UNIVERSITY OF CALIFORNIA
RIVERSIDE

The Evolution of Reproductive Mode and Its Effect on Speciation in Cyprinodontiform Fishes

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Evolution, Ecology, and Organismal Biology

by

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March 2017

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Acknowledgments

I thank my advisor, David Reznick, for his mentoring, his support, and his infectious passion for biology. Thank you to Drs. Mark Springer, Joel Sachs, Rich Cardullo, and J. Jaime Zúñiga-Vega for all of the advice and guidance they have given me throughout my dissertation work. A special thank you goes out to Drs. John Gatesy and Cheryl Hayashi for opening up their labs to me and assisting me with all of my molecular work. I'd like to thank the following people also gave me feedback and advice on my research: Rob Meredith, Crystal Chaw, Jim Starrett, Matt Collin, Liz Dlugosz, Tami Panhuis, Michael Guernsey, Pru Talbot, Ronald Bassar, Bart Pollux, Matt Collin, Paul De Ley, and Brad White. My research would not have been possible without the UC MEXUS Dissertation Fellowship, UC MEXUS small grants, and Newell Award.

I am grateful to all of the amazing members of the Reznick Lab who shared a research home with me; thank you to Mauricio Torres-Mejia, Andrew Furness, Yuridia Reynoso, Cynthia Dick, Jeffrey Arendt, Joshua Goldberg, Daniel Goldberg, Samantha Levell, Robert Prather, and Kristin Edwards. In particular, I thank Andrew Furness, Cynthia Dick, and Jeffrey Arendt for the many hours they spent helping me over the past five and half years. My time at UCR would not have been as fulfilling without the ability to share my research with enthusiastic undergraduates. I thank Johnny Huynh, Chris Tiet, Jordan Jew, Adanma Nwachuku, Tasnim Rahman, William Tedjo, Sugjit Singh, Kevin Khuu, Michelle Rodriguez, and especially Anthony Nguyen and Vyvian Ngo for all of their time and effort. Thank you to Claudia Olivera-Tlahuel, Alejandro Molina-Moctezuma, and Meaghan Weldele for assisting me in my field work.

I have made many friends during my time at UCR, but I would like to thank a few in particular: Andrew Furness, Steve Merkely, Gerald Claghorn, Elizabeth Rosen, Zoe Thompson, Jim Starrett, and Sarah Lillian. Lastly, I thank my mom, my dad, and the many members of my family who were nothing but supportive as I pursued my dreams.

I dedicate this dissertation to Sarah Robins Morrison - my wife, my support, and my favorite field assistant.

ABSTRACT OF THE DISSERTATION

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University of California, Riverside, March 2017
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There is remarkable diversity in the form and function of vertebrate reproductive mode, and adaptive explanations for the vast differences among species have fallen short. Instead, parent-offspring conflicts provide a parsimonious framework that describes why evolutionary transitions occur from one mode to another, and how the differences among species change the nature of sexual selection and speciation. My dissertation examines the effect of reproductive mode on vertebrate evolution by examining two topics – the evolution of matrotrophy following a transition from oviparity to viviparity and the effect differences in reproductive mode have the evolution of reproductive isolation and the rate of speciation. Cyprinodontiformes, an order of small mostly freshwater fish, are notable for exhibiting a wide range of reproductive phenotypes. I make use of the repeated transitions from oviparity to viviparity and from lecithotrophy to matrotrophy in Cyprinodontiformes to test hypotheses that parent-offspring conflicts have driven the evolution of reproductive mode.

In chapter one I demonstrate eggs from three oviparous species from Cyprinodontiformes and one from Atherinomorpha are capable of acquiring molecules from their surrounding environment via pinocytosis, a property that predisposes them to the evolution of matrotrophy following the transition to viviparity. In chapter two I find evidence that post-zygotic reproductive incompatibilities are evolving faster among populations within the placental species *Poeciliopsis prolifica* than within two closely related non-placental species of *Poeciliopsis*. In the placental species, offspring size decreased significantly as a function of increasing interpopulation distance, but offspring from non-placental species suffered no such fitness loss. In chapter three I demonstrate that interspecific post-zygotic reproductive isolation evolves at an accelerated rate among viviparous species relative to oviparous species, and that estimated levels of post-zygotic isolation are higher among matrotrophic species than among lecithotrophic species at all genetic distances. Similarly, I find diversification rates estimated from molecular phylogenies to be significantly higher for viviparous taxa than oviparous taxa, but marginally higher for lecithotrophic species than for matrotrophic species. As a whole, the results of this dissertation are consistent with hypotheses that parent-offspring conflicts have played a part in the evolution of vertebrate reproductive mode, and that variation in the nature of conflicts among taxa influence speciation.

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Introduction

Reproductive adaptations represent some of the most striking morphological differences observed among mammals. Since Aristotle, people have categorized animals by their modes of reproduction, particularly in regards to viviparity and oviparity (Thompson 1910). Characterizing the reproductive adaptations and determining what adaptive significance, if any, they serve to organisms has been a topic of considerable interest for Biologists (Wourms 1981; Blackburn et al. 1985; Wourms et al. 1988; Blackburn 1999). In the case of mammals, the striking diversity in the structure of the placenta and highly divergent phenotypes among closely related species has confounded attempts to define how they represent adaptations (Wourms et al. 1988; Crespi and Semeniuk 2004). As a result, a mounting body of literature suggests parent-offspring conflicts have played a crucial role in the evolution of vertebrate reproductive mode (Crespi and Semeniuk 2004). The intensity and nature of parent-offspring conflicts is predicted to vary among taxa that differ in their mode of reproduction (Furness et al. 2015). In oviparous taxa, conflicts are limited because mothers have full control over maternal provisioning. In viviparous taxa, internal development provides offspring a window of opportunity to influence the levels of provisioning they receive (Zeh and Zeh 2000; Crespi and Semeniuk 2004). The transition to livebearing creates an arena where fetomaternal conflicts can take place, and promotes the subsequent evolution of post-fertilization active provisioning (Crespi and Semeniuk 2004; Furness et al. 2015). Conflicts not only drive the major changes in vertebrate reproductive mode, but the nature of conflicts change in response to evolutionary transitions (Zeh and Zeh 2000;

Furness et al. 2015). The intensity of parent-offspring conflicts influences how quickly post-zygotic reproductive barriers evolve within species (Rice 1997; Gavrilets 2000; Zeh and Zeh 2008). Therefore, post-zygotic reproductive isolation is expected to evolve rapidly within placental livebearing species, at intermediate rates within non-placental livebearing species, and slowest within egg-laying species. In this dissertation, I take advantage of the diversity of reproductive adaptations present in Cyprinodontiform fishes to explore the role of conflict in driving the evolution of matrotrophy following a transition from oviparity to viviparity, and examine how rates of post-zygotic reproductive isolation and speciation vary among oviparous, lecithotrophic viviparous, and matrotrophic viviparous taxa.

Study System

Cyprinodontiformes is an order of fish consisting mostly of freshwater species native to the American and African continents (Nelson et al. 2016). The order is made up of ~1254 species and includes the livebearers and killifish. The order is particularly notable for exhibiting a diversity of reproductive adaptations, including egg-laying and live-bearing species, internal and external fertilizers, species with and without maternal provisioning, and species with reproductive adaptations analogous to the mammalian placenta (Wourms 1981; Blackburn et al. 1985; Wourms et al. 1988). Viviparity and matrotrophy have both evolved numerous times within Cyprinodontiformes (Blackburn 2005; Reznick et al. 2007; Pollux et al. 2009), allowing for multiple independent comparisons to be made among groups that differ in reproductive mode. I make use of

the unique evolutionary history of Cyprinodontiformes to examine the effect of different modes of reproduction on evolution among closely related taxa.

Dissertation Chapters

The purpose of this dissertation is to examine how parent-offspring conflict has influenced the evolution of reproductive mode and subsequently speciation in species of Cyprinodontiform fishes. Specifically, I ask the following three questions: 1.) Are fish eggs in species of Cyprinodontiformes preadapted to evolve matrotrophy following the transition from egg laying to livebearing? 2.) Does reproductive mode affect how quickly reproductive barriers evolve among populations within species of *Poeciliopsis*? 3.) Are rates of reproductive isolation evolution and macroevolutionary diversification different among species that differ in their mode of reproduction?

In chapter one, I demonstrate that eggs from three oviparous species of Cyprinodontiformes (and one species from the Superorder Atherinomorpha, which includes the Cyprinodontiformes) are capable of acquiring molecules from their surrounding environment via mechanisms of active transport. When incubated in the presence of radiolabeled amino acids, eggs from all four species are able to transport the label across the egg membrane against a concentration gradient. Saturating the radiolabel with an unlabeled analog could inhibit the rate of amino acid uptake. In addition, I show that rates of uptake are similar when competed against L-Leucine and the biologically uncommon entiaomer D-Leucine, which suggests that the transport mechanism is non-specific. Lastly I demonstrate that pinocytosis is the most likely mechanism of uptake, because eggs are capable of transporting large biologically inert molecules across the

membrane, and this transport was inhibited at temperatures that are known to hinder endocytosis. The rates of uptake observed in the embryos of these oviparous species are comparable to rates of uptake found in embryos of viviparous species that lack maternal provisioning after their mother had been injected with radiolabeled amino acids. I argue that the uptake observed in these viviparous lecithotrophic species are a product of the properties of the egg that were retained through the transition from oviparity to viviparity. Finally, I argue the ability for eggs in Cyprinodontiformes to acquire molecules from the external environment predisposes them to parent-offspring conflicts once the transition from oviparity to viviparity occurs, and increases the probability that matrotrophy will evolve following the transition to viviparity.

In chapter two, I quantify the levels of reproductive isolation among populations within species through a series of reciprocal hybrid crosses, and compare rates of reproductive barrier formation among placental and non-placental species of *Poecilipsis*. Within the placental species, hybrid offspring suffer fitness costs in the form of significantly reduced body sizes as a function of increasing interpopulation genetic distance. There was no loss of fitness associated with increasing genetic distance in non-placental species, as offspring from all crosses are phenotypically similar to one another. There is no evidence of inviability or sterility in any of the offspring, regardless of species or cross type. Offspring from interpopulation crosses in the placental species are 30-40% smaller than offspring from within population crosses, which falls outside of the average variation found within natural populations. Smaller body sizes are strongly associated with lower fitness and survival in other Poeciliid species, and the reduction in

body size observed in this study likely represents a barrier to reproduction. The evolution of placentation is predicted to lead to increased parent-offspring conflicts. I argue the accelerated rate of post-zygotic barrier formation within the placental species relative to the non-placental species of *Poeciliopsis* supports this hypothesis, and further builds the case that reproductive mode could play a role in the process of speciation.

