2015)
Poeciliopsis monacha	32.5	1.26	−0.516	2.00	0.61	0.140	Thibault & Schultz (1978), Pollux *et al.* (2014)
Poeciliopsis viriosa	37.93	0.43	−1.726	2.23	0.93	0.329	Pollux *et al.* (2014), This study
Poeciliopsis turneri	48.5	3.39	0.496	3.00	41.40	2.642	Thibault & Schultz (1978), Pollux *et al.* (2014)
Poeciliopsis gracilis	30.34	0.92	−0.705	1.88	0.84	0.057	Frías-Alvarez *et al.* (2014), Molina-Moctezuma (2015)
Poeciliopsis turrubarensis	39.38	1.58	−0.677	2.12	1.05	0.251	Zúñiga-Vega *et al.* (2007)
Poeciliopsis latidens	39.71	1.04	−1.240	1.12	0.86	−0.526	Pollux *et al.* (2014), This study
Poeciliopsis fasciata	30.78	1.92	0.264	1.34	0.91	−0.355	Pollux *et al.* (2014), This study
Poeciliopsis baenschi	25.62	1.28	−0.015	1.64	0.98	−0.122	Molina-Moctezuma (2011), This study
Heterophallus milleri	23.76	1.22	0.055	1.00	0.74	−0.624	Riesch *et al.* (2011)
Gambusia panuco	26.86	1.16	−0.222	1.00	0.79	−0.622	A. Ader, J. J. Zúñiga-Vega & J. Johnson, unpubl. data
Gambusia vittata	24.53	1.16	−0.059	1.00	1.29	−0.601	Weldele, Zúñiga-Vega & Johnson (2014)
Gambusia sexradiata	24.19	1.81	0.615	1.00	1.74	−0.582	Riesch *et al.* (2010a), R. Riesch, unpubl. data
Gambusia eurystoma	23.63	5.74	4.584	1.00	0.76	−0.624	Riesch *et al.* (2010a), R. Riesch, unpubl. data
Gambusia hubbsi	28.39	3.22	1.731	1.00	0.86	−0.619	Riesch *et al.* (2013)
Gambusia yucatana	23.45	0.8	−0.344	1.00	0.53	−0.633	This study
Gambusia holbrooki	31.7	0.97	−0.750	1.00	0.64	−0.629	Meffe (1990), Pollux *et al.* (2014)
Gambusia affinis	35	1.9	−0.051	1.00	0.62	−0.629	Stearns (1983), Pollux *et al.* (2014)

Table 1. *Continued*

Species	Female size (mm)	Offspring size (mg)	Offspring size (residuals)	Superfetation (no. of broods)	MI	PC1	Reference(s)
Gambusia speciosa	28.58	0.94	−0.562	1.00	0.45	−0.637	This study
Gambusia aurata	23.82	0.66	−0.510	1.00	0.82	−0.621	This study
Belonesox belizanus	99.4	6.9	0.450	1.00	0.70	−0.626	Turner & Snelson (1984), Pollux *et al.* (2014)
Pseudoxhiphophorus jonesii	48.10	4.36	1.494	1.00	0.65	−0.628	This study
Priapella chamulae	30.3	2.31	0.688	1.00	0.71	−0.626	Riesch *et al.* (2012)
Priapella olmecae	44.38	2.96	0.356	1.00	0.76	−0.623	This study
Poecilia mexicana	41.63	3.26	0.846	1.00	0.63	−0.629	Riesch, Plath & Schlupp (2010b), Pollux *et al.* (2014)
Poecilia sulphuraria	26.4	3.73	2.380	1.00	0.69	−0.627	Riesch *et al.* (2010a)
Poecilia butleri	41.77	1.64	−0.784	1.00	2.30	−0.558	Zúñiga-Vega *et al.* (2011)
Poecilia (*Micropoecilia*) *bifurca*	14.45	0.48	−0.035	1.20	55.05	1.836	Pires *et al.* (2010), Pollux *et al.* (2014)
Poecilia (*Micropoecilia*) *branneri*	19.16	0.62	−0.224	4.77	86.41	5.915	Pires *et al.* (2010), Pollux *et al.* (2014)
Poecilia (*Micropoecilia*) *parae*	23.33	0.67	−0.465	1.03	6.75	−0.346	Pires *et al.* (2010), Pollux *et al.* (2014)
Poecilia (*Micropoecilia*) *picta*	21.3	0.75	−0.243	1.00	0.78	−0.623	Pires *et al.* (2010), Pollux *et al.* (2014)
Poecilia (*Acanthophacelus*) *reticulata*	17.8	0.86	0.111	1.00	0.66	−0.628	Pires *et al.* (2010), Pollux *et al.* (2014)
Poecilia (*Acanthophacelus*) *wingei*	27.1	1.86	0.461	1.00	0.84	−0.620	Pires *et al.* (2010), Pollux *et al.* (2014)
Phalloceros caudiomaculatus	29.62	0.8	−0.775	1.00	2.14	−0.565	Arias & Reznick (2000), Pollux *et al.* (2014)
Phalloceros anisophallos	29.9	0.79	−0.804	1.00	2.80	−0.537	Almeida-Silva & Mazzoni (2014)
Xenodexia ctenolepis	47	3.57	0.781	4.13	3.38	1.896	Reznick *et al.* (2007b), Pollux *et al.* (2014)

Offspring size corresponds to the dry mass at the last stage of development. Given that larger species produce significantly larger offspring, we also show the residuals from a linear regression between female length and offspring mass. The column PC1 shows the species-specific scores in the first principal component that represents a combined measure of superfetation and matrotrophy.

at fertilization (Wourms, Grove & Lombardi, 1988; Trexler, 1997; Reznick *et al.*, 2002; Marsh-Matthews, 2011). Thus, MI represents the change in embryonic dry mass during development, with an MI value higher than 1 indicating an increase in embryo mass and lower than 1 indicating a decrease in embryo mass. An MI equal to 1 indicates that embryo mass remains constant from fertilization to parturition. When information was available for more than one population of a single species we used average values as input in our comparative analyses.

For some species (i.e. *Brachyrhaphis rhabdophora*, *B. episcopi*, *Gambusia vittata*, *G. panuco*, *Heterandria formosa*, *Poecilia butleri*, *Poeciliopsis baenschi*, *P. gracilis*, *P. infans* and *P. turrubarensis*), the authors who have published life-history descriptions provided us with their raw data, and we conducted a regression between stage of development and

log-transformed embryo dry mass. From this regression, we estimated the dry mass at the last developmental stage as a proxy for size at birth. In addition, for three of these species (*B. episcopi*, *G. panuco* and *P. baenschi*), we also used this regression to estimate the dry mass at fertilization [stage 4 according to Haynes (1995) or the equivalent stage in other classification methods] and then calculated an MI value as explained above (for most species MI values were available in Pollux *et al.*, 2014).

In addition, we quantified the same variables (standard length of reproductive females, dry mass of embryos at the last stage of development and degree of superfetation) from preserved females of the following species: *Brachyrhaphis holdridgei, Poeciliopsis fasciata, P. latidens*, *P. occidentalis*, *P. pleurospilus*, *P. viriosa*, *Gambusia yucatana*, *G. speciosa*, *G. aurata*, *Pseudoxiphophorus jonesii* and *Priapella olmecae* (Table 1). These specimens were preserved in ethanol at the National Collection of Fishes (Instituto de Biología, Universidad Nacional Autónoma de México). We dissected 20 reproductive females per species. Before dissection, we measured the standard length (mm) of all females with a digital caliper. Upon dissection, we removed the ovary and separated embryos into distinct broods based on stage of development (according to Haynes, 1995). We quantified the degree of superfetation as the number of broods at different developmental stages within each female. We dried each brood of embryos at 55 °C overnight, and then weighed embryos to the nearest 0.01 mg. To obtain an estimate of the dry mass at the last stage of development, we ran a regression between developmental stage and log-transformed embryo dry mass. In addition, for those species whose MI was not available in the literature (*G. yucatana*, *G. speciosa*, *G. aurata*, *Poecilipsis pleurospilus*, *Pseudoxiphophorus jonesii* and *Priapella olmecae*) we also used this regression to estimate dry mass at fertilization and then calculated an MI as explained above. In total, we obtained data for 44 poeciliid species (Table 1).

Accounting for potential confounding factors

Given that larger species could produce larger offspring and/or have higher degrees of superfetation or matrotrophy simply as a consequence of a larger body size (Reznick & Miles, 1989), we conducted linear regressions (using species-specific average values) between female length and offspring mass, between female length and superfetation, and between female length and MI. Offspring mass increased significantly with species-specific female size ($F = 31.7$, d.f. = 1, 42, $P < 0.0001$). Therefore, in all comparative analyses we used the residuals of this regression as size-corrected estimates of offspring size. In contrast, neither superfetation nor matrotrophy were affected by species-specific female size (superfetation: $F = 0.003$, d.f. = 1, 42, $P = 0.96$; matrotrophy: $F = 1.7$, d.f. = 1, 42, $P = 0.19$) and therefore we used the raw (unadjusted) values of number of simultaneous broods and MI.

Another potential confounding factor is the way in which specimens were originally preserved. Some species were preserved in formalin, whereas others were preserved in ethanol (Supporting Information, Table S1). Ethanol can extract lipids from the tissues and therefore might bias the estimates of offspring mass and MI (Shields & Carlson, 1996). We tested this potential confounding effect by means of general linear models using offspring dry mass and MI as response variables, the preservation method (formalin or ethanol) as a main factor and female length as a covariate. We also included in these models the interaction between preservation method and female length. Neither the preservation method nor its interaction with female length significantly affected offspring dry mass (preservation method: $F = 0.45$, d.f. = 1, 40, $P = 0.51$; interaction: $F = 0.10$, d.f. = 1, 40, $P = 0.76$) or MI (preservation method: $F = 0.14$, d.f. = 1, 40, $P = 0.71$; interaction: $F = 0.31$, d.f. = 1, 40, $P = 0.58$). Again here, female length had a highly significant effect on offspring mass ($F = 12.07$, d.f. = 1, 40, $P = 0.001$), whereas it did not affect MI ($F = 1.29$, d.f. = 1, 40, $P = 0.26$). Therefore, most of the variation among species in offspring mass was explained by the size of adult females, with no detectable effect of the preservation method on either offspring mass or MI.

To further examine the potential confounding effect of the preservation method, we implemented all our comparative analyses using only the 33 species that were preserved in ethanol (Table S1). All results were qualitatively similar to those obtained using the complete data set (44 species). Therefore, we report here the results obtained with the 44 species and those from the subset of 33 species in the Supporting Information.

A combined measure of superfetation and matrotrophy

We hypothesized that the combined effect of superfetation and at least moderate matrotrophy may allow for increases in size at birth in poeciliid fishes. Therefore, we conducted a principal components analysis on our species-specific values for degree of superfetation and MI. This analysis resulted in a single principal component with an eigenvalue > 1 (1.59) in which both superfetation and MI had high loadings (0.89 for both variables). Hence, we consid-

ered this principal component as a combined measure of superfetation and matrotrophy. Species with high positive scores corresponded to those with the highest degrees of superfetation and the largest amounts of post-fertilization maternal provisioning (e.g. *Poecilia branneri*: 4.77 simultaneous broods and MI = 86.41), whereas those with relatively high negative scores corresponded to lecithotrophic species without superfetation (e.g. *Gambusia speciosa*: lacks superfetation and MI = 0.45; Table 1). This combined measure of superfetation and matrotrophy was used as input in our comparative analyses.

Phylogeny and branch lengths

We used a well-resolved phylogeny of the family Poeciliidae that was reconstructed by Pollux *et al.* (2014) using a large molecular data set (20 nuclear and eight mitochondrial markers). We pruned their topology to our genera and species of interest. Although this phylogenetic reconstruction did not include all the 44 species that we considered in our study, we used it as the main representation of the relationships among genera. However, it did not include the genus *Heterophallus*. Therefore, the phylogenetic position of *H. milleri* with respect to other genera was based on Doadrio *et al.* (2009). Phylogenetic relationships within genera, among those species that were not included in the topology of Pollux *et al.* (2014), were obtained from the following studies: Mojica, Meyer & Barlow (1997) (*Brachyrhaphis*), Lydeard, Wooten & Meyer (1995), Langerhans *et al.* (2012) (*Gambusia*), Ptacek & Breden (1998) (*Poecilia*), Meredith *et al.* (2010) (*Poecilia*, subgenus *Micropoecilia*), Morales-Cazan & Albert (2012) (*Poeciliopsis*) and Meyer, Schories & Schartl (2011) (*Priapella*). The composite phylogeny that we used for comparative analyses is shown in Fig. 1.

Phylogenetic comparative methods require information about the expected amount of evolutionary change along each branch of the phylogenetic tree (i.e. branch lengths; Harvey & Pagel, 1991). Given that we based our comparative analyses on a composite phylogeny, we lacked branch lengths in units of DNA sequence divergence. Therefore, we used a conservative approach that consisted of generating 1000 possible sets of branch lengths in units of time by means of computer simulation, assuming a standard branching process of speciation (Martins, 1996). We conducted each analysis on each of these 1000 phylogenies and report average results. For analyses for which the calculation of average results across 1000 phylogenies was not automated, we generated five sets of branch lengths and report the average results across those five phylogenies.

Phylogenetic comparative methods

We used the program COMPARE 4.6b (Martins, 2004) to implement different phylogenetic comparative methods. To compare offspring size (adjusted for female length) between species with different degrees of superfetation and matrotrophy we used the adaptation-inertia model of Hansen (1997). This comparative method accounts for phylogenetic relatedness when analysing the relationship between a phenotypic trait (e.g. offspring size) and a particular selective force. Hansen (1997) described this as a model of evolutionary change based on strong stabilizing selection in which phenotypes evolve towards an adaptive optimum. This model accounts for competing selective forces that might result in different optimal phenotypic values depending on the particular evolutionary context. We implemented this method twice, with each implementation representing a different hypothesis. First, we tested the hypothesis that superfetation accompanied by at least moderate matrotrophy would be enough to facilitate the evolution of larger offspring. For this purpose, we classified our species into one of the following two groups, which we considered alternative reproductive modes: (1) species with superfetation and at least moderate matrotrophy (i.e. all superfetating species with an MI > 1), and (2) species without superfetation, species with superfetation and lecithotrophy (MI < 0.8) and species with superfetation and incipient matrotrophy ($0.8 \leq MI \leq 1$). We treated these two groups as alternative evolutionary forces and estimated how much of the variation across species in offspring size can be explained by the relative time each species has evolved with either of these two reproductive modes (r^2).

In the second implementation we tested the hypothesis that the combination of superfetation and extensive matrotrophy would provide even further opportunities for the evolution of larger offspring. Thus, we classified our species into one of the following two groups (alternative reproductive modes): (1) species with superfetation and extensive matrotrophy (i.e. all superfetating species with an MI > 5), and (2) all other species. Again, we estimated the proportion of interspecific variance in offspring size that can be explained by these two alternative evolutionary forces (r^2).

This method also allowed us to estimate optimal values of offspring size for species that exhibit these different combinations of the presence/absence of superfetation and relative amount of matrotrophy. Based on our hypotheses, we expected a larger optimal size at birth for superfetating species that exhibit moderate or extensive matrotrophy compared with that for non-superfetating species, superfetating

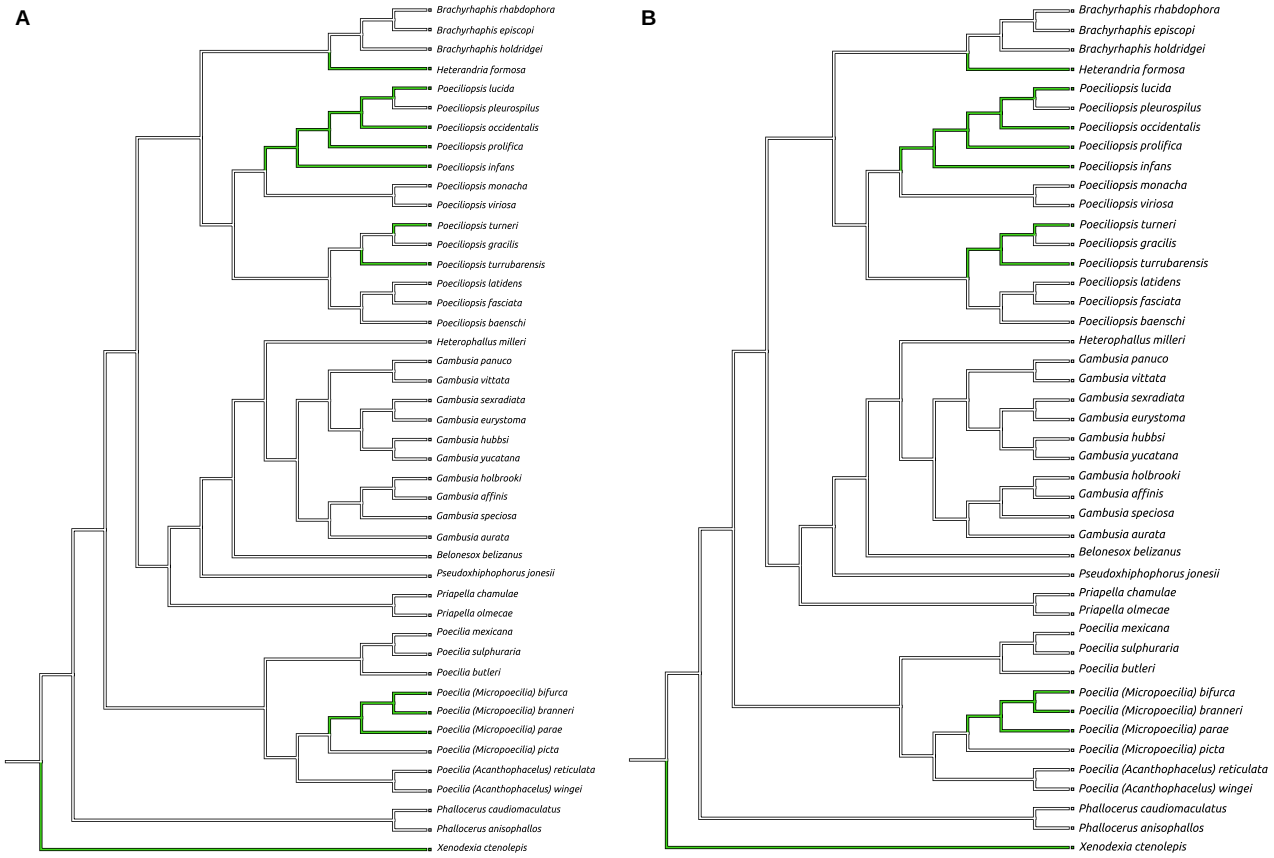


Figure 1. Composite phylogeny showing the relationships among 44 species of the family Poeciliidae as well as estimated ancestral states of the combined presence of superfetation and at least moderate matrotrophy (i.e. matrotrophy index > 1) based on parsimony. Green branches indicate presence of superfetation and at least moderate matrotrophy, whereas white branches indicate absence of superfetation, superfetation with lecithotrophy or superfetation with incipient matrotrophy. Both trees (A, reconstruction 1; B, reconstruction 2) were equally parsimonious.

species with lecithotrophy and superfetating species with incipient matrotrophy. Given that we adjusted our data to account for interspecific variation in female length, we actually expected a positive optimal value for superfetating species with moderate or extensive matrotrophy (a positive residual indicates a larger offspring size than that expected for a given female length) and a negative optimal value for non-superfetating species, superfetating species with lecithotrophy and superfetating species with incipient matrotrophy (a negative residual indicates a smaller offspring size than that expected for a given female length).

Hansen's (1997) method requires previous knowledge about the evolutionary history of the putative selective agent across the phylogeny. Thus, we reconstructed ancestral states for (1) the presence of superfetation with at least moderate matrotrophy and (2) the presence of superfetation with extensive matrotrophy using maximum likelihood and parsimony approaches implemented in the program MESQUITE 3.0 (Maddison & Maddison, 2009).

To estimate the magnitude of the relationship between offspring size and our combined measure of superfetation and matrotrophy we used different phylogenetic comparative methods. The first was Felsenstein's (1985) independent contrasts (FIC). This method solves the statistical problem of non-independence in the data due to shared ancestry and assumes that traits evolve along a phylogeny at a constant rate. Therefore, this mode of evolution can be described by Brownian motion, in which phenotypes diverge under random genetic drift. Hence, the expected phenotypic difference between sister species grows proportional to the time since they shared a common ancestor. From this method, we obtained a phylogenetically corrected correlation coefficient (r). We compared this with a Pearson correlation coefficient calculated using the uncorrected data (i.e. a non-phylogenetic approach referred to as TIPS;

Martins & Garland, 1991). The TIPS method assumes that traits adapt immediately to the local environment, leaving behind no trace of phylogenetic relationships.

We also used the phylogenetic generalized least squares method (PGLS) (Martins & Hansen, 1997), which uses an Ornstein–Uhlenbeck (OU) process as the underlying model of evolution. An OU process assumes that traits evolve under stabilizing selection. Similar to the FIC approach, PGLS estimates the relationship between two traits by means of a phylogenetically corrected correlation coefficient, r. In addition, PGLS estimates an additional parameter, α, which may vary between 0 and 15.5 (in COMPARE). When α is small, the rate of local adaptation is very slow and phenotypes appear to evolve by Brownian motion, which results in high phylogenetic signal. In contrast, when α is large the rate of adaptive change is fast and phenotypes evolve without a strong phylogenetic signal. Given that PGLS is a least squares regression model, we used our combined measure of superfetation and matrotrophy as predictor variable (X) and size-corrected offspring size as response variable (Y).

Finally, we used the phylogenetic mixed model (PMM) (Lynch, 1991; Housworth, Martins & Lynch, 2004), which also estimates the correlation between two traits after accounting for phylogenetic relatedness among species. The PMM assumes that phenotypes are the result of a linear combination of gradually accumulated evolutionary changes that have occurred along the phylogenetic history of the species and rapid evolutionary changes that probably resulted from adaptive responses to sudden changes in the environment. Thus, this model estimates the relative proportion of gradual Brownian-motion-like change (h^2, defined as phylogenetic heritability) for both traits (offspring size and the combined measure of superfetation and matrotrophy). When h^2 approaches one, most of the interspecific variation in the phenotypes is explained by the phylogenetic history. When h^2 approaches zero, phenotypes show little trace of phylogenetic signal and evolutionary changes have occurred faster than expected by Brownian motion (Housworth *et al.*, 2004). Using PMM, we also calculated h^2 separately for degree of superfetation and MI.

RESULTS

Ancestral reconstructions of superfetation and matrotrophy

The parsimony analysis resulted in two equally parsimonious ancestral reconstructions of superfetation and at least moderate matrotrophy, referred to herein as reconstructions 1 and 2 (Fig. 1). In reconstruction 1, the combination of superfetation and MI values > 1 originated in several occasions: once within the subgenus *Micropoecilia,* three times within the genus *Poeciliopsis*, with one loss (in *P. pleurospilus*), and once in *Hererandria formosa* (Fig. 1A). Reconstruction 2 was quite similar, with the only difference being two origins (instead of three) within *Poeciliopsis* and two losses (in *P. pleurospilus* and *P. gracilis*) (Fig. 1B). In both reconstructions we found uncertainty in whether the common ancestor of the family exhibited superfetation and at least moderate matrotrophy or not. If it did, then *Xenodexia ctenolepis* retained the ancestral state. If not, an additional independent origin occurred in *X. ctenolepis*.

The maximum likelihood reconstruction also indicated an origin of superfetation with at least moderate matrotrophy within the subgenus *Micropoecilia*, two origins within *Poeciliopsis* and two losses (in *P. pleurospilus* and *P. gracilis*), one additional origin in *H. formosa*, and high uncertainty with respect to the presence or absence of this reproductive mode in the common ancestor of the entire family (proportional likelihoods: 0.53 for superfetation and at least moderate matrotrophy and 0.47 for any other strategy) (Fig. 2). Again, *X. ctenolepis* represents either an additional independent origin or retention of an ancestral state. Note that the proportional likelihoods of the ancestral nodes in the group comprising *P. turneri*, *P. gracilis* and *P. turrubarensis* indicated high uncertainty with respect to the presence or absence of superfetation and MI values > 1 (Fig. 2). Given all this uncertainty, we decided to implement Hansen's (1997) method using both parsimony reconstructions 1 and 2 (the maximum likelihood reconstruction summarized these two) and assuming either presence or absence of superfetation and at least moderate matrotrophy in the most ancestral node. All results were qualitatively similar. Thus, for simplicity we report here only the results using reconstruction 1 and assuming that the ancestor of the family exhibited both superfetation and an MI > 1.

Regarding the ancestral reconstructions of superfetation and extensive matrotrophy, the parsimony analysis resulted in a single most parsimonious tree and identified four evolutionary origins (Fig. 3A). The first occurred within the subgenus *Micropoecilia*, particularly in the common ancestor of *Poecilia* (*Micropoecilia*) *bifurca*, *P.* (*M.*) *branneri*, and *P.* (*M.*) *parae*, the second in *Poeciliopsis turneri*, the third in *P. prolifica* and the fourth in *Heterandria formosa*. The maximum likelihood reconstruction gave identical results with minimum uncertainty with respect to the presence of superfetation and extensive matrotrophy in ancestral nodes (Fig. 3B).

Figure 2. Composite phylogeny showing the relationships among 44 species of the family Poeciliidae as well as estimated ancestral states of the combined presence of superfetation and at least moderate matrotrophy (i.e. matrotrophy index > 1) based on maximum likelihood. Circles depict proportional likelihoods for the presence (green) and absence (white) of superfetation and at least moderate matrotrophy.

Potential evolutionary relationships between offspring size and the combination of superfetation and matrotrophy

Interspecific variation in offspring size could not be explained well by the evolution of superfetation and at least moderate matrotrophy (Hansen's $r^2 = 0.05$) (Table 2). Contrary to what we expected, the optimal offspring size (after adjusting for female length) for species with superfetation and MI values > 1 was −0.38, whereas that for non-superfetating species, superfetating species with lecithotrophy and superfetating species with incipient matrotrophy was 0.16.