In chapter 3, I combine a meta-analysis with comparative phylogenetic methods to examine the effect of reproductive mode on rates of post-zygotic reproductive isolation evolution and macroevolutionary diversification in Cyprinodontiformes. I build a dataset of all known interspecific reciprocal hybrid crosses performed between species of Cyprinodontiformes, and then model the level of reproductive isolation between species as a function of genetic distance and reproductive mode. Consistent with predictions of conflict driven evolution of reproductive mode, I find post-zygotic reproductive isolation evolves faster in viviparous taxa than oviparous taxa. Rates of post-zygotic evolution are not accelerated in matrotrophs, but estimated levels of post-zygotic isolation are higher among matrotrophs than lecithotrophs at all levels of interspecific genetic distances. A fossil-calibrated phylogeny was also built using all available sequence data for Cyprinodontiformes. I use the tree to estimate macroevolutionary rates of speciation, extinction, and net diversification among species that differ in reproductive mode. Overall, rates of speciation and diversification are higher for viviparous taxa than oviparous taxa. The patterns among oviparity, lecithotrophic viviparity, and matrotrophic viviparity are less clear and depend on the method used. Under a MuSSE model speciation, extinction, and diversification rates do not significantly differ among the

modes of reproduction; however, speciation rates were estimated to be higher for viviparous lecithotrophs relative oviparous taxa in RevBayes. Overall, the transition from oviparity to viviparity does appear to accelerate how quickly post-zygotic reproductive isolation evolves, and in turn increases speciation rates in livebearing taxa. Matrotrophy appears to play some role in the evolution of reproductive barriers, but this effect does not influence patters at macroevolutionary scales.

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Active transport across the egg membrane of Cyprinodont fish as a preadaptation for placental evolution.

Abstract

Teleost fishes have evolved livebearing via egg retention 14 times, and placentation/matrotrophy has evolved within 12 of those lineages. In contrast, squamate reptiles have evolved livebearing over 115 times, but only two to four of those lineages are known to have evolved matrotrophy. One hypothesis is that the probability of this transition is caused by differences in their eggs. I evaluated whether four egg-laying species in Atherinomorpha are capable of acquiring molecules from their surrounding environment via mechanisms of active transport. If so, then retained eggs can acquire resources from their mother, which would initiate a mechanism for maternal provisioning and predispose them to the evolution of matrotrophy. Embryos of all four species accumulate amino acids across the egg membrane and against a concentration gradient. Uptake rates were inhibited by competing radiolabeled amino acids against unlabeled versions of themselves. The transport mechanism is non-specific, as rates of uptake were equal in L-Leucine and its biologically uncommon enantiomer D-Leucine. Eggs are capable of transporting larger microspheres across the egg membrane, but such transport is inhibited at temperatures below $4^{O}C$, indicating transport occurs via pinocytosis. The rate of radiolabel uptake is comparable to rates observed in live-bearing species that lack maternal provisioning, as expected if viviparity begins with retained eggs. Conflict theory predicts that such properties will facilitate the embryo-parent arms race that leads to the evolution of active provisioning following a transition to livebearing.

Introduction

The evolution of complex adaptations, such as vertebrate eyes, presents a challenge because they are composed of several intricate and highly specialized components that only appear functional in the context of an entire system. Thus the challenge lies in explaining how each individual component could have evolved in the absence of the others. Darwin himself struggled with this issue, but posed that "organs of extreme perfection and complication" could arise in a gradual stepwise fashion as long as each intermediate step was favored by natural selection (Darwin 1859). Under this scenario, complex adaptations result from a series of contingent events, where each event is facilitated by the ones that preceded it and the series of events sum to the evolution of a complex adaptation (Gregory 2008). One consequence of serial evolution is that the range of potential adaptations that a species can evolve is constrained by its evolutionary history (Blount et al. 2008).

Matrotrophy, most commonly observed in placental mammals (Mossman 1991), describes complex physiological adaptations that allow the active transfer of nutrients from mother to developing offspring throughout the course of gestation (Wourms 1981). Matrotrophy is a multifaceted mode of sexual reproduction and represents a modern-day endpoint in a series of evolutionary transitions that have occurred in vertebrates (oviparity with external fertilization ➔ oviparity with internal fertilization ➔ viviparity ➔ matrotrophic viviparity – modified from Furness et al. 2015; Crespi and Semeniuk 2004. Viviparity and matrotrophic viviparity have evolved numerous times throughout the animal kingdom, however the frequency of transitions from the former to the latter

has not been uniform across taxonomic groups. In Osteichthyan fish, viviparity has evolved independently at least 14 times and 12 of those times the subsequent evolution of matrotrophic viviparity occurred (Blackburn 1999; Blackburn 2015). Within those twelve lineages the evolution of matrotrophy has in some instances occurred multiple times (Reznick et al. 2002; Reznick et al. 2007). In contrast, viviparity has evolved over 115 times in squamate reptiles, but the transition to placentation is estimated to have taken place in only two to four of those lineages (depending on the phenotype of the common ancestor, (Pyron and Burbrink 2014)), and all within a single family (Blackburn 1999; Pyron and Burbrink 2014; Blackburn 2015). Other anamniotes show patterns similar to Osteichthyan fish, with the transition from viviparity to matrotrophic viviparity having occurred 6 out of 8 times in modern amphibians (Blackburn 2015; Wake 2015), and 5 out of 9 times in Chondrichthyes (Blackburn 2015). It is less clear how squamate reptiles compare to the other amniotes given viviparity has never evolved in Aves, and that both viviparity and matrotrophy have a single origin in mammals (Blackburn 1999; Springer et al. 2003). On the whole, these patterns raise the question of why the evolution of viviparity so often leads to the evolution of matrotrophy in some groups but not others. One possibility is that the evolution of matrotrophy represents a series of contingent events and that the divergent outcomes between modern taxa are the byproduct of differences that arose during intermediate stages of the series.

Here I propose the increased incidences of matrotrophy in Osteichthyan fish is a function of the properties of their eggs, and provide a series of tests designed to examine

whether the Cyprinodontiform (Superclass: Osteichthyes) egg is pre-adapted to facilitate the evolution of matrotrophy following the transition to viviparity.

Within the fish Order Cyprinodontiformes there have been multiple independent origins of viviparity and matrotrophy (Wourms 1981; Wourms et al. 1988; Blackburn 1999; Reznick et al. 2002; Pollux et al. 2009). The levels of maternal provisioning among the viviparous species, estimated by the Matrotrophy Index (dry weight of offspring at birth/dry weight of embryo at fertilization), ranges from less than 1 (newborn offspring are lighter than fertilized eggs) to greater than 100 (Wourms 1981; Wourms et al. 1988; Reznick et al. 2002). Species with a matrotrophy index of less than one are characterized as lecithotrophic (Reznick et al. 2002), meaning yolk feeding, because the absence of weight gain during development suggests all or most maternal investment is made in the egg prior to fertilization, or that embryos receive little or no post-fertilization provisioning from their mothers (Wourms 1981). Species with a matrotrophy index greater than one are classified as matrotrophic, meaning mother feeding, because the measurable weight gain suggests the developing offspring receives more resources than is provided in the pre-packaged yolk (Wourms et al. 1988). While the embryos in lecithotrophic species do not appear to receive active provisioning from their mothers after fertilization, some evidence suggests they may still have access to maternal resources during development. When gravid females from lecithotrophic species with MI values in the vicinity of 0.6 to 0.7 (Family: Poeciliidae) were injected in the caudal musculature with radiolabeled amino acids, embryos showed detectable levels of radioactivity within a few hours (Marsh-Matthews et al. 2001; Marsh-Matthews et al.

2005; Marsh-Matthews and Deaton 2006; Riesch et al. 2010). Similarly, when females were injected with large, biologically inert fluorescent microspheres, developing embryos were found to fluoresce (DeMarais et al. 2005). Despite the lack of active provisioning in lecithotrophic species, these results suggest that internally developing eggs are still able to acquire organic and inorganic molecules from their surrounding environment. Active transport, the movement of molecules across a cell membrane against a concentration gradient and assisted by the consumption of ATP, has been previously observed in eggs of oviparous fish species (Terner 1968; Siebers and Rosenthal 1977). Trout (Terner 1968) and herring (Siebers and Rosenthal 1977) have both been observed using active transport to internalize amino acids from their surrounding environment. It is possible then that the developing eggs of lecithotrophic livebearers are capable of active transport, and that trait predisposes them to the evolution of placentation following the transition to livebearing. To test the generality of the capacity of fish eggs to acquire resources from their surrounding environment, I tested eggs from three oviparous species of Cyprinodontiformes and a fourth species from the superorder Atherinomorpha, which includes the Cyprinodontiformes. Viviparity and matrotrophy has evolved multiple times within the Order Cyprinodontiformes, therefore any properties I observe among the oviparous species were probably shared with the predecessors to the livebearing lineages. My experiments characterize the type of active transfer, if any, that is present within the Cyprinodontiform egg. Experiment 1 was designed to assess whether or not the eggs were capable of internalizing amino acids from their external environment, and concentrating them against a gradient. I then addressed two hypotheses to characterize

the mechanism of uptake: 1.) If uptake is achieved by active transport that involves cell membrane proteins that are specific in the molecules transported through the cell membrane, then biologically common and uncommon isomers of unlabeled amino acids should differ in their ability to inhibit uptake (Experiment 2), 2.) If instead the uptake is achieved via a general mechanism capable of transferring larger molecules, like pinocytosis, then uptake should be seen when eggs are exposed to larger biologically inert fluorescent microspheres (Experiment 3). Finally, I consider the evolutionary implications of these experiments, which indicate that the Cyprinodontiform egg is capable of actively concentrating molecules against a concentration gradient via pinocytosis.

Methods

Egg Collection and Staging

Experiments were performed on three species of egg-laying Cyprinodontiformes (*Nothobranchius furzeri*, *Rivulus hartii*, *Cyprinodon variegatus*) and one egg-laying species from the Superorder Atherinomorpha (*Oryzias latipes*). Stocks of male and female *O. latipes*, *N. furzeri*, and *R. hartii* were maintained in 20-50 gallon stock tanks and fed twice daily. Fertilized eggs were collected daily, and stored in 96 mL Conex plastic cups filled with Yamamoto solution (NaCl, 0,75%, KCl, 0,02%, CaCl2, 0,02%; (Yamamoto 1939)) until experimentation. Fertilized eggs of *C. variegatus* were provided by another investigator (Steve Munch - Stony Brook University). *C. variegatus* eggs were collected and stored in saltwater inside of a cool storage container (20-22 ^{o}C) for 24

hours or seven days prior to experimentation. Eggs were characterized by their age (in days) as well as stage of development two hours before experimentation (Wourms 1972; Iwamatsu and Ohta 1974). For *O. latipes*, *C. Variegatus*, and *N. furzeri*, 6-10 eggs of the same age (and within 1 stage of each other) were grouped into a single well in incubation experiments, while only one egg per well was used for *R. hartii* because the eggs are larger (~3mm diameter in *R. hartii* vs. 1-1.5mm diameter in other species). For the temperature, amino acid competition, and microsphere experiments eggs were alternatively grouped into three stage ranges simplified from (Wourms 1972): early (stages 1-13; pre-somite stages), intermediate (stages 13-20; pre-pigment stages), and late (stages 36-43; pre-hatching stages).

Incubation in radiolabeled amino acids (Experiment 1)

Eggs were transferred into wells of a 48-well Falcon™ Tissue Culture Plate containing 300µL of water filtered through a 0.2µm polyethersulfone membrane. Prior to this study I monitored response of embryos left within multiwall plates and found that eggs from all four species could be maintained in 300µL of filtered water for up to 6 hours without any observed impact to their heart rate or subsequent development. Multichannel pipettes were used to simultaneously deliver 300µL of ^{14}C-glycine radiolabel in filtered water [20nCi/mL] resulting in an activity level of 6 nCi in a total volume of 600µL for each well. Experimental eggs were incubated in the radiolabel solution in a 22-24ºC water bath for 30 minutes. Within each 48-well plate, negative control eggs were incubated in filtered water without radiolabel, and were used to evaluate possible sample contamination or measurement error of radioactivity detectors.