Similarly, the evolution of superfetation accompanied by extensive matrotrophy did not explain the interspecific variation in offspring size (Hansen's $r^2 = 0.01$) (Table 2). Again, the estimated optimal values were opposite to our prediction. The optimal offspring size for species with superfetation and MI values > 5 was −0.26, whereas that for all other species was 0.05.

The evolutionary relationship between degrees of superfetation and matrotrophy treated as a combined continuous variable (i.e. the species-specific scores in the first principal component) and offspring size was very weak, as indicated by the small, non-significant correlation coefficients obtained from FIC, PGLS and PMM (0.23, 0.07 and −0.21, respectively; $P > 0.13$ in all cases) (Table 2). A non-phylogenetic approach also indicated a weak correlation (TIPS $r = -0.10$, $P = 0.50$). According to PGLS, the combination of superfetation and matrotrophy explained only 8% of the interspecific variation in offspring size ($r^2 = 0.08$). All these analyses conducted on the subset of 33 species that were preserved in ethanol yielded equivalent results (Supporting Information, Table S2).

Phylogenetic signal in superfetation, matrotrophy and offspring size

PGLS estimated a large value of the parameter α (15.4), which indicates that the species-specific offspring sizes have been pulled strongly towards optimal values. Therefore, the evolution of this trait cannot be explained very well by the phylogenetic history alone. Consistent with this result, the phylogenetic heritability of offspring size was low ($h^2 = 0.22$), retaining only weak evidence of the phylogeny. In contrast, the combination of superfetation and matrotrophy has tracked the phylogeny more closely, resulting in a remarkably high phylogenetic heritability ($h^2 = 0.86$) (Table 2). Separately, superfetation and MI values also had high phylogenetic heritabilities ($h^2 = 0.81$ and 0.87, respectively). We obtained similar estimates of phylogenetic signal for offspring size, superfetation and matrotrophy from the subset of 33 species that were preserved in ethanol (Table S2).

DISCUSSION

Superfetation and matrotrophy have not facilitated the evolution of larger offspring

We have demonstrated here that the combination of superfetation and matrotrophy does not exhibit an evolutionary relationship with offspring size, regardless of whether superfetation and matrotrophy were treated as a dichotomous variable (presence or absence of superfetation and moderate or extensive matrotrophy) or as a continuous variable (degrees of superfetation and matrotrophy combined into a single measure). Thus, neither of our two hypotheses was supported by our results. In fact, the estimated

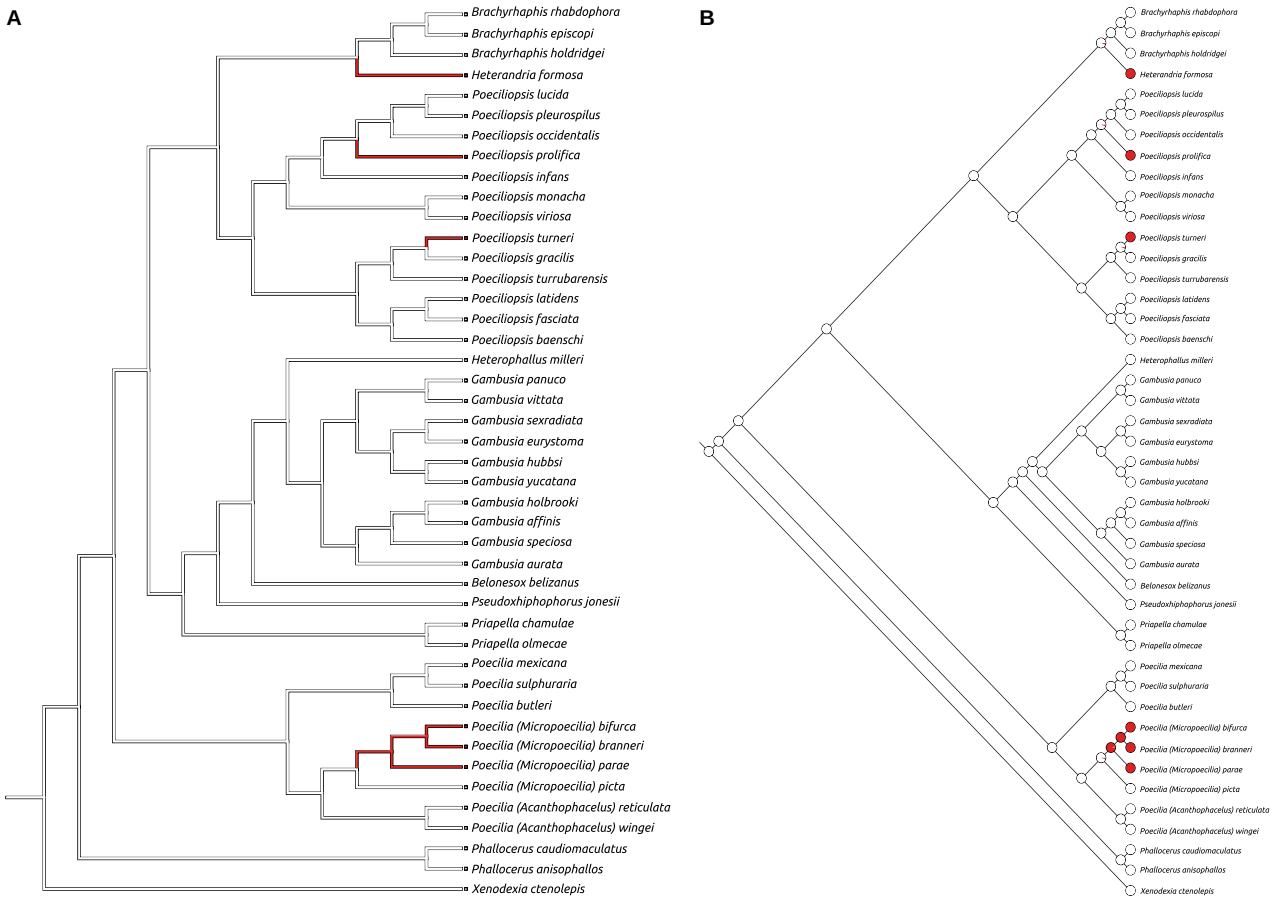


Figure 3. Composite phylogeny showing the relationships among 44 species of the family Poeciliidae as well as estimated ancestral states of the combined presence of superfetation and extensive matrotrophy (i.e. matrotrophy index > 5) based on (A) parsimony and (B) maximum likelihood. Red branches in A indicate presence of superfetation and extensive matrotrophy. Circles in B depict proportional likelihoods for the presence (red) and absence (white) of superfetation and extensive matrotrophy.

Table 2. Phylogenetic estimates of the magnitude of the evolutionary relationship between offspring size and the combination of superfetation and matrotrophy calculated using different phylogenetic comparative methods

Hansen's method					
SF and MI > 1	SF and MI > 5	FIC	TIPS	PGLS	PMM
$r^2 = 0.05$	$r^2 = 0.01$	$r = 0.23$	$r = -0.10$	$r = 0.07$ $r^2 = 0.08$ $\alpha = 15.4$	$r = -0.21$ Offspring size $h^2 = 0.22$ SF and MI $h^2 = 0.86$ SF $h^2 = 0.81$ MI $h^2 = 0.87$

FIC, Felsenstein's independent contrasts; TIPS, non-phylogenetic approach; PGLS, phylogenetic generalized least squares method assuming an Ornstein–Uhlenbeck model of evolution; PMM, phylogenetic mixed model; SF, superfetation; MI, matrotrophy index; r, correlation coefficient; r^2, proportion of the variation among species in offspring size explained by superfetation and matrotrophy; α, a measure of the strength of selection pulling offspring size towards optimal values; h^2, phylogenetic heritability.

optimal sizes at birth for species with superfetation and moderate or extensive matrotrophy were smaller than those for species without superfetation, species with superfetation and lecithotrophy, and species with superfetation and incipient matrotrophy (although this effect was weak). This result was contrary to expectation. Pires *et al.* (2011b) found a similar result using only six species of the genus *Poeciliopsis*, all of which exhibit superfetation. Larger MI values were associated with smaller offspring. Reznick & Miles (1989), in a preliminary examination that did not account for phylogenetic relatedness, also found that superfetation (which they noted was strongly linked to matrotrophy) was not associated with larger offspring.

Our estimates of phylogenetic heritability also confirmed that offspring size has evolved at a different pace in comparison with superfetation and matrotrophy, rather than in a correlated way as we expected. Offspring size has evolved flexibly at a faster pace, leaving behind little trace of the phylogeny. In contrast, the combination of superfetation and matrotrophy has evolved at a rate that has been proportional to the time elapsed between speciation events. When we analysed the degree of superfetation and MIs separately, we confirmed that both traits evolved for the most part by Brownian motion. This is the main reason why most of the interspecific variation in the degrees of superfetation and matrotrophy can be explained by the phylogenetic history. However, we highlight that the particular combination of superfetation and extensive matrotrophy has arisen recently and independently in three distinct species (*Heterandria formosa*, *Poeciliopsis prolifica* and *P. turneri*) with little evidence of the presence of this particular reproductive mode in ancestral nodes. In contrast, within the subgenus *Micropoecilia*, superfetation and extensive matrotrophy arose in the common ancestor of *Poecilia* (*Micropoecilia*) *bifurca*, *P.* (*M.*) *branneri* and *P.* (*M.*) *parae* and these three species inherited this reproductive mode (Fig. 3). Apparently, these distinct and relatively recent evolutionary events that resulted in the co-occurrence of superfetation and extensive matrotrophy did not promote parallel changes in offspring size.

We note here an important caveat. Superfetating species appear to produce smaller broods (Reznick & Miles, 1989; Pollux *et al.*, 2009), thus potentially providing additional space within the reproductive tract if at least moderate matrotrophy is present. However, the potential for this additional space within the reproductive tract, which we hypothesized could be used to produce larger offspring, depends on total fecundity remaining similar to that of non-superfetating and lecithotrophic species. If the selective forces that favour the presence of superfetation and matrotrophy also promote increased fecundity, then the additional space could be used to produce more embryos (by means of increasing the number of simultaneous broods) rather than larger offspring.

We found no evidence of an effect of superfetation and matrotrophy on offspring size at the interspecific level. However, several studies have demonstrated that these three traits may vary widely among populations within particular species (Trexler, 1985; Riesch, Martin & Langerhans, 2013; Frías-Alvarez *et al.*, 2014). Therefore, a large offspring size as a result of increased levels of superfetation and matrotrophy at the intraspecific level is still a possibility that deserves future field and experimental tests.

Offspring size as a local adaptation

If offspring size is not influenced by the combination of superfetation and moderate or extensive matrotrophy, then other factors must have caused the observed variation in size at birth among species of the family Poeciliidae. Our results indicated a low phylogenetic signal in offspring size because there was more interspecific variation than expected by Brownian motion. Therefore, the evolution of offspring size in poeciliid fishes appears to be driven primarily by local adaptation to particular environmental conditions.

Several studies in poeciliids demonstrated adaptive responses of offspring size to local conditions. For example, Leips & Travis (1999) found that offspring of *Heterandria formosa* from high-density populations were 45% larger than offspring from low-density populations. High population density is characterized by high levels of competition and, under these conditions, large offspring are more likely to survive to maturity (Schrader & Travis, 2012). This same positive effect of population density and intraspecific competition on offspring size has been observed in the guppy, *Poecilia reticulata* (Bashey, 2006, 2008).

Predation is also an important selective force for the evolution of offspring size (Gordon *et al.*, 2009; Schrader & Travis, 2012). The size at birth of *Poecilia reticulata* differs between populations inhabiting two types of predation environments. Female *P. reticulata* from populations sympatric with predators that feed on small juvenile fish produce larger newborns relative to females from populations that do not experience this predation pressure. Larger newborns reach adult sizes faster than smaller newborns and spend less time at body sizes susceptible to higher predation rates. In contrast, when coexisting with predators that prey on large adult fish, females of *P. reticulata* increase their reproductive

output by producing many smaller offspring (Reznick, 1982; Reznick & Endler, 1982; Reznick *et al.*, 1990). Two species in the genus *Brachyrhaphis* (*B. episcopi* and *B. rhabdophora*) show a similar pattern of predator-mediated divergence in offspring size (Johnson & Belk, 2001; Jennions & Telford, 2002). Additional studies in *Gambusia hubbsi* and *Poecilia vivipara* also have suggested local selective effects of predation on offspring size (Downhower, Brown & Matsui, 2000; Gomes & Monteiro, 2007). Apparently, high rates of cannibalism on juveniles may also select for larger offspring sizes as observed in *Poeciliopsis monacha* (Thibault, 1974; Weeks & Gaggiotti, 1993).

Additionally, toxic compounds in the water (e.g. hydrogen sulfide) could be selective agents for offspring size. Species such as *Poecilia sulphuraria* and *Gambusia eurystoma* that inhabit water bodies with high concentrations of hydrogen sulfide produce relatively large newborns. This is presumably because a large body size results in a relatively low surface/volume ratio, which in turn implies that less surface area per volume of body tissue is exposed to the toxin. In addition, larger offspring probably have lower metabolic rates and, hence, lower oxygen consumption compared with smaller offspring (Riesch *et al.*, 2010a). Some studies have documented effects of water salinity on offspring size with contradictory results. In *Gambusia affinis, G. holbrooki* and *Heterandria formosa*, water bodies with high salinities appear to promote smaller offspring (Stearns & Sage, 1980; Brown-Peterson & Peterson, 1990; Alcaraz & García-Berthou, 2007; Martin *et al.*, 2009). In contrast, in *Poecilia vivipara* high salinity is associated with larger offspring (Gomes & Monteiro, 2007). The mechanisms by which salinity favours large offspring in some species and small offspring in other species remain unknown. In addition to factors that promote adaptive changes, offspring size might also exhibit phenotypic plasticity as a result of food availability (Grether & Kolluru, 2011). In *Brachyrhaphis episcopi*, females produce larger offspring during the wet season when food is more abundant (Jennions *et al.*, 2006). All these studies have demonstrated that in poeciliid fishes, offspring size may exhibit adaptive and plastic responses to a wide array of environmental conditions, thereby explaining the fluid evolutionary changes that we have detected here.

Adaptive value of superfetation and matrotrophy

The combination of superfetation and moderate or extensive matrotrophy has not facilitated the evolution of larger offspring in poeciliid fishes. Several authors have suggested an evolutionary link between superfetation and matrotrophy and have proposed other potential benefits of their concurrent presence (Reznick & Miles, 1989; Pollux *et al.*, 2009, 2014; Trexler & DeAngelis, 2010; Meredith *et al.*, 2011). First, matrotrophy may increase total fecundity because initial egg size is small and females may fertilize a relatively large number of eggs without a large initial energetic investment. In contrast, lecithotrophic species are limited in the initial number of eggs that females can fertilize because a large amount of resources are needed to produce fully yolked eggs (Trexler, 1997; Trexler & DeAngelis, 2003, 2010; Marsh-Matthews *et al.*, 2005; Marsh-Matthews, 2011). Thus, total fecundity may be limited in lecithotrophic species. However, matrotrophic females need constant food supply to actively transfer nutrients to developing embryos. Otherwise, matrotrophy could become a disadvantage if enough resources are not available to sustain developing offspring. Hence, matrotrophy could increase total fecundity in environments where food resources are constant and abundant. According to a theoretical model developed by Trexler & DeAngelis (2003, 2010), in these predictable and stable environments, the previous presence of superfetation would facilitate the evolution of matrotrophy. Recently, some studies have conducted empirical tests of this 'resource-availability' hypothesis (Trexler, 1997; Marsh-Matthews & Deaton, 2006; Banet & Reznick, 2008; Banet, Au & Reznick, 2010; Pollux & Reznick, 2011; Riesch *et al.*, 2013).

Second, matrotrophy and superfetation could reduce the locomotory cost imposed by viviparity. During pregnancy, females gain mass and volume, which negatively affects escape response and swimming performance due to increased drag forces on the female body (Plaut, 2002; Ghalambor, Reznick & Walker, 2004; Langerhans & Reznick, 2010). Matrotrophy could reduce this reproductive burden because mature ova are small and thus initial space requirements are low (Thibault & Schultz, 1978; Marsh-Matthews, 2011). Superfetation might provide additional advantages because it further reduces reproductive allocation by allowing females to divide the total number of developing embryos into smaller broods (Thibault & Schultz, 1978; Zúñiga-Vega *et al.*, 2010). Therefore, both matrotrophy and superfetation could result in thinner bodies without a reduction in the total number of offspring produced because females bear a combination of small early-stage embryos and fewer large late-stage embryos compared with lecithotrophic and non-superfetating species that must bear many large embryos (Miller, 1975; Thibault & Schultz, 1978; Zúñiga-Vega *et al.*, 2007, 2010; Pollux *et al.*, 2009; Pires *et al.*, 2011a).

Selective environments where a streamlined body shape is needed such as high-velocity water systems or habitats where fish must swim fast to escape from abundant predators might promote the evolution of both reproductive strategies. Zúñiga-Vega *et al.* (2007) provided evidence that supports this hypothesis using *Poeciliopsis turrubarensis* as a model system. However, they focused only on the adaptive value of superfetation without any emphasis on spatial variation in matrotrophy. Additional tests of this 'locomotor cost' hypothesis are needed, analysing the effects of the concurrent presence of matrotrophy and superfetation on body streamlining and fitness in these particular selective environments.

Third, moderate and extensive matrotrophy have coevolved with specialized morphological structures that facilitate the active transfer of nutrients from mother to embryos (i.e. placentas; Turner, 1940; Jollie & Jollie, 1964; Knight *et al.*, 1985; Grove & Wourms, 1994; Kwan *et al.*, 2015). This enhanced physiological communication through placental tissues could facilitate the rise of parent–offspring conflicts during pregnancy (i.e. individual embryos demand a greater investment and mothers attempt to optimize the allocation to each offspring; Trivers, 1974; Zeh & Zeh, 2000, 2008; Crespi & Semeniuk, 2004; Schrader & Travis, 2008). Parent–offspring conflicts during embryo development may drive a shift from pre-copulatory female mate choice to post-copulatory mechanisms of sexual selection (Pollux *et al.*, 2014). The capacity to control the amount of resources that are devoted to each offspring through matrotrophy and placentas would allow females to allocate more resources to those offspring that carry the best paternal genes. In addition, superfetation could further enhance post-copulatory female selection by facilitating multiple paternity because each brood may be fertilized by a different male (Travis, Trexler & Mulvey, 1990; Zane *et al.*, 1999; Soucy & Travis, 2003). This potential to produce temporally overlapping, mixed-paternity broods might also increase genetic variability of the offspring (Macías-Garcia & González-Zuarth, 2005). Pollux *et al.* (2014) used phylogenetic comparative methods and a large data set (94 species) to suggest that the correlated evolution of superfetation and matrotrophy in the family Poeciliidae has allowed placental females to gain control over paternity, thereby relying to less extent on pre-copulatory mechanisms of sexual selection.

Additional theoretical models have attempted to explain the fitness benefits of superfetation and matrotrophy, although considering them separately (Thibault, 1974; Downhower & Brown, 1975; Travis *et al.*, 1987; Zúñiga-Vega *et al.*, 2010; Pires *et al.*, 2011b; Bassar, Auer & Reznick, 2014). However, their correlated evolution strongly suggests that the adaptive benefits of superfetation depend, at least to some degree, on the concurrent presence of matrotrophy and/or vice versa (Reznick & Miles, 1989; Pollux *et al.*, 2009, 2014; Trexler & DeAngelis, 2010; Meredith *et al.*, 2011). Therefore, further theoretical and empirical studies must focus on the combination of both traits when examining their potential adaptive value.

ACKNOWLEDGEMENTS

This research was supported by the Mexican Research Council (Consejo Nacional de Ciencia y Tecnología, CONACyT) through a doctorate scholarship awarded to C.O.-T. (368782/245650) and through grant SEP-CONACyT-129675. A.G.O.K. was supported by a Common Themes in Reproductive Diversity training grant (NIH-NICD 5T32HD049336-10). This paper is a requisite for C.O.-T. to obtain the PhD degree in the Posgrado en Ciencias Biológicas of Universidad Nacional Autónoma de México. We thank Alecandria Ader, Alejandro Molina-Moctezuma, Jerald Johnson, Joseph Travis, Matthew Schrader, Mark Belk, Michael Jennions, Patricia Frías-Alvarez and Rüdiger Riesch for providing us with their data sets and Norma Moreno-Mendoza and Maricela Villagrán-Santa Cruz for academic advice. We also thank Patricia Frías-Alvarez, Marcelo Pires and the anonymous reviewers for their helpful comments.

REFERENCES

Alcaraz C, García-Berthou E. 2007. Life history variation of invasive mosquitofish (*Gambusia holbrooki*) along a salinity gradient. *Biological Conservation* **139:** 83–92.

Almeida-Silva PH, Mazzoni R. 2014. Life history aspects of *Phalloceros anisophallos* Lucinda, 2008 (Osteichthyes, Poeciliidae) from Córrego Andorinha, Ilha Grande (RJ, Brazil). *Studies on Neotropical Fauna and Environment* **49:** 1–8.

Arias AL, Reznick DN. 2000. Life history of *Phalloceros caudiomaculatus*: a novel variation on the theme of live-bearing in the family Poeciliidae. *Copeia* **2000:** 792–798.

Banet AI, Reznick DN. 2008. Do placental species abort offspring? Testing an assumption of the Trexler–DeAngelis model. *Functional Ecology* **22:** 323–331.

Banet AI, Au AG, Reznick DN. 2010. Is mom in charge? Implications of resource provisioning on the evolution of the placenta. *Evolution* **64:** 3172–3182.

Bashey F. 2006. Cross-generational environmental effects and the evolution of offspring size in the Trinidadian guppy *Poecilia reticulata*. *Evolution* **60:** 348–361.

Bashey F. 2008. Competition as a selective mechanism for larger offspring size in guppies. *Oikos* **117:** 104–113.

Bassar RD, Auer SK, Reznick DN. 2014. Why do placentas evolve? A test of the life-history facilitation hypothesis in two clades in the genus *Poeciliopsis* representing two independent origins of placentas. *Functional Ecology* **28:** 999–1010.

Brockelman WY. 1975. Competition, the fitness of offspring, and optimal clutch size. *American Naturalist* **109:** 677–699.

Brown-Peterson N, Peterson MS. 1990. Comparative life history of female mosquito fish, *Gambusia affinis*, in tidal freshwater and oligohaline habitats. *Environmental Biology of Fishes* **27:** 33–41.

Cheong RT, Henrich S, Farr JA, Travis J. 1984. Variation in fecundity and its relationship to body size in a population of the least killifish, *Heterandria formosa* (Pisces: Poeciliidae). *Copeia* **1984:** 720–726.

Cooley LR, Foighil DÓ. 2000. Phylogenetic analysis of the Sphaeriidae (Mollusca: Bivalvia) based on partial mitochondrial 16S rDNA gene sequences. *Invertebrate Biology* **119:** 299–308.

Crespi B, Semeniuk C. 2004. Parent–offspring conflict in the evolution of vertebrate reproductive mode. *American Naturalist* **163:** 635–653.

Doadrio I, Perea S, Alcaraz L, Hernandez N. 2009. Molecular phylogeny and biogeography of the Cuban genus *Girardinus* Poey, 1854 and relationships within the tribe Girardinini (Actinopterygii, Poeciliidae). *Molecular Phylogenetics and Evolution* **50:** 16–30.

Downhower JF, Brown L. 1975. Superfetation in fishes and the cost of reproduction. *Nature* **256:** 345.

Downhower JF, Brown LP, Matsui ML. 2000. Life history variation in female *Gambusia hubbsi*. *Environmental Biology of Fishes* **59:** 415–428.

Felsenstein J. 1985. Phylogenies and the comparative method. *American Naturalist* **125:** 1–15.

Fraser EA, Renton RM. 1940. Observation on the breeding and development of the viviparous fish *Heterandria formosa*. *Quarterly Journal of Microscopical Science* **81:** 479–520.

Frías-Alvarez P, Macías Garcia C, Vázquez-Vega LF, Zúñiga-Vega JJ. 2014. Spatial and temporal variation in superfoetation and related life history traits of two viviparous fishes: *Poeciliopsis gracilis* and *P. infans*. *Naturwissenschaften* **101:** 1085–1098.

Ghalambor CK, Reznick DN, Walker JA. 2004. Constraints on adaptive evolution: the functional trade-off between reproduction and fast-start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). *American Naturalist* **164:** 38–50.

Gomes JL Jr, Monteiro LR. 2007. Size and fecundity variation in populations of *Poecilia vivipara* Block & Schneider (Teleostei; Poeciliidae) inhabiting an environmental gradient. *Journal of Fish Biology* **71:** 1799–1809.

Gordon SP, Reznick DN, Kinnison MT, Bryant MJ, Weese DJ, Räsänen K, Millar NP, Hendry AP. 2009. Adaptive changes in life history and survival following a new guppy introduction. *American Naturalist* **174:** 34–45.

Grether GF, Kolluru GR. 2011. Evolutionary and plastics responses to resource availability. In: Evans JP, Pilastro A, Schlupp I, eds. *Ecology and evolution of poeciliid fishes*. Chicago: The University of Chicago Press, 28–37.

Grove BD, Wourms JP. 1994. The follicular placenta of the viviparous fish, *Heterandria formosa*. I. Ultrastructure and development of the embryonic absorptive surface. *Journal of Morphology* **209:** 265–284.

Gunn JS, Thresher RE. 1991. Viviparity and the reproductive ecology of clinid fishes (Clinidae) from temperate Australian waters. *Environmental Biology of Fishes* **31:** 323–344.

Hansen TF. 1997. Stabilizing selection and the comparative analysis of adaptation. *Evolution* **51:** 1341–1351.

Harvey PH, Pagel MD. 1991. *The comparative method in evolutionary biology*. Oxford: Oxford University Press.