Following the 30-minute incubation period, all liquids were removed from the wells and each well was rinsed twice by pipetting 300µL of filtered water in and out of the well ten times. Eggs were immediately transferred into liquid scintillation vials containing 600µL of tissue solubilizer (Solvable^{TM}, Perkin Elmer) and incubated overnight at 60^{o} C. To test the effectiveness of the rinsing protocol, two empty wells in each plate were filled with 600µL of the radiolabeled solution (matching the volume of experimental wells) for 30 minutes and then rinsed alongside the experimental wells. In lieu of eggs, 30µL of water from the second rinse of the control wells was transferred into the scintillation vials. Neither the egg controls nor the rinse controls ever showed levels of radioactivity above background levels, and were therefore combined as a single control in all analyses. Once eggs were fully solubilized, samples were allowed to cool to room temperature before adding 5.4mL of Ultima Gold^{TM} LSC Cocktail (Perkin Elmer). The counts-per-minute (CPM) for each vial was measured over ten minutes using a Beckman Coulter LS6500 Multipurpose Scintillation Counter calibrated for ^{14}C. I repeated the experiments in *O. latipes* using ^{14}C-Leucine to rule out the possibility that my observations were a product of the small molecular size of Glycine and not applicable to other amino acids.

The levels of radioactivity observed within each scintillation vial were used to estimate the molecular concentration of amino acid within each egg relative to the incubation medium. Given that the specific activity of the radiolabel was known (55 mCi/mmol), the observed CPM values were converted into Curies (ci) and then used to calculate the number of mmol within each vial. Molarity was estimated by assuming the number of moles calculated was contained within the total volume of the eggs that were

de-solubilized (mmol/mL). The radioactive concentration for the incubation medium (50 nCi/mL) was known; therefore I estimated the molarity of the incubation medium as the expected number of moles in volume equal to that of the eggs. The molarity within the eggs was then compared to the expected molarity in an equal volume of incubation medium.

Amino Acid Competition Experiments (Experiment 2)

Amino-acid competition assays were performed to test if rates of radiolabel uptake could be inhibited through direct competition with unlabeled "cold" amino acids. Unlabeled Leucine was mixed into solution with ^{14}C-Leucine at molecular ratios of 2.5, 12.5, 31.25, 62.5, 125, and 187.5:1. Eggs were then incubated in the mixed solution and radioactivity levels measured using the same experimental protocols as the previous experiments.

Radiolabeled ^{14}C-Leucine was also competed against two isomers of unlabeled Leucine in order to evaluate the specificity of active transport. Though identical in chemical formula, L-Leucine is an essential amino acid vital for organism function while D-Leucine is biologically uncommon isomer and typically does not travel across the membrane via the same specific transport channels when those channels are present (Schneider et al. 1979; Hoshino and Kageyama 1980; Hosie et al. 2002). If transport is specific, then only L-Leucine is expected to compete with the radiolabeled Leucine. If it is non-specific, L- and D-Leucine would compete equally with radiolabeled Leucine. Unlabeled L- and D-Leucine were pipetted into separate solutions with ^{14}C-Leucine at molecular ratios ranging from 1:2 to1000:1. Two hours prior to experimentation, eggs of

similar stages and ages (in days) were grouped together. Eggs were then incubated in either L-Leucine or D-Leucine mixtures and radiation levels measured using the same protocols as before.

Fluorescent Microsphere Experiment (Experiment 3)

To assess the endocytotic capabilities of the eggs I incubated them in filtered water with 0.04μm diameter carboxylate-modified red-orange fluorescent microspheres (FluoSpheres®, Life Technologies™). Microspheres were diluted in filtered water to a concentration of $1.18x10^{13}$ beads/mL. Individual eggs from *R. hartii* were incubated in the well of a 96-well Falcon™ Tissue Culture Plates containing 300μL of the diluted microspheres. Plates were incubated at room temperature (22-24°C) for 30 minutes as in the previous experiments. Control eggs were incubated in filtered water without microspheres in the same plate for the same duration as the experimental treatment. After the second rinsing, eggs were immediately fixed in 2% glutaraldehyde. Eggs were visualized using an Atto Pathway HT High Throughput Automated Confocal Microscope. Images of the eggs were taken using transmitted light and then a 580nm arc lamp (565/580nm excitation/emission maxima), and subsequently superimposed on one another for analysis.

Previous research shows that endocytosis can be inhibited at low temperatures (Wright and Oparka 1989); therefore I repeated the experiment at decreased temperatures. Four hours prior to the experiment eggs were grouped into early-, intermediate-, and late-stages and then evenly split between two separate 96-well plates. One plate, along with the microsphere mixture and filtered water, was left on ice within a

6°C walk-in cooler for 2 hours prior to experimentation. The second plate and relevant liquids were simultaneously left at room temperature. Experimental and control eggs (no microspheres) in both temperature treatments were simultaneously incubated, rinsed, and photographed using the aforementioned protocol.

Statistical Analysis

Mean levels of radioactivity for each egg were analyzed using Mixed Models (GLMM) in R package MASS (2005; Venables and Ripley 2013). Experimental treatment and embryo age were included as fixed effects in all models. For the competition experiments, the ratio of cold label to radiolabel was included as fixed effect. To control for any variation among replicates of an experiment, experiment date was included as a random effect. In cases where the age of the embryo had no significant effect, the model was rerun with embryo age included as a random effect.

All models used a gamma distribution with a log link to account for the fact that radioactivity data is positive with a variance that is near constant on a log scale. Shapiro-Wilkes tests were performed for all of the data subsets to test for normality.

Results

Active transport of amino acids

Eggs from all four species had significantly higher levels of radioactivity in the Glycine incubation treatment relative to both control treatments (Figure 1.1a, Table 1.1). Eggs from *O. latipes* also had significantly higher levels of radioactivity in the Leucine incubation treatment relative to the control treatment (Figure 1.1a, Table 1.1).

The concentrations of radiolabel inside the eggs, estimated from the total radioactivity in the scintillation vials and the volume of the eggs, were 2.8 to 11 times higher within the egg than in the incubation medium (Table 1.1).

Table 1.1. Results of GLMMs evaluating mean levels of radioactivity (CPM/egg) as a function of experimental treatment with embryo age and experiment date included as random effects. The final column shows the mean amino acid concentrations observed within the egg relative to the concentration in an equal volume of the incubation medium.

Species	Amino Acid	$\chi 2$	df	Pr (> $\chi 2$)	[Egg]:[Solution]
O. latipes	Glycine	210.63	1	< 0.0001	11:1
O. latipes	Leucine	128.51	1	< 0.0001	9.4:1
R. hartii	Glycine	502.44	1	< 0.0001	4.5:1
N. furzeri	Glycine	252.39	1	< 0.0001	2.8:1
C. variegatus	Glycine	43.10	1	< 0.0001	9.6:1

Embryo age had a significant positive effect on the observed levels of radioactivity in the Glycine incubation treatment but not in the control treatment for both *C. variegatus* (age x treatment: Chi-square = 4.85, df = 1, P = 0.028) and *O. latipes* (age x treatment: Chi-square = 7.29, df = 1, P = 0.007). This pattern was not observed in *R. hartii* (age x treatment: Chi-square = 0.925, df = 1, P = 0.335) or *N. furzeri* (age x treatment: Chi-square = 1.67, df = 1, P = 0.19).

Cold Label Competition Assays

Mean levels of radioactivity within the eggs decreased in response to increased concentrations of unlabeled Glycine in the experimental treatment relative to the control treatment (Figure 1.2, Figure 1.5). Increasing concentrations of unlabeled Leucine also decreased mean levels of radioactivity observed in *R. hartii* eggs in experimental

treatments. D-Leucine was as effective as L-Leucine in inhibiting uptake of the radiolabeled Leucine (Figure 1.3).

Uptake of Fluorescent Microspheres

Fluorescent microspheres were detected within the eggs of *R. hartii*, *N. furzeri*, and *O. latipes* following a 30-minute incubation in the presence of microspheres. I did not detect any fluorescence in either control group. Fluorescence was observed on the external surface of the egg as well as within the egg, often concentrated within the yolk sac. This pattern was only observed for incubation temperatures above 4°C. Eggs incubated in microspheres at temperatures below 4°C only showed fluorescence on the external surface of the egg, never within the egg or concentrated in the yolk sak (Figure 1.4, Supplementary Information).

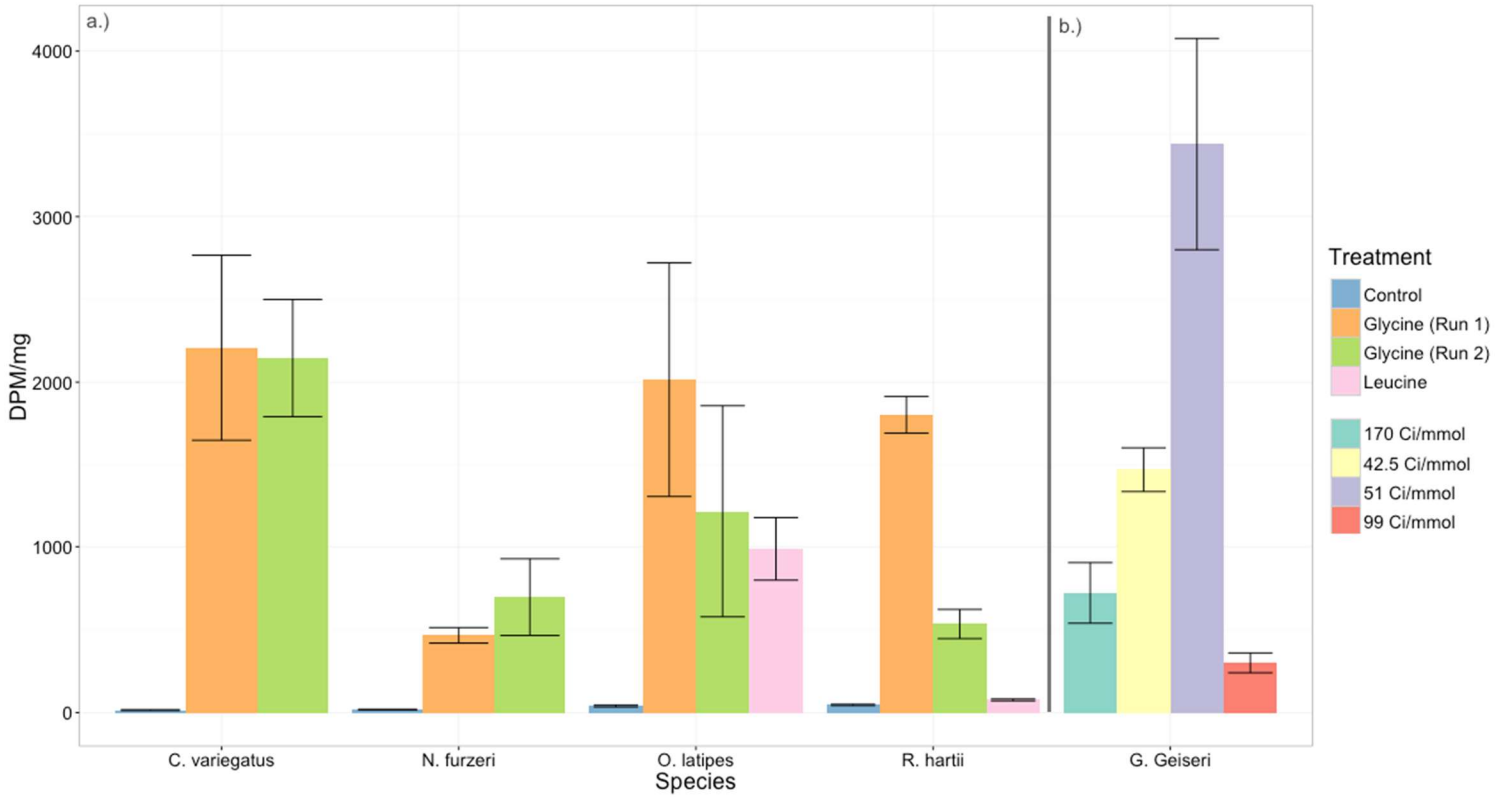

Figure 1.1. Mean and standard errors of observed levels of radiation in embryos from (a) four egg-laying species (this study) and (b) one live-bearing species (Marsh-Matthews et al. 2001; Marsh-Matthews et al. 2005) of Cyprinodontiformes, after correcting for embryo mass and radiolabel concentration. These comparisons are conservative because the exposure times were longer and the specific activities of the radiolabel were higher in G. geiseri studies than in the three egg-laying species