Haynes JL. 1995. Standardized classification of poeciliid development for life-history studies. *Copeia* **1995:** 147–154.

Housworth EA, Martins EP, Lynch M. 2004. The phylogenetic mixed model. *American Naturalist* **163:** 84–96.

Jennions M, Telford S. 2002. Life-history phenotypes in populations of *Brachyrhaphis episcopi* (Poeciliidae) with different predator communities. *Oecologia* **132:** 44–50.

Jennions MD, Wong BB, Cowling A, Donnelly C. 2006. Life-history phenotypes in a live-bearing fish *Brachyrhaphis episcopi* living under different predator regimes: seasonal effects? *Environmental Biology of Fishes* **76:** 211–219.

Johnson JB, Belk MC. 2001. Predation environment predicts divergent life-history phenotypes among populations of the livebearing fish *Brachyrhaphis rhabdophora*. *Oecologia* **126:** 142–149.

Jollie WP, Jollie LG. 1964. The fine structure of the ovarian follicle of the ovoviviparous poeciliid fish, *Lebistes reticulatus*. I. Maturation of follicular epithelium. *Journal of Morphology* **114:** 479–501.

Knight FM, Lombardi J, Wourms JP, Burns JR. 1985. Follicular placenta and embryonic growth of the viviparous four-eyed fish (*Anableps*). *Journal of Morphology* **185:** 131–142.

Kwan L, Fris M, Rodd FH, Rowe L, Tuhela L, Panhuis TM. 2015. An examination of the variation in maternal placentae across the genus *Poeciliopsis* (Poeciliidae). *Journal of Morphology* **276:** 707–720.

Langerhans RB, Reznick DN. 2010. Ecology and evolution of swimming performance in fishes: predicting evolution with biomechanics. In: Domenici P, Kapoor BG, eds. *Fish locomotion: an eco-ethological perspective*. Boca Ratón, FL: Science Publishers, 200–248.

Langerhans RB, Gifford ME, Domínguez-Domínguez O, García-Bedoya D, DeWitt TJ. 2012. *Gambusia quadruncus* (Cyprinodontiformes: Poeciliidae): a new species of mosquitofish from east-central México. *Journal of Fish Biology* **81:** 1514–1539.

Leips J, Travis J. 1999. The comparative expression of life-history traits and its relationship to the numerical dynamics of four populations of the least killifish. *Journal of Animal Ecology* **68:** 595–616.

Leips J, Richardson JM, Rodd FH, Travis J. 2009. Adaptive maternal adjustments of offspring size in response to conspecific density in two populations of the least killifish, *Heterandria formosa*. *Evolution* **63:** 1341–1347.

Lombardi J, Wourms JP. 1985. The trophotaenial placenta of viviparous goodeid fish. I. Ultrastructure of the internal ovarian epithelium, the maternal component. *Journal of Morphology* **184:** 277–292.

Lydeard C, Wooten MC, Meyer A. 1995. Molecules, morphology, and area cladograms: a cladistic and biogeographic analysis of *Gambusia* (Teleostei: Poeciliidae). *Systematic Biology* **44:** 221–236.

Lynch M. 1991. Methods for the analysis of comparative data in evolutionary biology. *Evolution* **45:** 1065–1080.

Macías-Garcia C, González-Zuarth CA. 2005. Reproductive behavior of viviparous fish and intersexual conflict. In: Uribe MC, Grier HJ, eds. *Viviparous fishes*. Homestead, FL: New Life Publications, 289–302.

Maddison WP, Maddison DR. 2009. Mesquite: a modular system for evolutionary analysis. Version 2.1. Available at: http://mesquiteproject.wikispaces.com

Marsh-Matthews E. 2011. Matrotropy. In: Evans JP, Pilastro A, Schlupp I, eds. *Ecology and evolution of poeciliid fishes*. Chicago, IL: The University of Chicago Press, 28–37.

Marsh-Matthews E, Deaton R. 2006. Resources and offspring provisioning: a test of the Trexler–DeAngelis model for matrotrophy evolution. *Ecology* **87:** 3014–3020.

Marsh-Matthews E, Brooks M, Deaton R, Tan H. 2005. Effects of maternal and embryo characteristics on post-fertilization provisioning in fishes of the genus *Gambusia*. *Oecologia* **144:** 12–24.

Martin SB, Hitch AT, Purcell KM, Klerks PL, Leberg PL. 2009. Life history variation along a salinity gradient in coastal marshes. *Aquatic Biology* **8:** 15–28.

Martins EP. 1996. Conducting phylogenetic comparative studies when the phylogeny is not known. *Evolution* **50:** 12–22.

Martins EP 2004. COMPARE, version 4.6 b. Available at: http://www.indiana.edu/~martinsl/compare

Martins EP, Garland T Jr. 1991. Phylogenetic analyses of the correlated evolution of continuous characters: a simulation study. *Evolution* **45:** 534–557.

Martins EP, Hansen TF. 1997. Phylogenies and the comparative method: a general approach to incorporating phylogenetic information into the analysis of interspecific data. *American Naturalist* **149:** 646–667.

Meffe GK. 1990. Offspring size variation in eastern mosquitofish (*Gambusia holbrooki*: Poeciliidae) from contrasting thermal environments. *Copeia* **1990:** 10–18.

Meredith RW, Pires MN, Reznick DN, Springer MS. 2010. Molecular phylogenetic relationships and the evolution of the placenta in *Poecilia* (*Micropoecilia*) (Poeciliidae: Cyprinodontiformes). *Molecular Phylogenetics and Evolution* **55:** 631–639.

Meredith RW, Pires MN, Reznick DN, Springer MS. 2011. Molecular phylogenetic relationships and the coevolution of placentotrophy and superfetation in (*Poecilia*) (Poeciliidae: Cyprinodontiformes). *Molecular Phylogenetics and Evolution* **59:** 148–157.

Meyer MK, Schories S, Schartl M. 2011. Description of *Priapella lacandonae* sp. n.–a new poeciliid fish from the río Tulijá basin, Grijalva system, Chiapas, Mexico (Teleostei: Cyprinodontiformes: Poeciliidae). *Vertebrate Zoology* **61:** 91–97.

Miller RR. 1975. Five new species of Mexican poeciliid fishes of the genera *Poecilia*, *Gambusia*, and *Poeciliopsis*. *Occasional Papers of the Museum of Zoology University of Michigan* **672:** 1–44.

Mojica CL, Meyer A, Barlow GW. 1997. Phylogenetic relationships of species of the genus *Brachyrhaphis* (Poeciliidae) inferred from partial mitochondrial DNA sequences. *Copeia* **1997:** 298–305.

Molina-Moctezuma A. 2011. Influencia de la depredación sobre las características de historias de vida y la dinámica poblacional de *Poeciliopsis baenschi*. Bachelor's Thesis, Universidad Nacional Autónoma de México.

Molina-Moctezuma A. 2015. Relación entre la disponibilidad de recursos y el nivel de transferencia de nutrientes entre madres y embriones en dos especies de peces vivíparos. Master's Thesis, Universidad Nacional Autónoma de México.

Morales-Cazan A, Albert JS. 2012. Monophyly of Heterandriini (Teleostei: Poeciliidae) revisited: a critical review of the data. *Neotropical Ichthyology* **10:** 19–44.

Mossman HW. 1991. Classics revisited: comparative morphogenesis of the fetal membranes and accessory uterine structures. *Placenta* **12:** 1–5.

Pires MN, McBride KE, Reznick DN. 2007. Interpopulation variation in life-history traits of *Poeciliopsis prolifica*: implications for the study of placental evolution. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* **307:** 113–125.

Pires MN, Arendt J, Reznick DN. 2010. The evolution of placentas and superfetation in the fish genus *Poecilia* (Cyprinodontiformes: Poeciliidae: subgenera *Micropoeciliia* and *Acanthophacelus*). *Biological Journal of the Linnean Society* **99:** 784–796.

Pires MN, Banet AI, Pollux BJA, Reznick DN. 2011a. Variation and evolution of reproductive strategies. In: Evans JP, Pilastro A, Schlupp I, eds. *Ecology and evolution of poeciliid fishes*. Chicago, IL: The University of Chicago Press, 28–37.

Pires MN, Bassar RD, McBride KE, Regus JU, Garland T, Reznick DN. 2011b. Why do placentas evolve? An evaluation of the life-history facilitation hypothesis in the fish genus *Poeciliopsis*. *Functional Ecology* **25:** 757–768.

Plaut I. 2002. Does pregnancy affect swimming performance of female Mosquitofish, *Gambusia affinis*? *Functional Ecology* **16:** 290–295.

Pollux BJA, Reznick DN. 2011. Matrotrophy limits a female's ability to adaptively adjust offspring size and fecundity in fluctuating environments. *Functional Ecology* **25:** 747–756.

Pollux BJA, Pires MN, Banet AI, Reznick DN. 2009. Evolution of placentas in the family Poeciliidae: an empirical study of macroevolution. *Annual Review of Ecology, Evolution, and Systematics* **40:** 271–289.

Pollux BJA, Meredith RW, Springer MS, Reznick DN. 2014. The evolution of the placenta drives a shift in sexual selection in livebearing fish. *Nature* **513:** 233–236.

Ptacek MB, Breden F. 1998. Phylogenetic relationships among the mollies (Poeciliidae: *Poecilia*: *Mollienesia* group) based on mitochondrial DNA sequences. *Journal of Fish Biology* **53:** 64–81.

Reznick DN. 1982. The impact of predation on life history evolution in Trinidadian guppies: genetic basis of observed life history patterns. *Evolution* **36:** 1236–1250.

Reznick DN, Endler JA. 1982. The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). *Evolution* **36:** 160–177.

Reznick DN, Miles DB. 1989. A review of life history patterns in Poeciliid fishes. In: Meffe GK, Snelson FF, eds. *Ecology and evolution of livebearing fishes (Poeciliidae)*. Englewood Cliffs, NJ: Prentice Hall, 125–148.

Reznick DN, Bryga H, Endler JA. 1990. Experimentally induced life-history evolution in a natural population. *Nature* **346:** 357–359.

Reznick DN, Mateos M, Springer MS. 2002. Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science* **298:** 1018–1020.

Reznick DN, Meredith R, Collette BB. 2007a. Independent evolution of complex life history adaptations in two families of fishes, live-bearing halfbeaks (Zenarchopteridae, Beloniformes) and Poeciliidae (Cyprinodontiformes). *Evolution* **61:** 2570–2583.

Reznick DN, Hrbek T, Caura S, De Greef J, Roff D. 2007b. Life history of *Xenodexia ctenolepis*: implications for life history evolution in the family Poeciliidae. *Biological Journal of the Linnean Society* **92:** 77–85.

Riesch R, Plath M, García de León FJ, Schlupp I. 2010a. Convergent life-history shifts: toxic environments result in big babies in two clades of poeciliids. *Naturwissenschaften* **97:** 133–141.

Riesch R, Plath M, Schlupp I. 2010b. Toxic hydrogen sulfide and dark caves: life-history adaptations in a livebearing fish (*Poecilia mexicana*, Poeciliidae). *Ecology* **91:** 1494–1505.

Riesch R, Colston TJ, Joachim BL, Schlupp I. 2011. Natural history and life history of the Grijalva gambusia *Heterophallus milleri* Radda, 1987 (Teleostei: Poeciliidae). *Aqua* **17:** 95–102.

Riesch R, Martin RA, Bierbach D, Plath M, Langerhans RB, Arias-Rodriguez L. 2012. Natural history, life history, and diet of *Priapella chamulae* Schartl, Meyer & Wilde 2006 (Teleostei: Poeciliidae). *Aqua* **18:** 95–102.

Riesch R, Martin RA, Langerhans RB. 2013. Predation's role in life-history evolution of a livebearing fish and a test of the Trexler–DeAngelis model of maternal provisioning. *American Naturalist* **181:** 78–93.

Roellig K, Menzies BR, Hildebrandt TB, Goeritz F. 2011. The concept of superfetation: a critical review on a 'myth' in mammalian reproduction. *Biological Reviews* **86:** 77–95.

Schrader M, Travis J. 2008. Testing the viviparity-driven-conflict hypothesis: parent–offspring conflict and the evolution of reproductive isolation in a poeciliid fish. *American Naturalist* **172:** 806–817.

Schrader M, Travis J. 2012. Assessing the roles of population density and predation risk in the evolution of offspring size in populations of a placental fish. *Ecology and Evolution* **2:** 1480–1490.

Schultz RJ. 1961. Reproductive mechanism of unisexual and bisexual strains of the viviparous fish *Poeciliopsis*. *Evolution* **15:** 302–325.

Scrimshaw NS. 1944. Superfetation in poeciliid fishes. *Copeia* **1944:** 180–183.

Shields PA, Carlson SR. 1996. Effects of formalin and alcohol preservation on lengths and weights of juvenile sockeye salmon. *Alaska Fishery Research Bulletin* **3:** 81–93.

Smith CC, Fretwell SD. 1974. The optimal balance between size and number of offspring. *American Naturalist* **108:** 499–506.

Soucy S, Travis J. 2003. Multiple paternity and population genetic structure in natural populations of the poeciliid fish, *Heterandria formosa*. *Journal of Evolutionary Biology* **16:** 1328–1336.