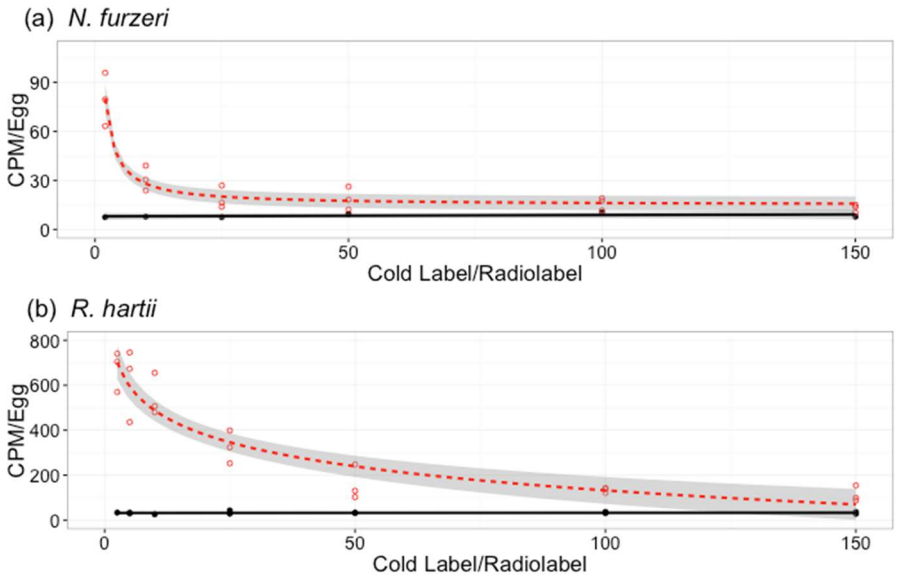

Figure 1.2. Fitted regression lines and 95% confidence intervals showing the influence of increased additions of unlabeled Glycine to the mean levels of radioactivity in eggs from N. furzeri and R. hartii when incubated in the presence (open circles/dashed line) and absence (closed circles/solid line) of radiolabeled Glycine (Dilution x Treatment (a): $\chi 2 = 4.78$, df = 1, P = 0.029; Dilution x Treatment (b): $\chi 2 = 3.49$, df = 1, P = 0.061).

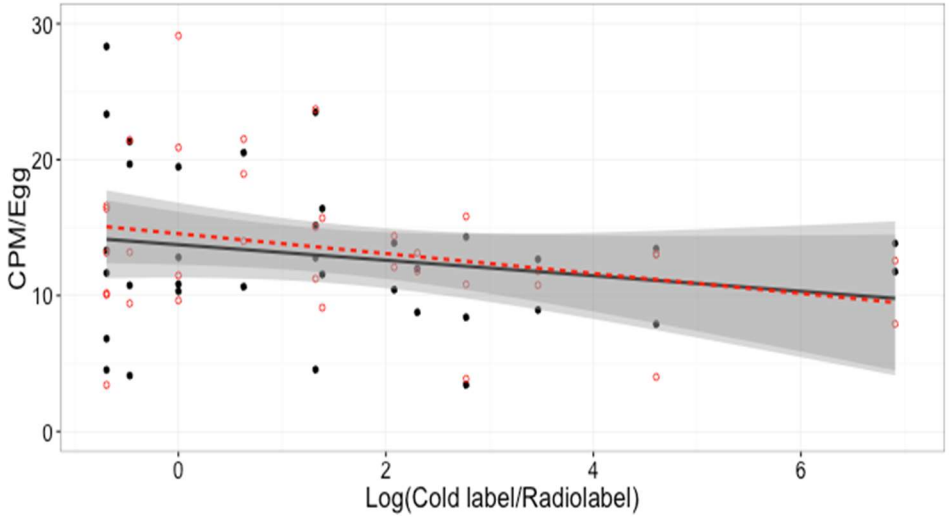

Figure 1.3. Fitted regression lines and 95% confidence intervals (shaded) showing similar levels of radioactivity in eggs of *R. hartii* when competed against increasing concentrations of L-Leucine (open circles/dashed line) and D-Leucine (closed circles/solid line; t = 0.664, df = 49, P = 0.51). The x-axis is log transformed for clarity.

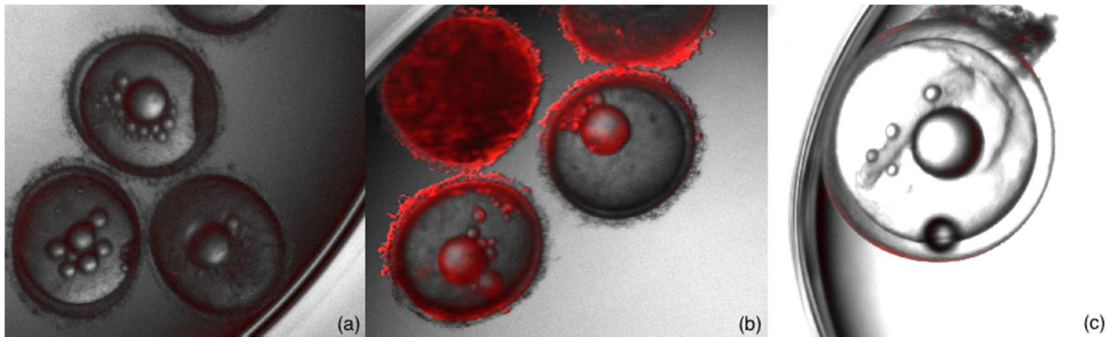

Figure 1.4. Overlaid confocal microscopy images taken with ambient light and a 565/580 nm excitation lamp: (a) *O. latipes* eggs from the negative control which were not exposed to the microsphere and show no fluorescence, (b) *O. latipes* embryos from the exposure treatment showing fluorescence concentrated internally and on the outer surface of the egg, (c) an embryo of *R. hartii* that was exposed to radiolabel at sub-4°C temperatures with fluorescence only on the outer surface of the egg.

Discussion

The results of this study support my hypothesis that fertilized eggs of Cyprinodontiform fishes are capable of amino acid active transport (Table 1.1). Regardless of species or organic molecule, eggs in the experimental incubation treatments contained higher levels of radioactivity than control groups. The washing protocol appeared sufficient in removing radiolabel from adhering to exposed surfaces since activity levels of both control groups were consistent with natural background radioactivity. Moreover, the molarity of both amino acids within each egg was 2.8-11 times higher (Table 1.1) than an equivalent volume of the incubation medium, indicating that each egg is capable of acquiring organic molecules against a concentration gradient.

Observed rates of active transport were variable across species, developmental stage, temperature, and amino acid concentration. All four species showed varying levels

of transport, with the fastest rate of transport observed in Japanese Medaka (*O. latipes*) and the slowest rate observed in the Turqoise Killifish (*N. furzeri*), when controlling for egg size. Interestingly, rates of transport were elevated in older embryos of *O. latipes* and *C. variegatus*, but not in *R. hartii* and *N. furzeri*. The results of *R. hartii* and *N. furzeri* are inconsistent with previous work that found rates of uptake to be elevated in older herring (*Clupea harengus*) embryos (Siebers and Rosenthal 1977). This pattern may be explained by the biology of the two species; *N. furzeri* is an annual killifish capable of reducing its metabolic rate by undergoing developmental diapause (Furness et al. 2015), while *R. hartii* can also exhibit delayed hatching phenotypes (Furness 2015). If the levels of uptake in Cyprinodontiform eggs are influenced by the metabolic rate of the embryo, then later stage embryos of *R. hartii* and *N. furzeri* undergoing developmental arrest would be expected to show reduced levels of uptake. Lastly, uptake of the radiolabeled amino acid decreased in response to increased concentrations of the unlabeled versions of the same amino acid (Figure 1.1, Figure 1.4), indicating that rates of uptake can be inhibited in these eggs. In this case, the inhibition points to an upper limit to whatever mechanism is transport amino acids across the surface of the egg, but does not rule out any of the possible explanations.

Active transport across an egg membrane can occur via a number of mechanisms, including ion pumps, exocytosis, and endocytosis (Lodish et al. 2000), but the results of this study suggest that Cyprinodon eggs are concentrating molecules through the non-selective mechanism of pinocytosis. I observed similar rates of competitive inhibition for both D- and L-Leucine (Figure 1.3). D-Leucine is a biologically uncommon enantiomer,

therefore I predict that unlabeled D-Leucine would not inhibit the uptake of radiolabeled L-Leucine if transport occurs across highly specific transport channels, as has been observed in *Pseudomas* and *Rhizobium* (Hoshino and Kageyama 1980; Hosie et al. 2002). The similar rates of inhibition in the presence of unlabeled D- and L-Leucine, along with the presence of a non-specific binding region in the competition curve (Figure 1.5), points to the presence of a non-specific transport mechanism. Moreover, bulk transport via pinocytosis can be inhibited by saturating the surrounding medium with an analogous molecule as was observed in this study (Bronner and Kleinzeller 1978). This conclusion is also supported by the results of the microsphere experiments. Microspheres are biologically inert and larger than molecules (40nm in diameter vs. 0.8nm diameter of amino acid) that can pass through cell membrane channels, and are most likely transferred via endocytotic pathways (Mellman 1996). Eggs from all three species examined were capable of microsphere uptake, and microspheres were found concentrated in the yolk (Figure 1.4b). Previous work in mammals and plants revealed that pinocytosis, the most common mechanism for transferring particles ~40nm in diameter, can be inhibited at lower temperatures (Wright and Oparka 1989; Wolkers et al. 2003). In support of a pinocytotic mechanism, I found uptake of the microspheres ceased when eggs were incubated at temperatures below 4°C (Figure 1.4c). Specifically, eggs incubated in microspheres at sub-4°C would have microspheres adhered to their outer shell but did not contain microspheres internally (Figure 1.4c). At low temperatures, it is likely that free-floating microspheres adhere to the eggs naturally, but are not transported across the egg membrane due to the inhibition of pinocytosis.