Stearns SC. 1976. Life-history tactics: a review of the ideas. *Quarterly Review of Biology* **51:** 3–47.

Stearns SC. 1983. The evolution of life-history traits in mosquitofish since their introduction to Hawaii in 1905: rates of evolution, heritabilities, and developmental plasticity. *American Zoologist* **23:** 65–75.

Stearns SC, Sage RD. 1980. Maladaptation in a marginal population of the mosquito fish, *Gambusia affinis*. *Evolution* **34:** 65–75.

Thibault RE. 1974. Genetics of cannibalism in a viviparous fish and its relationship to population density. *Nature* **251:** 138–140.

Thibault RE, Schultz RJ. 1978. Reproductive adaptations among viviparous fishes (Cyprynodontiformes: Poeciliidae). *Evolution* **32:** 320–333.

Travis J, Farr JA, Henrich S, Cheong RT. 1987. Testing theories of clutch overlap with the reproductive ecology of *Heterandria formosa*. *Ecology* **68:** 611–623.

Travis J, Trexler JC, Mulvey M. 1990. Multiple paternity and its correlates in female *Poecilia latipinna* (Poeciliidae). *Copeia* **1990:** 722–729.

Trexler JC. 1985. Variation in the degree of viviparity in the sailfin molly, *Poecilia latipinna*. *Copeia* **1985:** 999–1004.

Trexler JC. 1997. Resource availability and plasticity in offspring provisioning: embryo nourishment in sailfin mollies. *Ecology* **78:** 1370–1381.

Trexler JC, DeAngelis DL. 2003. Resource allocation in offspring provisioning: an evaluation of the conditions favoring the evolution of matrotrophy. *American Naturalist* **162:** 574–585.

Trexler JC, DeAngelis DL. 2010. Modeling the evolution of complex reproductive adaptations in poeciliid fishes: matrotrophy and superfetation. In: Uribe MC, Grier HJ, eds. *Viviparous fishes II*. Homestead, FL: New Life Publications, 231–240.

Trivers RL. 1974. Parent–offspring conflict. *American Zoologist* **14:** 249–264.

Turner CL. 1937. Reproductive cycles and superfetation in poeciliid fishes. *Biological Bulletin* **72:** 145–164.

Turner CL. 1940. Pseudoamnion, pseudochorion, and follicular pseudoplacenta in poeciliid fishes. *Journal of Morphology* **67:** 59–89.

Turner JS, Snelson FF Jr. 1984. Population structure, reproduction and laboratory behavior of the introduced *Belonesox belizanus* (Poeciliidae) in Florida. *Environmental Biology of Fishes* **10:** 89–100.

Weeks SC, Gaggiotti OE. 1993. Patterns of offspring size at birth in clonal and sexual strains of *Poeciliopsis* (Poeciliidae). *Copeia* **1993:** 1003–1009.

Weldele ML, Zúñiga-Vega JJ, Johnson JB. 2014. Life history of *Gambusia vittata* (Pisces: Poeciliidae). *Southwestern Naturalist* **59:** 449–460.

Wourms JP. 1981. Viviparity: the maternal fetal relationships in fishes. *American Zoologist* **21:** 473–515.

Wourms JP, Grove BD, Lombardi J. 1988. The maternal–embryonic relationship in viviparous fishes. In: Hoar WS, Randall DJ, eds. *Fish physiology: the physiology of developing fish*. New York: Academic Press, 1–134.

Zane L, Nelson WS, Jones AG, Avise JC. 1999. Microsatellite assessment of multiple paternity in natural populations of a live-bearing fish, *Gambusia holbrooki*. *Journal of Evolutionary Biology* **12:** 61–69.

Zeh DW, Zeh JA. 2000. Reproductive mode and speciation: the viviparity-driven conflict hypothesis. *BioEssays* **22:** 938–946.

Zeh JA, Zeh DW. 2008. Viviparity-driven conflict. *Annals of the New York Academy of Sciences* **1133:** 126–148.

Zúñiga-Vega JJ, Reznick DN, Johnson JB. 2007. Habitat predicts reproductive superfetation and body shape in the livebearing fish *Poeciliopsis turrubarensis*. *Oikos* **116:** 995–1005.

Zúñiga-Vega JJ, Macías-Garcia C, Johnson JB. 2010. Hypotheses to explain the evolution of superfetation in viviparous fishes. In: Uribe MC, Grier HJ, eds. *Viviparous fishes II*. Homestead, FL: New Life Publications, 241–254.

Zúñiga-Vega JJ, Suárez-Rodríguez M, Espinosa-Pérez H, Johnson JB. 2011. Morphological and reproductive variation among populations of the Pacific molly *Poecilia butleri*. *Journal of Fish Biology* **79:** 1029–1046.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. List of the species that we used for comparative analyses showing the method of preservation (ethanol or formalin).

Table S2. Phylogenetic estimates of the magnitude of the evolutionary relationship between offspring size and the combination of superfetation and matrotrophy calculated using different phylogenetic comparative methods. These estimates were obtained from analyses conducted on data from the 33 species that were preserved in ethanol.

Table S1. List of the species that we used for comparative analyses showing the method of preservation (ethanol or formalin).

Species	Preservation method
Brachyrhaphis rhabdophora	Ethanol
Brachyrhaphis episcopi	Formalin
Brachyrhaphis holdridgei	Ethanol
Heterandria formosa	Formalin
Poeciliopsis lucida	Ethanol
Poeciliopsis pleurospilus	Ethanol
Poeciliopsis occidentalis	Ethanol
Poeciliopsis prolifica	Formalin
Poeciliopsis infans	Ethanol
Poeciliopsis monacha	Ethanol
Poeciliopsis viriosa	Ethanol
Poeciliopsis turneri	Ethanol
Poeciliopsis gracilis	Ethanol
Poeciliopsis turrubarensis	Ethanol
Poeciliopsis latidens	Ethanol
Poeciliopsis fasciata	Ethanol
Poeciliopsis baenschi	Ethanol
Heterophallus milleri	Formalin
Gambusia panuco	Ethanol
Gambusia vittata	Ethanol
Gambusia sexradiata	Formalin
Gambusia eurystoma	Formalin

Gambusia hubbsi	Formalin
Gambusia yucatana	Ethanol
Gambusia holbrooki	Ethanol
Gambusia affinis	Ethanol
Gambusia speciosa	Ethanol
Gambusia aurata	Ethanol
Belonesox belizanus	Ethanol
Pseudoxhiphophorus jonesii	Ethanol
Priapella chamulae	Formalin
Priapella olmecae	Ethanol
Poecilia mexicana	Formalin
Poecilia sulphuraria	Formalin
Poecilia butleri	Ethanol
Poecilia (Micropoecilia) bifurca	Ethanol
Poecilia (Micropoecilia) branneri	Ethanol
Poecilia (Micropoecilia) parae	Ethanol
Poecilia (Micropoecilia) picta	Formalin
Poecilia (Acanthophacelus) reticulata	Ethanol
Poecilia (Acanthophacelus) wingei	Ethanol
Phalloceros caudiomaculatus	Ethanol
Phalloceros anisophallos	Ethanol
Xenodexia ctenolepis	Ethanol

Table S2. Phylogenetic estimates of the magnitude of the evolutionary relationship between offspring size and the combination of superfetation and matrotrophy calculated using different phylogenetic comparative methods. These estimates were obtained from analyses conducted on data from the 33 species that were preserved in ethanol.

Hansen's method		FIC	TIPS	PGLS	PMM
SF and MI > 1	SF and MI > 5				
		r = 0.21	r = 0.13	r = 0.15	r = 0.07
r^2 = 0.002	r^2 = 0.01			r^2 = 0.02	
				α = 12.04	
					Offspring size h^2 = 0.52
					SF and MI h^2 = 0.98
					SF h^2 = 0.88
					MI h^2 = 0.86

FIC, Felsenstein's independent contrasts; TIPS, non-phylogenetic approach; PGLS, phylogenetic generalized least squares method assuming an Ornstein-Uhlenbeck model of evolution; PMM, phylogenetic mixed model; SF, superfetation; MI, matrotrophy index; r, correlation coefficient; r^2, proportion of the variation among species in offspring size explained by superfetation and matrotrophy; α, a measure of the strength of selection pulling offspring size towards optimal values; h^2, phylogenetic heritability.

DISCUSIÓN GENERAL

La superfetación es una estrategia reproductora que evolucionó independientemente en diferentes géneros de la familia Poeciliidae (Turner, 1937; Reznick y Miles, 1989; Gunn y Thresher, 1991; Scrimshaw, 1994; Reznick *et al*., 2007). Es interesante estudiar esta estrategia debido a las ventajas adaptativas que le confiere a las especies que la presentan (e.g. forma más hidrodinámica, reparten su esfuerzo reproductor y mayor número de crías; Zúñiga-Vega *et al*., 2007, 2010). Recientemente, se demostró que la superfetación y la matrotrofía han evolucionado en conjunto (Meredith *et al*., 2011; Pollux *et al*., 2014). La matrotrofía es un importante aprovisionamiento materno que ha evolucionado y ha sido estudiado ampliamente en poecílidos. Sin embargo, varios estudios únicamente se han enfocado en conocer el efecto por separado de la matrotrofía y la superfetación sobre diferentes aspectos reproductores en poecílidos (i.e. ornamentaciones, longitud de gonopodio e índice de selección sexual; Pollux *et al*., 2014). Por lo que un aspecto interesante por conocer son las consecuencias sobre los mecanismos morfológicos y fisiológicos, que permiten que funcionen estas dos estrategias en conjunto. Por lo tanto, mi tesis se enfocó en conocer algunas de las consecuencias de la superfetación y la matrotrofía en diferentes aspectos de la vida de los poecílidos. En especial, examiné las consecuencias en aspectos reproductores (como el nivel de complejidad de la placenta folicular y retención de esperma) y cambios en las historias de vida (como el tamaño del embrión al nacer).

¿En qué difieren las placentas de especies de peces que superfetan con respecto a las placentas de especies que no superfetan? ¿Altos niveles de matrotrofía están asociados con placentas más complejas?

En el primer capítulo observé que la placenta folicular en todas las especies de poecílidos se dividió en dos capas principales que anteriormente habían sido observadas por Jollie y Jollie (1964): la capa folicular interna y la capa folicular externa. La capa folicular interna contiene las células foliculares aplanadas con elementos citoplasmáticos evidentes como como vesículas, aparato de Golgi, extenso retículo endoplásmico y mitocondrias que indican alta actividad metabólica. La capa folicular externa se compone principalmente de tejido conjuntivo laxo con vasos sanguíneos y fibrillas de colágeno, estas estructuras ayudan a la protección de las células foliculares y el embrión. En las placentas de todas las especies identificamos estructuras que aparentemente facilitan el transporte de nutrientes y gases. Particularmente, en la capa folicular interna se encuentran prolongaciones de la membrana o microvellosidades orientados hacia el embrión que incrementan la superficie de contacto para el intercambio de sustancias (como nutrientes, gases y deshechos). Estas estructuras de la placenta folicular fueron observadas anteriormente en *Poecilia reticulata* y *Heterandria formosa* (Jollie y Jollie, 1964; Grove y Wourms, 1994).

Poeciliopsis presidionis fue un caso particular, la estructura de su placenta es diferente a las otras especies observadas con anterioridad (*Poecilia reticulata,* Jollie y Jollie, 1964; *Heterandria formosa*, Grove y Worms, 1994). No en lo referente a que está constituida por las mismas dos capas (interna y externa), sino en cuanto al grosor y estructura de ambas capas. Pude examinar con detalle la organización general de este tipo

de híper-placenta (estructura con características placentarias más desarrolladas para el intercambio de nutrientes entre madre y sus embriones). La híper-placenta consiste primordialmente en el plegamiento del epitelio de la capa folicular interna, con un tipo celular que no es plano sino columnar. El plegamiento de la capa folicular interna es seguido por el plegamiento de la capa folicular externa, destacando la entrada de los capilares sanguíneos hasta el límite epitelial. Otra diferencia muy marcada entre esta especie y todas las demás es que la capa externa presenta mayor grosor del tejido conjuntivo y múltiples capas de fibrillas de colágeno. Este tipo de morfología placentaria se ha observado también en otras especies con matrotrofía extensa como *Poeciliopsis turneri* y *P. retropinna* por medio de microscopía óptica (Kwan *et al*., 2015). Sin embargo, estas últimas especies no fueron analizadas detalladamente con microscopía electrónica como en el presente estudio, por lo que no me fue posible comparar sus componentes celulares con los que yo observé en *P. presidionis*. Con base a mis resultados y los de Kwan *et al*. (2015) es posible concluir que a partir de ciertos valores de matrotrofía (*P. presidionis* MI=21.5, *P. turneri* MI=41.4, *P. retropinna* MI=117; Reznick *et al*., 2002; Kwan *et al*., 2015) exista la necesidad de estructuras placentarias muy complejas (híper-placentas) para facilitar la transferencia de sustancias activas entre la madre y el embrión a lo largo del desarrollo.