Why do fish eggs have the ability to absorb materials from the external environment? Previous work suggests that the rates of uptake in fish eggs are not high enough to supplement an embryo's diet in any meaningful way (Terner 1968; Siebers and Rosenthal 1977). Instead, it may be that active transport serves as a means of gaining information about the external environment. For example, eggs of other teleost fish have been shown to vary their hatching rates when exposed to predator cues (Sih and Moore 1993; Jones et al. 2003). Active transport on the external surface of the teleost egg would potentially be a way of sensing the external environment, such as the remnants of a predation event.

Active transport across the egg membrane in this study was observed in oviparous species of Cyprinodontiformes, however mounting literature suggests that this trait may be retained in viviparous species (Marsh-Matthews et al. 2001; DeMarais et al. 2005; Marsh-Matthews et al. 2005; Marsh-Matthews and Deaton 2006). Multiple studies have observed the uptake of radiolabeled amino acids in internally developing embryos shortly after injecting gravid females (Marsh-Matthews et al. 2001; Marsh-Matthews et al. 2005; Marsh-Matthews and Deaton 2006; Riesch et al. 2010). These experiments were performed on *Poecilia mexicana* and several species of *Gambusia,* all viviparous lecithotrophic fish species (Marsh-Matthews et al. 2001; Reznick et al. 2002; Marsh-Matthews et al. 2005; Marsh-Matthews and Deaton 2006; Riesch et al. 2010). These authors interpreted the uptake of label as the presence of matrotrophy, however my results suggest that this uptake may instead be a property of the egg that was retained from an egg-laying ancestor. I evaluated this possibility by creating a conceptual model

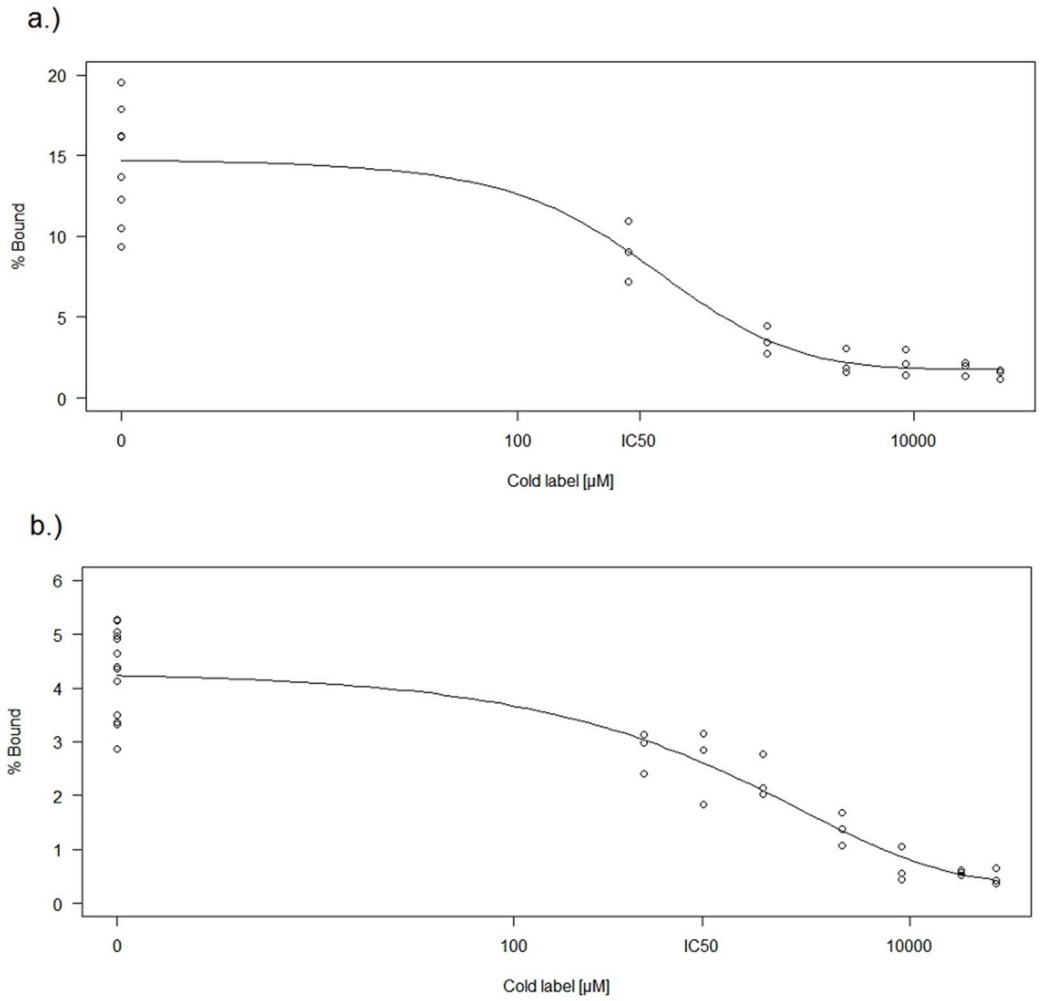

Figure 1.5. Dose-response curves showing the influence of increased additions of unlabeled Glycine to the mean levels of radioactivity in eggs from *N. furzeri* and R. *hartii* when incubated in the presence of radiolabeled Glycine. The continued presence of signal at the highest concentrations indicates the presence of a non-specific binding region.

in which the total volume of a gravid female was treated as a volume of water that radiolabel was added to and assumed that the radiolabel was uniformly distributed throughout this volume. Molar concentrations were estimated by using the specific activity of the radiolabeled amino acid from observed levels of radioactivity into the number of amino acid molecules in the volume of each egg. I compared the estimated

concentration of label in the dissected embryos from those studies with the concentration observed in the embryos of my study, after correcting for the concentrations of radiolabel I estimated in my conceptual model. The degree to which label was concentrated in the dissected embryos was in the same range as those observed in my eggs (Figure 1.1b). Moreover, the injection studies used longer exposure times and radiolabel with a higher specific activity (Marsh-Matthews et al. 2001; Marsh-Matthews et al. 2005), which makes the direct comparison between my study and theirs conservative since these differences in conditions lead to the expectation of higher concentrations of label in the livebearing fish if all things are equal. These similarities argue that active transport via pinocytosis is a property of the Cyprinodonitform egg that is retained through the evolutionary transition from egg laying to livebearing. These results also argue that using radiolabel uptake in livebearing species does not prove the presence of matrotrophy because there are potential mechanisms for developing embryos to acquire the radiolabel in the absence of maternal provisioning.

A growing body of work suggests the evolution of placentation represents the most recent step in a series of reproductive evolutionary transitions in reproductive mode (Crespi and Semeniuk 2004; Furness et al. 2015). Here I argue that these transitions represent a contingent series of evolutionary events that only proceed when the requisite conditions are met in the preceding steps. The contingent nature of these sequential adaptations potentially explains the discrepancy among lineages in the probability that matrotrophy evolves from lecithotrophic ancestors. The discrepancy between teleost fishes (12 out of 14) and squamate reptiles (2-4 out of 115) (Blackburn 1999) is

particularly striking. Teleost egg membranes, like those of the Cyprinodontiformes included in this study, are capable of actively acquiring resources from their external environment, which is formed by the body fluids of their mother. This property means that the egg that is retained in the earliest stages in the evolution of viviparity has the ability to acquire nutrients from its mother. This ability lays the foundation for the parent-offspring conflict that is the proposed mechanism behind the evolution of complex forms of matrotrophy, including placentas (Trivers 1974; Haig 1993; Haig 1997, 1999). In contrast, in order for a squamate to evolve viviparity, it must first suppress the development of tissues (e.g., the egg shell) that evolved to isolate and protect the embryo from its surrounding environment. If the amniotic membranes that enclose the developing embryo were incapable of active transport, the same foundation of active transfer and trigger for the initiation of intergenomic conflict would not be present.

A second possible explanation is that teleost eggs are much smaller than eggs from squamate reptiles, and consequently will have a larger surface area to volume ratio. Eggs with a larger surface area to volume ratio will be more efficient at transporting molecules across the egg surface relative to the size of the embryo. This would be particularly relevant if efficient eggs were predisposed to evolving a mechanism of maternal provisioning. I plotted the surface area and volume of the eggs in this study to some egg-laying squamates that are close relatives of lecithotrophic live-bearing species, and found the eggs in my species to have a much greater ratio (Figure S1.1). Though circumstantial, what evidence I do have indicates that the size of the egg may influence the propensity for matrotrophy to evolve in live-bearing species. Regardless of which egg

properties are responsible, I suggest that these differences between the starting points of viviparity in teleosts vs. squamates may contribute to the observed differences in the rate at which livebearing lineages evolve matrotrophy.

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Reproductive mode affects rates of reproductive barrier formation among species of Poeciliopsis

Abstract

The evolution of placentation sets the stage for intergenomic conflicts to play out between mothers and offspring over the optimal levels of maternal investment, because it provides offspring with opportunities to potentially manipulate their mothers into providing more resources. Parent-offspring conflicts can lead to the evolution of reproductive isolation among populations when conflicts are resolved in different ways. Since conflicts are more intense in placental species, post-zygotic reproductive isolation is predicted to evolve more rapidly in placental species than in non-placental species. I tested this hypothesis by performing a series of interpopulation crosses within closely related placental and non-placental species of *Poeciliopsis*. I did not observe any inviability or sterility of offspring among any of the populations crossed. In terms of offspring size, however, offspring fitness declined rapidly as a function of interpopulation genetic distance within the placental species, but did not differ among populations within the non-placental species. I show that the decrease in offspring size observed in the placental species falls outside the range of normal variation, and likely represents a major fitness cost. my results are consistent with the predictions of conflict-driven speciation because negative epistatic interactions are evolving more quickly among populations in the placental species than either non-placental species. I discuss how the results of this study continue to argue that parent-offspring conflicts have played a role in the evolution of reproductive isolation and vertebrate reproductive mode.

Introduction

The evolution of reproductive isolation among groups of organisms is central to the process of speciation (Coyne and Orr 2004). As a result, considerable interest has been paid to the types of reproductively isolating barriers that exist between species (Coyne and Orr 1989, Coyne and Orr 2004), the evolutionary processes that facilitate the evolution of reproductive isolation (Bolnick and Near 2005, Schluter 2009, Maan and Seehausen 2011), and the rate at which barriers evolve (Coyne and Orr 1989, Bolnick and Near 2005, Rabosky and Matute 2013). One of the insights gleaned from this body of research is that the types of reproductive barriers and the rates at which they evolve can differ among taxa. Within *Drosophila*, pre- and post-zygotic reproductive isolation evolve at equal rates under allopatric conditions, but pre-zygotic reproductive barriers evolve faster in sympatry (Coyne and Orr 1989). Similarly, prezygotic barriers appear to evolve more quickly than postzygotic barriers in birds (Price and Bouvier 2002, Fitzpatrick 2004, Rabosky and Matute 2013) and African Lake Cichlids (Stelkens, Young et al. 2010). In contrast, mammals evolve post-zygotic reproductive barriers five to ten times faster than birds, reptiles, and amphibians (Wilson, Maxson et al. 1974, Prager and Wilson 1975, Fitzpatrick 2004). Furthermore, the rates at which post-zygotic reproductive isolation evolve within mammals differ depending on the structure of the placental connection between mother and offspring (Capellini, Venditti et al. 2011). These observations suggest that the relative importance of pre- and post-zygotic barriers not only differs among taxa, but potentially as a function of the biological differences among organisms.

The viviparity-driven conflict hypothesis (VDCH) proposes that reproductive mode influences the evolution of reproductive isolation as a consequence of differences in the levels of parent-offspring conflict experienced among taxa with dissimilar reproductive adaptations (Trivers 1974, Zeh and Zeh 1996). Parent-offspring conflict describes the evolutionary conflict of interests that occur between mothers and offspring over optimal levels of maternal provisioning (Trivers 1974). A mother is equally related to all of her offspring, thus her evolutionarily optimal strategy is to distribute resources evenly among them. In contrast, each individual offspring is more closely related to itself than its mother or siblings causing selection to favor adaptations that enable the offspring to obtain more investment than is optimal for its mothers to give (Trivers 1974). This inequality between what is optimal for the mother and optimal for the offspring results in an intraspecific arms race (Dawkins and Krebs 1979). This conflict can lead to the evolution of post-zygotic reproductive isolation when rapid coevolution between mothers and offspring within a population leads to incidental evolutionary divergence between allopatric populations when conflicts are resolved in different ways (Dawkins and Krebs 1979, Gavrilets and Hayashi 2005, Rice, Linder et al. 2005, Hayashi, Vose et al. 2007, Crespi and Nosil 2013). Moreover, since parent-offspring conflicts are continuous and selection is acting directly on reproductive traits, post-zygotic reproductive barriers are predicted to evolve more rapidly from parent-offspring conflicts than other mechanisms of mutation-order speciation (Zeh and Zeh 2000). In oviparous species without parental care, parent-offspring conflict is limited because mothers have complete control over maternal provisioning (Zeh and Zeh 2000, Crespi and Semeniuk 2004). In viviparous

species that lack placentas and fully provision eggs prior to fertilization internal development provides a window of opportunity for parent-offspring conflicts because the developing embryo potentially has access to maternal resources (Zeh and Zeh 2000, Crespi and Semeniuk 2004). The evolution of matrotrophy (post-fertilization maternal provisioning) intensifies conflict because the direct physiological connection between mothers and offspring and the transfer of nutrients from mother to developing young increases the number of avenues where conflict can take place (Crespi and Semeniuk 2004). The intensified conflict that accompanies the evolution of viviparity is thus predicted to cause accelerated rates of evolution of post-zygotic reproductive isolation (Zeh and Zeh 2000, Crespi and Semeniuk 2004, Zeh and Zeh 2008). The subsequent evolution of matrotrophy is predicted to further magnify this conflict and further accelerate the evolution of post-zygotic reproductive isolation (Crespi and Semeniuk 2004, Crespi and Nosil 2013, Furness, Morrison et al. 2015).

Existing evidence is circumstantial but consistent with the predictions of the VDCH. Fetal manipulation of the maternal environment is well documented in humans (Haig 1993, Haig 1999, Haig 1999, Crespi and Semeniuk 2004), and analogous patterns have been observed in a wide spectrum of viviparous taxa (Guillette 1991, Crespi and Semeniuk 2004, Schrader 2009, Ala-Honkola, Friman et al. 2011). Furthermore, placental morphology is highly divergent among closely related mammal species, suggesting that the organ is a product of tight coevolution between mothers and offspring within taxa, causing divergence to rapidly evolve among taxa (Crespi and Semeniuk 2004, Elliot and Crespi 2006, Capellini, Venditti et al. 2011).

In the placental fish species *Heterandria formosa* (family: Poeciliidae), significant levels of post-zygotic reproductive isolation were observed among populations separated for only 10,000 years (Schrader and Travis 2008). In contrast, interpopulation crosses performed in the non-placental Poeciliid species *Gambusia affinis* and *Poecilia reticulata* found weak or non-existent post-zygotic barriers (Reznick 1981, Reznick 1982). Similarly, little to no post-zygotic isolation appears to exist between species of oviparous Centrarchids (sunfish) that diverged over 6 MYA (Bolnick and Near 2005). The average time since common ancestry in successfully hybridizing species of Teleost fish was significantly greater for oviparous species (~35 mya) than for viviparous species (~10 mya; (Coleman, Harlin-Cognato et al. 2009)). In *Heterandria formosa*, the strength of post-zygotic reproductive isolation between populations is predicted by how divergent the populations are in offspring size at birth (Schrader and Travis 2008, Schrader, Fuller et al. 2013). Post-zygotic reproductive isolation thus appears to evolve quickly in placental species, but all studies to date make broad comparisons among distantly related species. Here I examine the influence of reproductive mode on rates of reproductive isolation in three species of *Poeciliopsis*, and provide a direct comparison between closely related placental and non-placental species.

The fish family Poeciliidae is a group of small Neotropical fish that exhibit a wide variety of reproductive adaptations (Rosen and Bailey 1963, Lucinda and Reis 2005). Multiple species within the family have follicular pseudoplacentas, a matrotrophic adaptation analogous to the mammalian placenta (Turner 1940, Reznick, Mateos et al. 2002). In non-placental species, females fully provision eggs prior to fertilization, so

developing offspring rely solely on the resources present in the yolk, thereby reducing the potential for conflict. Within the genus *Poeciliopsis*, placentation has evolved multiple times resulting in multiple clades that contain closely related placental and non-placental taxa (Reznick, Mateos et al. 2002, Mateos 2005, Pollux, Pires et al. 2009), creating a unique opportunity to make direct comparisons of the evolution of reproductive isolation between placental and non-placental sister taxa. Here, I perform intraspecific crosses and look for evidence of reproductive incompatibilities among populations. I predict that, as genetic distance among populations increase, there will be a more rapid rate of decline of fitness in crosses in species with placentas than in species without placentas. Reduced fitness can take the form of decreased fecundity, decreased offspring viability, decreased offspring size, or an increase in length of pregnancy

Methods

Three species of *Poeciliopsis* were used in this study. I selected *Poeciliopsis prolifica* and *Poeciliopsis infans* to represent a paired comparison of closely related placental and non-placental species from the same clade, respectively (Reznick, Mateos et al. 2002, Pollux, Meredith et al. 2014). *Poeciliopsis gracilis*, was included as an additional non-placental species from a separate clade (Reznick, Mateos et al. 2002). I established population stock tanks in 20-gallon aquaria at the University of California Riverside by collecting 15 pregnant females and five adult males from 4 populations of *P. prolifica*, 4 populations of *P. infans*, and 2 populations of *P. gracilis* (Table S2.1) between 2014-2015. Fish from an additional population of *P. gracilis* collected in 2004

and maintained in 3 stock tanks were also included (Table S2.1). To ensure allopatry, I collected each population from a different river (Figure 2.1; Table S2.1). Schrader et al. (2013) found in their crosses among populations of *H. formosa* that differences among populations in offspring size cause reductions in the viability of offspring from hybrid crosses. I exploited existing data for *P. infans* and *P. gracilis* (Frías-Alvarez, Macías Garcia et al. 2014) to choose sites with large difference in late-stage embryo size. I recorded measures of water quality, temperature, canopy cover, and piscine species richness at each collection site (Table S2.1).

Wild-caught adults were housed in 19 or 38 L aquaria with clumps of aquatic moss (*Vesicularia dubyana*) to provide cover for newborn offspring. I removed F1 offspring daily and reared them in group tanks. The anal fine of male Poeciliids metamorphose into the gonopodium, the intromittent organ, over a period of weeks (Turner 1941). I used this metamorphosis to identify the sex of individuals while they were immature, then moved them into single-sex aquaria to rear them to maturity. Lab-born male tanks were seeded with two females from the same population to stimulate sperm production. As lab-born females approached sexual maturity, I isolated them into individual 2-gallon tanks containing gravel and aquatic moss, and fed ad libitum. Once individual lab-born females reached sexual maturity, I used them in a single intrapopulation or interpopulation cross.

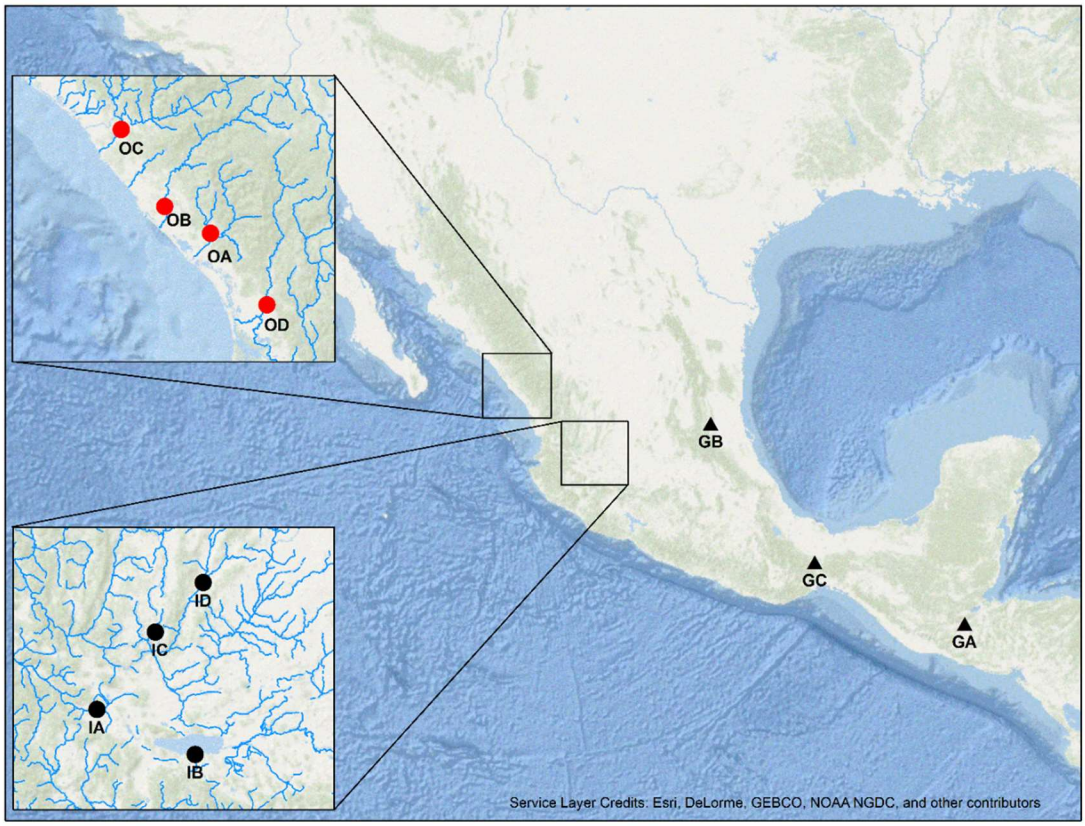

Figure 2.1. Waterway map of collection localities for *Poeciliopsis prolifica* (red circles), *P. infans* (black circles), and *P. gracilis* (black triangles) showing the restriction of sites to separate river systems.

I performed three crosses in all possible interpopulation cross directions, and six intrapopulation crosses within each population as a control. I placed lab-born adult males into the tanks of isolated virgin females for 7 days, after which time they were removed. I monitored the mated females daily in order to record the latency time (in days) between mating and producing their first brood. Once females gave birth to their first brood, daily monitoring continued for a period of 60 days, after which time females were euthanized using MS-222 and preserved in 95% ethanol. I removed newborn offspring from the female tanks daily to ensure individuals were less than 24-hours old at the time of

collection. The first 10 offspring born to a given female were immediately euthanized in MS-222 and preserved in 95% ethanol. All remaining offspring of both sexes were placed in a 2-gallon stock tank and allowed to reach sexual maturity. I monitored these tanks to see if the offspring that resulted from interpopulation crosses produced offspring of their own as a measure of hybrid sterility.