Hasta la fecha no se había examinado la posible relación entre la superfetación y la complejidad de las placentas en especies de la familia Poeciliidae. Mi estudio es el primero en demostrar que las especies superfetadoras aparentemente tienen características de las placentas que reflejan un mayor nivel de complejidad en comparación con las especies que no superfetan. Las especies con superfetación mostraron mayor área del folículo materno,

más microvellosidades y de mayor longitud en comparación con las especies sin superfetación. Mi primera hipótesis en esta sección argumenta que la superfetación requiere una regulación diferencial por parte de las hembras de las tasas de desarrollo de embriones en distintos estadios (Turner, 1937, 1940; Scrimshaw, 1944). Por lo tanto, las hembras de especies que superfetan deberían tener una placenta más compleja comparada con especies que no superfetan. Esta hipótesis se cumplió parcialmente, ya que observé únicamente una tendencia de características morfológicas más desarrolladas y complejas en las especies que superfetan en comparación con las especies que no superfetan.

Por otro lado, con respecto a la asociación entre matrotrofía y el nivel de complejidad de las placentas encontré evidencia de que altos niveles de matrotrofía también se relacionan con placentas más complejas. Es decir, el área del folículo materno, el número de microvellosidades, el número de vesículas y el tamaño de estas vesículas tienden a aumentar conforme aumenta el índice matrotrófico. Estas ultraestructuras placentarias sugieren la transferencia activa y continua de nutrientes entre la madre y el embrión (Turner, 1940; Jollie y Jollie, 1964; Grove y Wourms, 1994). La segunda hipótesis planteada para esta sección fue que, debido a que la matrotrofía implica la transferencia activa de nutrientes por parte de la madre hacia sus embriones a lo largo de todo el desarrollo (Wourms, 1981; Marsh-Matthews, 2011), las hembras de especies con altos niveles de matrotrofía tendrían una placenta más compleja en comparación con las hembras de especies con lecitotrofía o matrotrofía incipiente. Mis resultados apoyaron esta hipótesis debido que las especies con altos niveles de matrotrofía presentaron características placentarias más complejas y presumiblemente ayudan a maximizar la

transferencia activa de nutrientes. Consistente también con esta hipótesis, Kwan y colaboradores (2015) mostraron que el grosor del folículo materno crece conforme aumenta el nivel matrotrófico en ocho especies de poecílidos. En resumen, con base en el presente trabajo es posible concluir que existe un mayor nivel de complejidad en las placentas de especies que exhiben alta matrotrofía.

Antes de esta investigación no había estudios sobre la superfetación y su relación con la complejidad de la placenta. Por lo que el primer capítulo de esta tesis tiene una relevancia importante porque pude identificar las posibles consecuencias de la superfetación y la matrotrofía sobre la estructura celular de la placenta folicular materna. Sin embargo, aún quedan varios aspectos por conocer sobre la placentación en poecílidos. Primero, en mi proyecto de investigación se trabajó con un tamaño de muestra pequeño, lo que implica que nuestras relaciones biológicas pueden tener poco poder estadístico. Se necesitan estudios adicionales con más especies y con un mayor número de muestras por especie para fortalecer la evidencia de las relaciones observadas entre la superfetación, la matrotrofía y el nivel de placentación. Segundo, en la presente investigación tomé en cuenta sólo algunas características ultraestructurales que facilitan la trasferencia de nutrientes (microvellosidades y vesículas) y algunas medidas de la placenta folicular materna (área y grosor del folículo materno; de acuerdo con Jollie y Jollie, 1964 y Grove y Wourms, 1994). Sin embargo, hace falta examinar otras ultraestructuras placentarias como la cantidad de mitocondrias y aparatos de Golgi y su posible asociación con la presencia de altos niveles de superfetación y matrotrofía. Tercero, en esta tesis mostré cómo algunas ultraestructuras de la placenta folicular, específicamente en la parte materna de las

placentas, se encuentran relacionadas con la superfetación y matrotrofía. Sin embargo, hasta la fecha no se han investigado las características ultraestructurales placentarias del tejido absorbente del embrión. Al igual que la placenta folicular materna, el tejido absorbente del embrión debería tener características ultraestructurales que optimicen la transferencia de nutrientes, tales como numerosas microvellosidades y vesículas.

¿Existen diferencias entre especies que superfetan y no superfetan en la capacidad de almacenar esperma?

En el segundo capítulo propuse que debido a que la superfetación implica una producción constante de embriones en diferentes estadios de desarrollo (Turner, 1937, 1940; Scrimshaw, 1994), se espera que las especies con superfetación requieran fertilizar constantemente a sus ovocitos. Por lo que, en las hembras de especies con superfetación deberían existir estructuras que almacenen espermatozoides, y que les permitan estar disponibles para fertilizar constantemente a diferentes grupos de embriones.

Encontré espermatecas solamente en ovarios de nueve de las 12 especies que examiné (*Gambusia panuco, Xiphophorus hellerii, Poecilia mexicana, Poeciliopsis prolifica, P. infans, P. viriosa, P. presidionis, P. gracilis y Heterandria formosa*). Estructuras similares que almacenan esperma debido al cierre de los pliegues ováricos se habían reportado con anterioridad en *Heterandria formosa* y *Poecilia reticulata* (Fraser y Renton, 1940; Kobayashi y Iwamatsu, 2002). En general, observamos que las espermatecas no se encuentran en un área específica dentro del ovario, sino que están distribuidas aleatoriamente, aunque

siempre en los límites cercanos a la luz del ovario. Las espermatecas están compuestas de epitelio ovárico engrosado y frecuentemente se localizan junto a túbulos de vasos sanguíneos. En general, las cabezas de los espermatozoides se encontraron orientadas hacia el epitelio ovárico y las colas hacia la luz de la espermateca. Este particular arreglo de los espermatozoides, podría indicar que el tejido ovárico interno nutre a los espermatozoides para mantenerlos viables. Potter y Kramer (2000) reportaron que las células del tejido ovárico que interactúan directamente con los espermatozoides presentan abundantes ribosomas, microvellosidades y desmosomas, que son características de células que transportan nutrientes.

Por otra parte, en la presente tesis encontré que existe una relación entre la presencia de superfetación con el número y el tamaño de las espermatecas. Las especies que presentan superfetación tienen mayor número de espermatecas y más grandes espermatecas en comparación con las especies que no superfetan, sin importar su historia filogenética. Por lo tanto, las especies superfetadoras destinan una mayor proporción del volumen total del ovario a la producción de espermatecas en comparación con las especies sin superfetación. Adicionalmente, en tres de las seis especies que no superfetan no encontré la presencia de espermatecas. La hipótesis que puse a prueba con respecto a este tema de investigación es que debido a la necesidad de fertilizar frecuentemente a distintos grupos de embriones, las hembras de las especies que superfetan deberían tener mayor capacidad de almacenar esperma en comparación con hembras de especies que no superfetan. Mis resultados apoyaron esta hipótesis y sugieren que el almacenamiento de esperma dentro de las espermatecas favorece la superfetación, ya que presumiblemente

facilita la disponibilidad constante de espermatozoides para fertilizar a las camadas que se desarrollan frecuentemente.

La relación entre la superfetación con el mayor número de las espermatecas puede deberse a que en las especies superfetadoras puede haber selección sexual post-copulatoria dentro de las espermatecas (elección femenina críptica, Birkhead y Pizzari, 2002; Evans y Pilastro, 2011). Es decir, estudios reportados recientemente han demostrado que en especies con superfetación es muy común la coerción sexual, por lo que una estrategia de elección post-copulatoria por parte de la hembra compensaría la falta de las hembras para escoger a su pareja (Macías-García y González-Zuarth, 2005; Pollux *et al*., 2014). Por lo que la retención de esperma en una espermateca puede ser fundamental, debido a que los espermatozoides más fuertes sobrevivirán en las espermatecas hasta ser usados por las hembras. Además, la competencia de esperma puede ocurrir en las espermatecas. Previos estudios han documentado la influencia de la hembra sobre la competencia espermática, a través del fluido ovárico que provoca cambios en la motilidad y supervivencia de los espermatozoides (Gasparini *et al*., 2010; Gasparini y Pilastro, 2011; Gasparini y Evans, 2013).

Este estudio es el primero que examina la asociación entre la capacidad de almacenar esperma y la presencia de superfetación. A partir de estos resultados surgieron otros temas que no se han resuelto. Por ejemplo, cuál es la posible función tienen los vasos sanguíneos que se encontraron rodeando a las espermatecas, sugiero que podría ser que sean necesarios para aportar nutrientes a los espermatozoides que se almacenan dentro de las espermatecas, debido a que los vasos sanguíneos transportan oxígeno y

nutrientes necesarios para las células (Schaller et al., 2008). Su cercanía a las espermatecas representa evidencia adicional de que el tejido ovárico de las hembras provee los medios necesarios para mantener viables a los espermatozoides durante periodos de tiempo relativamente prolongados. También se necesitan más estudios para conocer exactamente, cuáles son las sustancias nutritivas que reciben los espermatozoides dentro de las espermatecas, y cuáles son los mecanismos fisiológicos que permiten el transporte de estos nutrientes hacia el interior de las espermatecas. Otro aspecto importante por explorar es cuál es la duración de las espermatecas dentro del ovario. Existen estudios que sugieren una duración aproximada de la retención de esperma con base en simples observaciones sobre el tiempo que una hembra aislada de machos puede seguir produciendo crías. Sin embargo, ninguno de estos trabajos hizo una examinación fisiológica o anatómica (Clark y Aronson, 1951; Hildemann y Wagner, 1954; Hubbs, 1964, 1997; Greven, 2011).

¿La presencia conjunta de superfetación y matrotrofía promueven la producción de crías más grandes en especies de la familia Poeciliidae?

En el tercer capítulo, examiné la hipótesis de que la evolución conjunta de la superfetación y la matrotrofía en la familia Poeciliidae ha facilitado la evolución de crías más grandes. Debido a que las hembras con superfetación, reparten su esfuerzo reproductor produciendo pequeñas camadas que nacen frecuentemente (Reznick y Miles, 1989; Pollux *et al*., 2009; Zuñiga-Vega *et al*., 2010). Por lo que las hembras superfetadoras pudieran tener

mayor espacio disponible dentro de su ovario en comparación con hembras que no superfetan. Además, la matrotrofía implica que los embriones son pequeños en los primeros estadios de desarrollo debido a su escaso vitelo y más grandes en los últimos estadios del desarrollo (Wourms, 1981; Marsh-Matthews, 2011). En consecuencia, una combinación de la presencia de superfetación y alta matrotrofía podría promover el incremento del tamaño de las crías al nacer. Sin embargo, encontré que altos niveles superfetación y matrotrofía no tienen una relación con el tamaño de las crías al nacer. Por lo tanto, la hipótesis no fue apoyada por mis resultados. De hecho, los resultados que obtuve fueron contrarios a los que esperaba. El tamaño óptimo al nacer estimado para especies con superfetación y matrotrofía moderada o extensa fue menor comparado con el tamaño al nacer estimado para especies sin superfetación, especies con superfetación y lecitrotrofía y especies con superfetación y matrotrofía incipiente. Un estudio que es consistente con estos resultados es el de Pires y colaboradores (2011), quienes encontraron que altos valores de matrotrofía están asociados a crías de menor tamaño al nacer en seis especies del género *Poeciliopsis*. Sus resultados soportan la hipótesis de la facilitación de historias de vida (i.e. la matrotrofía facilita la evolución de algunos rasgos de la historia de vida), por lo que la evolución de alta matrotrofía promueve la maduración temprana de los embriones provocando crías de tamaño pequeño (Pires *et al*., 2011).

Otro resultado importante es que el tamaño de la cría en las especies que se analizaron tiene baja heredabilidad filogenética. Esto indica que la evolución del tamaño al nacer en poecílidos parece deberse principalmente a procesos de adaptación local a condiciones particulares del medio ambiente. Varios estudios apoyan este resultado, ya que

han demostrado que ciertos factores ambientales como alta competencia, alta depredación o presencia de sustancias tóxicas en el agua tienen como consecuencia cambios predecibles y diferencias entre sitios (intraespecíficas) en el tamaño óptimo de las crías (i.e. *Heterandria formosa*, *Poecilia reticulata, P. sulphuraria, Brachyrhaphis episcopi, B. rhabdophora, Gambusia eurystoma*; Reznick, 1982; Reznick y Endler, 1982; Reznick *et al*., 1990; Leips y Travis, 1999; Johnson y Belk, 2001; Jennions y Telford, 2002, Bashey, 2006, 2008; Riesch *et al*., 2010).