I took multiple measures from the outcome of each cross to serve as an index of fitness. The total number of offspring born to a given female over the 2-month monitoring period was recorded as a measure of fecundity. Preserved offspring were measured for body length (mm), wet weight (mg), and dry weight (mg) at birth. I dissected females and removed all embryonic tissue to record the total number of developing embryos and the wet weight (g) of all the reproductive tissue. Total reproductive tissue mass was analyzed as a proportion of female wet weight to account for differences in the size of individual females. I scored embryos as viable or inviable depending on whether or not they exhibited the typical phenotype of a normally developing embryo (Haynes 1995). Inviable embryos exhibited the characteristic markers of their developmental stage, but were typically smaller, duller in color, and more semisolid that viable embryos. I measured the focal females for body length (g) and wet weight (g) to control for the influence of female size on offspring size and number. To supplement the data on offspring size differences among the populations of *P. gracilis* and *P. infans* (Frías-Alvarez, Macías Garcia et al. 2014), I pooled and averaged all of the offspring resulting from intrapopulation crosses for each population of *P. prolifica*.

Sequencing and Bioinformatics

To assess genetic distance among the populations, I extracted DNA from the tail tissue of five male and five female wild-caught individuals from each population. . Extractions were performed using a Qiagen® DNeasy Blood & Tissue Kit, with two modifications to the spin-column protocol; I extended the duration of proteinase K treatment to 10 hours then incubated each sample in 8 µL of RNAse for 30-minutes at 37^{O}C. I quantified DNA concentrations with a Qubit 2.0 Fluorescence Reader, and each sample was adjusted to a concentration of 10ng/ µL using a Zymo Research DNA Clean & Concentrator™ kit. ddRAD sequencing was performed by the University of Texas at Austin Genomics Sequencing and Analysis Facility (GSAF), including enzyme digestion, size selection, adaptor ligation, and sequencing. I selected the EcoRI-MspI enzyme pair for digestion, and 200-300 base-pair fragments were retained for sequencing. 2x150bp reads of the digested samples were obtained using the Illumina HiSeq 2500 system.

I performed all demultiplexing, *de novo* assembly, and genetic distance calculations using the STACKS pipeline (Catchen, Hohenlohe et al. 2013). I used the process_radtags program to filter out low quality reads with raw phred scores below 10 (< 90% probability of being correct) and uncalled bases. The STACKS core modules ('ustacks', 'cstacks', 'sstacks', and 'populations') were executed through the denovomap.pl program with a minimum stack depth of 3 reads, a maximum number of mismatches allowed between loci within an individual (M) of 2, a maximum number of mismatches between loci in a catalog (m) of 1, and the deleveraging and highly repetitive

stack removal algorithms enabled. I used the 'populations' function to calculate pairwise F_{ST} estimates between all population combinations.

Statistical analysis

I analyzed measures of reproductive success using linear mixed effects models in the R package lme4 (Team 2013, Venables and Ripley 2013). Since the data came from three species representing two modes of reproduction, I accounted for the unbalanced design by including species as a random effect nested within reproductive mode in all models. Each dependent variable was initially analyzed as a function of reproductive mode and four covariates - interpopulation genetic distance (F_{ST}), the difference in offspring size between populations (Offspring Size Difference – OSD), the wet mass of the mother, and a principal component axis (PC1 = ~ 40%) that captured the most ecological variation among sites. OSD and F_{ST} were highly collinear (VIF > 5); therefore Type I SS ANOVAs were used to determine which of the two covariates explained more of the variation in the dependent variables when they each entered into the model first. F_{ST} explained more of the variation for all of dependent variables thus OSD was dropped from all of the final models. When models contained predictor variables that were independently insignificant ($P > 0.05$) and absent from any significant interactions, the models were rerun without those predictors.

Results

I did not detect any evidence of local adaptation to the ecological differences among collection sites. PC1 did not have a significant effect on any of the dependent variables, nor did it predict the genetic distance among populations (χ^2_1= 1.8526, P = 0.1735). Similarly, I did not observe a significant effect of maternal size on the average length (offspring length: χ^2_1 = 0.3380, P = 0.56101) and mass (Figure 2.2) of offspring at birth. Accordingly, PC1 and maternal wet mass were removed as predictors from all of the final models.

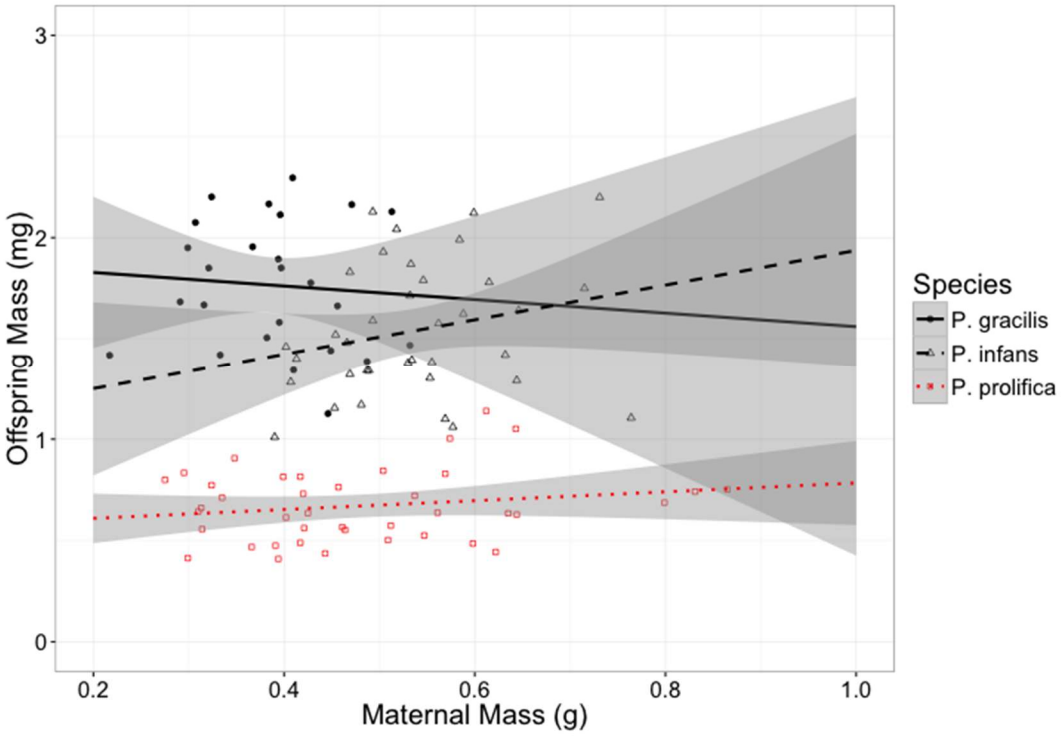

Figure 2.2. The average birth weight of offspring as a function of the weight of their mother for a placental (red) and non-placental (black) species of *Poeciliopsis* (maternal mass: χ^2_1= 1.7832, P = 0.181753). The shaded region represents the 95% confidence interval.

Irrespective of cross type, the placental species *Poeciliopsis prolifica* gave birth to more offspring on average than either non-placental species (Table 2.1), but the average size of an individual offspring was larger for non-placental species (Table 2.1). The time until the birth of the first offspring did not differ among placental and non-placental species (Table 2.1).

Individuals in every possible interpopulation cross direction were capable of successfully reproducing for all three species. Some females failed to reproduce in the initial cross or in subsequent crosses, but mating failure was independent of cross type ($\chi^2_{25} = 20.79$, $P = 0.704$). I did not detect any inviable or prematurely aborted embryos detected for any of the 108 crosses. Similarly, there was no evidence of sterility within the offspring that resulted from any of the 102 successful crosses.

Increasing interpopulation genetic distance led to smaller offspring (Figure 2.3) and the production of less reproductive tissue (Figure 2.4) in the placental species *P. prolifica*, but did not significantly affect either non-placental species (Table 2.1). The F_{ST} x Reproductive Mode interaction did not significantly affect the number of offspring born, the time until the birth of the first offspring, or the number of viable and inviable embryos dissected (Table 2.1). Within *P. prolifica*, offspring from interpopulation crosses were ~60% the size of offspring from intrapopulation crosses, and were significantly smaller in terms of terms of length ($F_{1,36} = 55.37$, $P < 0.0001$; Figure 2.5) and mass ($F_{1,36} = 101.856$, $P < 0.0001$; Figure 2.5).

The differences in offspring size between the two cross types was not influenced by the size of the mother for either mass ($F_{1,36}$ = 0.03, P = 0.762; Figure S2.1) or length ($F_{1,36}$ = 3.66, P = 0.0635; Figure S2.1) measure.

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Table 2.1. Effects of interpopulation genetic distance and reproductive mode on measures of reproductive success taken from intra- and interpopulation reciprocal crosses performed within *Poeciliopsis prolifica*, *P. infans*, and *P. gracilis*. The statistically significant chi-square values ($P < 0.05$) are highlighted in bold.

Effect	d.f.	Offspring length (cm)	Offspring dry mass (g)	Total Reproductive Tissue (g)	# of offspring	# of viable embryos	# of inviable embryos	Time to first birth (days)
F_{ST}	1	**12.1858**	3.4272	2.3959	0.4494	2.6734	2.6734	0.9002
Reproductive Mode	1	**33.5646**	**25.565**	0.8651	**13.6264**	0.0947	0.0947	0.9833
F_{ST} x Reproductive Mode	1	**53.0544**	**7.4704**	**5.0545**	0.7825	0.2844	0.2844	0.1283

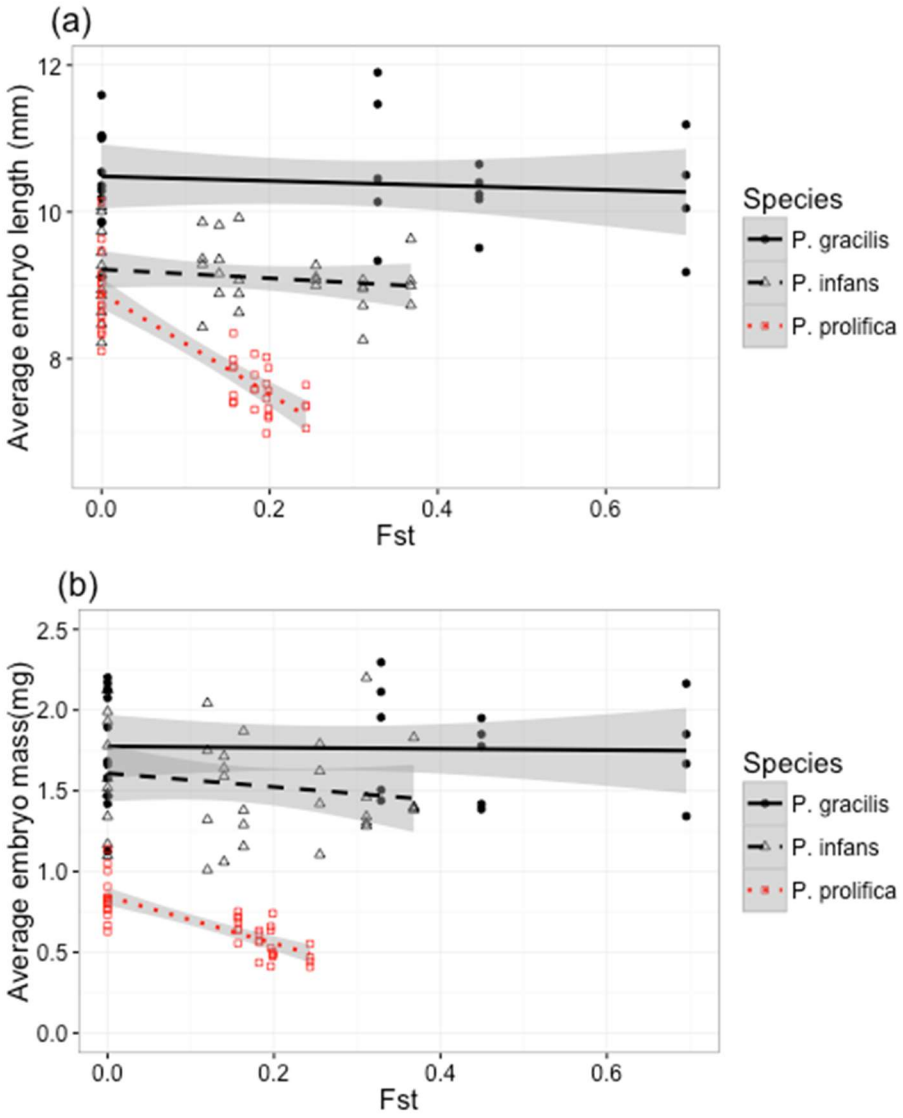

Figure 2.3. Birth length (a) and weight (b) of newborn offspring from intra- and interpopulation reciprocal crosses performed within a placental (red) and two non-placental (black) species of *Poeciliopsis,* viewed as a function of the genetic distance between their parent populations. The shaded region represents the 95% confidence interva