Aún hacen falta estudios enfocados en entender el significado adaptativo de la presencia conjunta de altos niveles de superfetación y matrotrofía y sus posibles efectos sobre otras características fisiológicas, conductuales y de historias de vida. Por ejemplo, una hipótesis que aún no se ha puesto a prueba, es que la combinación entre la matrotrofía y superfetación podrían reducir los costos locomotores asociados a la viviparidad (Pollux *et al*., 2009). Durante el embarazo, las hembras ganan peso y volumen, lo cual tiene efectos negativos en su respuesta de escape y en su rendimiento al nadar (Plaut, 2002; Ghalambor et al., 2004; Langerhans y Reznick, 2010). Entonces, las hembras con matrotrofía y superfetación podrían repartir su esfuerzo reproductor teniendo varias camadas de embriones en diferente estadio de desarrollo. Estas hembras tendrían pocos embriones de tamaño pequeño por estar en estadios tempranos, pocos embriones de tamaño mediano por estar en estadios intermedios y pocos embriones de tamaño grande por estar en estadios de desarrollo más avanzados. En consecuencia, esta repartición de camadas y los diferentes tamaños embrionarios provocaría la disminución del volumen total ocupado por

los embriones en comparación con especies que carecen de superfetación y matrotrofía (Pollux *et al*., 2009; Reznick y Miles, 1989)

Además, sería interesante comparar los resultados que hemos observado con especies de la familia Poeciliidae con otras familias de peces que también presentan superfetación (Clinidae y Zenarchopteridae; Gunn y Thresher, 1991; Reznick et al., 2007). Otras interrogantes que aún genera la superfetación y que necesitan ser estudiadas son: ¿Cuáles son los procesos fisiológicos que subyacen a la capacidad de procrear simultáneamente a varios grupos de embriones de diferentes estadios? particularmente, ¿Qué hormonas regulan este desarrollo embrionario diferencial? ¿Cómo se relaciona la superfetación con la selección sexual post-copulatoria? ¿Las especies con superfetación tienen mayores niveles de paternidad múltiple?

En la presente tesis logré examinar una variedad de consecuencias adaptativas de la superfetación y matrotrofía que van desde la evaluación de aspectos reproductores como la complejidad de algunas ultraestructuras placentarias (e.g. el área de folículo materno, el número y tamaño de microvellosidades, el número y tamaño de vesículas y el grosor del folículo materno) y la retención de esperma (presencia, tamaño y número de espermatecas), hasta rasgos en las historias de vida como el tamaño de la cría en algunas especies de la familia Poeciliidae. Estas consecuencias adaptativas podrían ser la base de nuevas investigaciones de los peces poecílidos y su peculiar modo reproductor que es la viviparidad.

CONCLUSIONES

- En todas las especies de poecílidos la placenta folicular se dividió en dos capas foliculares: la interna y la externa. La capa folicular interna contiene un epitelio de células foliculares. Mientras que la cara externa es de tejido conjuntivo laxo que predominan capilares sanguíneos y fibras de colágena en toda la capa. *Poeciliopsis presidionis* fue la especie que sobresalió debido a que esta presenta un plegamiento prominente en la capa folicular interna. Por su parte, la capa folicular externa tiene varias capas de fibra de colágena y sus capilares sanguíneos protruyen hacia la capa folicular interna.
- Demostré que las especies superfetadoras tienen características de las placentas que reflejan un mayor nivel de complejidad (mayor área folicular, más microvellosidades y de mayor longitud) en comparación con las especies que no superfetan.
- Encontré una asociación positiva entre la intensidad matrotrófica y el nivel de complejidad de las placentas. El área del folículo materno, el número de microvellosidades, el número de vesículas y el tamaño de estas vesículas aumentan conforme aumenta el índice matrotrófico. Estas características placentarias sugieren que la transferencia de nutrientes es activa entre la madre y el embrión.
- Observé espermatecas en los ovarios de nueve de las 12 especies que se examinaron (*Gambusia panuco, Xiphophorus hellerii, Poecilia mexicana, Poeciliopsis prolifica, P. infans, P. viriosa, P. presidionis, P. gracilis y Heterandria formosa*).

- Este estudio es el primero que examina la asociación entre la capacidad de almacenar esperma y la presencia de superfetación. Las especies que presentan superfetación tienen mayor número de espermatecas y espermatecas más grandes en comparación con las especies que no superfetan
- Encontré que altos niveles superfetación y matrotrofía no tienen una relación con el tamaño de las crías al nacer.
- El tamaño de la cría en las especies que se analizaron tiene baja heredabilidad filogenética. Esto indica que la evolución del tamaño al nacer en poecílidos parece deberse principalmente a procesos de adaptación local.
- Aún hacen falta estudios enfocados en entender el significado adaptativo de la presencia conjunta de altos niveles de superfetación y matrotrofía y sus posibles efectos sobre otras características fisiológicas, conductuales y de historias de vida.
- Logré examinar una variedad de consecuencias adaptativas de la superfetación y matrotrofía que van desde la evaluación de aspectos reproductores como la complejidad ultraestructural de la placenta y la retención de esperma, hasta rasgos en las historias de vida como el tamaño de la cría en algunas especies de la familia Poeciliidae.

LITERATURA CITADA

Bashey F. 2006. Cross-generational environmental effects and the evolution of offspring size in the Trinidadian guppy *Poecilia reticulata*. Evolution 60:348-361.

Bashey F. 2008. Competition as a selective mechanism for larger offspring size in guppies. Oikos. 117:104-113.

Birkhead, TR, Pizzari T. 2002. Postcopulatory sexual selection. Nature Reviews Genetics 3: 262–273.

Clark E, Aronson LR. 1951. Sexual behavior in the guppy, *Lebistes reticulatus* (Peters). Zoologica 36:49-66.

Evans JP, Pilastro A. 2011. Postcopulatory sexual selection. In J. P. Evans, A. Pilastro, & I. Schlupp (Eds.), Ecology and evolution of poeciliid fishes (pp. 197–208). Chicago, IL: The University of Chicago Press.

Fraser EA, Renton RM. 1940. Observation on the breeding and development of the viviparous fish. *Heterandria formosa*. Quarterly Journal of Microscopical Science 81: 479-520.

Gasparini C, Evans JP. 2013. Ovarian fluid mediates the temporal decline in sperm viability in a fish with sperm storage. PLoS One 8: e64431.

Gasparini C, Pilastro A. 2011. Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy. Proceedings of the Royal Society of London B: Biological 278: 2495–2501.

Gasparini C, Simmons, LW, Beveridge M, Evans JP. 2010. Sperm swimming velocity predicts competitive fertilization success in the green swordtail *Xiphophorus helleri*. PLoS One 5: e12146.

Ghalambor CK, Reznick DN, Walker JA. 2004. Constraints on adaptive evolution: the functional trade-off between reproduction and fast start swimming performance in the Trinidadian guppy (*Poecilia reticulata*). American Naturalist 164:38-50.

Greven H. 2011. Gonads, genitals, and reproductive biology. In: Evans JP, Pilastro A, Schlupp I, editors. Ecology and Evolution of Poeciliid Fishes. Chicago, Illinois: The University of Chicago Press. pp 3-17.

Grove BD, Wourms JP. 1994. The follicular placenta of the viviparous fish, *Heterandria formosa*, In: ultrastructure and development of the embryonic absorptive surface. Journal of Morphology 209: 265-284.

Gunn JS, Thresher RE. 1991. Viviparity and the reproductive ecology of clinid fishes (Clinidae) from temperate Australian waters. Environmental Biology of Fishes 31: 323-44.

Hildemann WH, Wagner ED. 1954. Intraspecific sperm competition in *Lebistes reticulatus*. Am Nat 88: 87-91.

Hubbs C. 1964. Interactions between a bisexual fish species and its gynogenetic sexual parasite. Bulletin Texas Memorial Museum 8:1-72.

Hubbs C. 1997. Sperm retention in poeciliids. Bulletin American Livebearers Association 146:9.

Jennions M, Telford S. 2002. Life-history phenotypes in populations of *Brachyrhaphis episcopi* (Poeciliidae) with different predator communities. Oecologia 132:44-50.

Johnson JB, Belk MC. 2001. Predation environment predicts divergent life-history phenotypes among populations of the livebearing fish *Brachyrhaphis rhabdophora*. Oecologia 126:142-149.

Jollie WP, Jollie LG. 1964. The fine structure of the ovarian follicle of the ovoviviparous poeciliid fish, *Lebistes reticulatus*. I. Maturation of follicular epithelium. Journal of Morphology 114: 479-501.

Kobayashi H, Iwamatsu T. 2002. Fine structure of the storage micropocket of spermatozoa in the ovary of the guppy *Poecilia reticulata*. Zoological Science 19:545-555.

Kwan L, Fris M, Rodd FH, Rowe L, Tuhela L, Panhuis TM. 2015. An examination of the variation in maternal placentae across the genus Poeciliopsis (Poeciliidae). Journal of Morphology 276: 707-720.

Langerhans RB, Reznick, DN. 2010. Ecology and evolution of swimming performance in fishes: predicting evolution with biomechanics. In: Domenici, P.; Kapoor, BG., editors. Fish locomotion: an eco-ethological perspective. Boca Ratón: Science Publishers,p. 200-248.

Leips J, Travis J. 1999. The comparative expression of life history traits and its relationship to the numerical dynamics of four populations of the least killifish. Journal of Animal Ecology 68:595-616.

Macías-Garcia C, Gonzalez-Zuarth CA. 2005. Reproductive behavior of viviparous fish and intersexual conflict. In M. C. Uribe & H. J. Grier HJ (Eds.), Viviparous fishes (pp. 289–302). Homestead, FL: New Life Publications.

Marsh-Matthews E. 2011. Matrotropy. In: Evans JP, Pilastro A, Schlupp I, eds. Ecology and evolution of poeciliid fishes. Chicago, IL: The University of Chicago Press, 28-37.

Meredith RW, Pires MN, Reznick DN, Springer MS. 2011. Molecular phylogenetic relationships and the coevolution of placentotrophy and superfetation in (*Poecilia*) (Poeciliidae: Cyprinodontiformes). Molecular Phylogenetics and Evolution 59: 148-157.

Pires MN, Bassar RD, McBride KE, Regus JU, Garland T, Reznick DN. 2011. Why do placentas evolve? An evaluation of the life-history facilitation hypothesis in the fish genus *Poeciliopsis*. Functional Ecology 25: 757-768.

Plaut I. 2002. Does pregnancy affect swimming performance of female Mosquitofish, *Gambusia affinis*? Functional Ecology 16:290-295.

Pollux BJA, Meredith RW, Springer MS, Garland T, Reznick DN. 2014. The evolution of the placenta drives a shift in sexual selection in livebearing fish. Nature, 513: 233-236.

Pollux, BJA, Pires MN, Banet AI, Reznick, DN. 2009. Evolution of placentas in the fish family Poeciliidae: an empirical study of macroevolution. Annual Review of Ecology, Evolution, and Systematics 40: 271-289.

Potter H, Kramer CR. 2000. Ultrastructural observations on sperm storage in the ovary of the platyfish, *Xiphophorus maculatus* (Teleostei: Poeciliidae): the role of the duct epithelium. Journal of Morphology 245: 110-129.

Reznick DN, Bryga H, Endler JA. 1990. Experimentally induced life-history evolution in a natural population. Nature 346:357-359.

Reznick DN, Endler JA. 1982. The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). Evolution 36:160-177.

Reznick DN, Meredith R, Collette BB. 2007. Independent evolution of complex life history adaptations in two families of fishes, live-bearing halfbeaks (Zenarchopteridae, Beloniformes) and Poeciliidae (Cyprinodontiformes). Evolution 61: 2570-83.

Reznick DN, Miles DB. 1989. Review of life history patterns in poeciliid fishes. In: Meffe GK, Snelson FF Jr., editors. Ecology and evolution of livebearing fishes (Poeciliidae). Prentice Hall, New Jersey, p. 125-148.

Reznick DN. 1982. The impact of predation on life history evolution in Trinidadian guppies: genetic basis of observed life history patterns. Evolution 36:1236-1250.

Riesch R, Plath M, García de León FJ, Schlupp I. 2010. Convergent life-history shifts: toxic environments result in big babies in two clades of poeciliids. Naturwissenschaften 97:133-141.

Schaller J, Gerber S, Kaempfer U, Lejon S, Trachsel C. 2008. Human blood plasma proteins: structure and function. John Wiley and Sons. Southern Gate, Chichester.

Scrimshaw NS. 1944. Superfetation in poeciliid fishes. Copeia 1944: 180-183.

Turner CL. 1937. Reproductive cycles and superfetation in poeciliid fishes. Biological Bulletin 72:145-164.

Turner CL. 1940. Pseudoamnion, pseudochorion, and follicular pseudoplacenta in poeciliid fishes. Journal of Morphology 67: 59-89.

Turner CL. 1947. Viviparity in teleost fishes. The Scientific Monthly 65: 508-518.

Villagrán SM, Méndez FR, Stewart JR. 2005. Placentation in the Mexican lizard *Sceloporus mucronatus* (Squamata: Phrynosomatidae). Journal of Morphology 264: 286-297.

Wourms JP. 1981. Viviparity: the maternal fetal relationships in fishes. American Zoologist 21: 473–515.

Zúñiga -Vega JJ, Macías-Garcia C, Johnson JB. 2010. Hypotheses to explain the evolution of superfetation in viviparous fishes. In: Uribe MC, Grier HJ, eds. Viviparous fishes II. Homestead, FL: New Life Publications, 241–254.

Zúñiga-Vega JJ, Reznick DN, Johnson JB. 2007. Habitat predicts reproductive superfetation and body shape in the livebearing fish *Poeciliopsis turrubarensis*. Oikos 116: 995-1005.