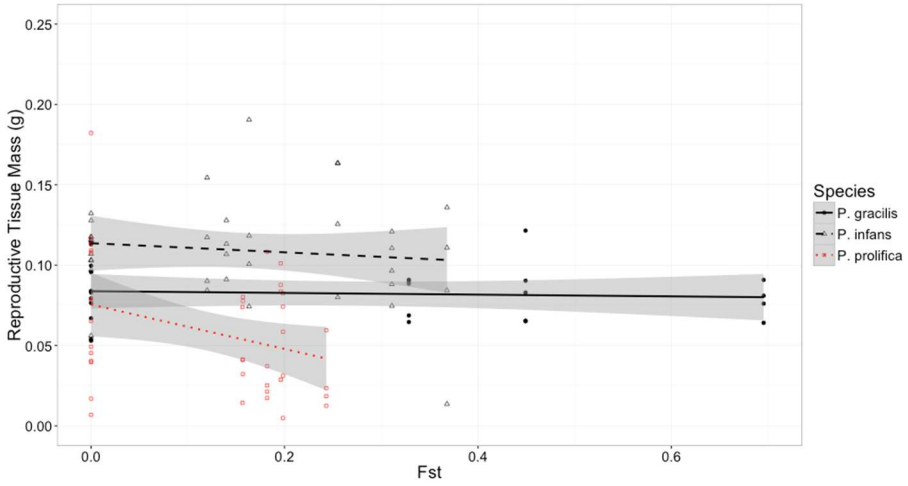

Figure 2.4. The amount of reproductive tissue, as a proportion of female size, produced by females from intra- and interpopulation reciprocal crosses performed within a placental (red) and two non-placental (black) species of *Poeciliopsis,* viewed as a function of the genetic distance between their parent populations. The shaded region represents the 95% confidence interval.

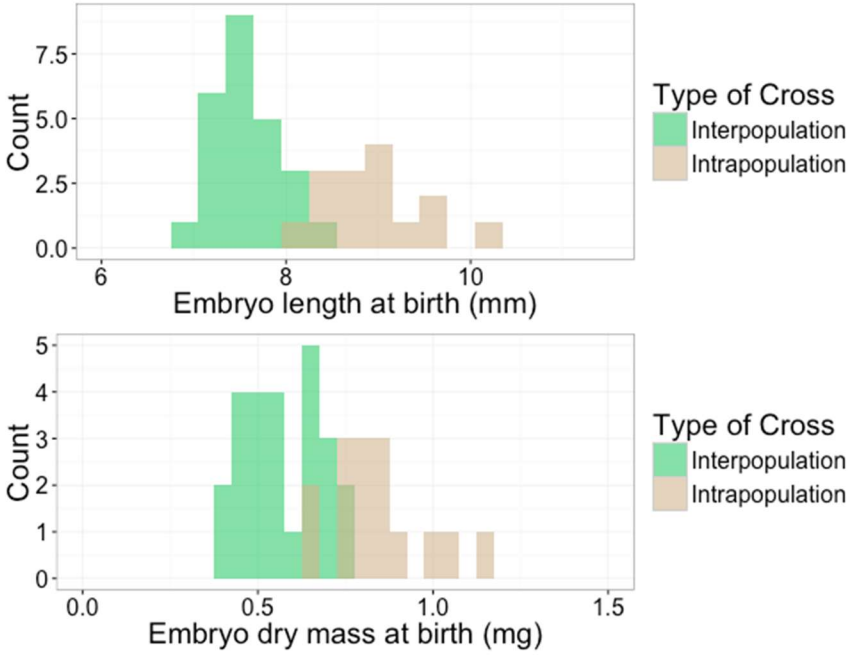

Figure 2.5. Histogram of the offspring length (top) and mass (bottom) at birth for offspring born from interpopulation (green) and intrapopulation (tan) mating crosses performed within the placental species *Poeciliopsis prolifica*.

Discussion

Offspring length and mass at birth decreased as a function of interpopulation F_{ST} in the placental species *P. prolifica*, but remained relatively constant in non-placental species *P. infans* and *P. gracilis* (Figure 2.3). If diminished offspring size is indicative of reproductive incompatibility, the patterns observed in this study supports the prediction that the evolution of placentation increases the likelihood reproductive isolation will evolve among geographically isolated populations, and therefore accelerates speciation. This assumes that undersized offspring have reduced fitness in comparison to normal-sized offspring. Offspring of *P. prolifica* from the most divergent crosses were 40-50% smaller than their counterparts from within-population crosses (Figure 2.5). The decrease in offspring size observed here is well below normal variation (Figure 2.5), and offspring this small would be statistical outliers in a natural setting. In addition, these size differences were independent of maternal size (Figure S2.1). This pattern suggests that the stark reduction in offspring body size is the product of mating individuals from highly divergent populations with some resulting imbalance between the allocation of resources by the mother and acquisition of resources by the embryo. In Poeciliids, larger offspring generally have higher fitness early in life, and differences in birth length smaller than those observed in this study can drastically influence mortality rates (Henrich 1988). In nature, extrinsic selection pressures would most likely disfavor the production of smaller offspring, and therefore serve as a reproductive barrier between these populations. The total mass of reproductive tissue produced by a female matched the patterns observed for offspring size (Figure 2.3, Figure 2.4). This difference is a function of producing smaller

offspring since the majority of the reproductive tissue is composed of developing embryos. Together, these results suggest females within the placental species suffer negative fitness consequences from mating with males from distantly related populations. Non-placental species suffer no such loss of fitness in association with crosses among populations.

Schrader et al.'s (2008) experimental study of *Heterandria formosa*, another species of Poeciliidae that represents an independent origin of placentation, revealed a different form of reproductive incompatibility in crosses among populations. In their case, incipient reproductive isolation was driven by differences among populations in the size of offspring they produced, rather than the genetic distances between them. Reproductive isolation was manifested with the production of inviable offspring with no observable differences in offspring size. One potential explanation is that the genetic basis of conflict resolution in *H. formosa* is different from *P. prolifica*. In *P. prolifica*, genes involved in conflict resolution, and hence in the mismatch of that resolution when different populations are hybridized, may only involve the quantity of maternal provisioning during development. In *H. formosa*, conflict resolution may invoke genes that play some fundamental role in governing how development proceeds. The important distinction between this project and the earlier work by Schrader et al. (2008) is that there study was performed on a single species. I included two non-placental species and show that species without placentas reveal no hint of reduced fitness as a function the genetic differences among parents. This interaction between fitness reduction and reproductive mode makes a stronger case that placentation is the cause of the loss of fitness.

One possible alternative explanation for the patterns observed in this study is that the variation in offspring size is being driven by environmental differences among the populations. The principal component axis that accounted for the most variation in ecological measures included every environmental measure I gathered. None of the interactions including PC1 were significant, and PC1 itself did not appear to significantly affect the variation in my measures. In general, the environmental differences that did exist across rivers resolved into species differences, rather than differences among populations within a species (i.e. populations of single species occur in ecologically similar streams). I also found that the size of the mother did not significantly affect the size of the offspring in this study (Figure 2.2). This result was consistent with a similar study in *Heterandria formosa*, where offspring viability was predicted by the characteristics of the mating cross and not the phenotype of the individual mother (Schrader and Travis 2008, Schrader, Fuller et al. 2013).

The results of this study are consistent with the Viviparity-driven conflict hypothesis because increasing genetic distance among populations resulted in the production of inferior offspring in the placental species but not in either non-placental species (Zeh and Zeh 2000, Crespi and Semeniuk 2004, Zeh and Zeh 2008, Furness, Morrison et al. 2015). An increasing body of evidence, including this study, suggests that the evolution of reproductive strategies that increase the connection between mother and developing offspring accelerates the evolution of post-zygotic reproductive isolation. At the microevolutionary level, the results presented here argue that reproductive incompatibilities evolve faster in a placental species than in a closely related non-

placental species. At the macroevolutionary level, a recent meta-analysis performed within the same Order (Cyprinodontiformes) determined that reproductive isolation evolves significantly faster between viviparous species than between oviparous species (Morrison et al. unpub). Furthermore, Helmstetter et al. (2016) have shown in the order Cyprinodontiformes that viviparous species have higher speciation and diversification rates than oviparous species. Diversification rates appear to be faster for viviparous species relative to oviparous species in other taxa, including lizards (Lambert and Wiens 2013) and snakes (Lynch 2009). Morrison et al (unpub.) also show that post-zygotic reproductive isolation evolves fastest in livebearing placental species, at intermediate rates in livebearing non-placental species, and slowest in egg laying species.

Given the results of these comparative studies, one might also expect to see some degree of interpopulation reproductive isolation in the non-placental species in the current study, given that they are livebearers. Placental species are predicted to evolve post-zygotic barriers faster than non-placental species, but the VDC generally predicts that post-zygotic barrier formation should be accelerated in all livebearing species. One possibility is that rates of evolution of post-zygotic reproductive isolation are still accelerated in viviparous taxa relative to oviparous taxa, but not fast enough to be evident in the timescales observed in this study. To this point, I did observe a negative effect of interpopulation genetic distance on offspring size in the two non-placental species (slope term for *P. gracilis* = -0.037; for *P. infans* = -0.423), suggestive of fitness loss in those species as well, but the slopes were not significantly different from zero. Given a longer

divergence time, or potentially more statistical power, it is possible that a detectable effect of viviparity exists.

A growing number of studies are beginning to corroborate one another, and highlight the importance of reproductive mode as a driver of speciation and macroevolutionary diversification. Unlike ecological speciation, which is driven by natural selection pressures that promote the evolution of optimal values, antagonistic coevolution is rarely resolved; therefore conflict may function as a perpetual force driving speciation. Further work may illuminate how ubiquitous conflict is in the origin of species and its relative importance in comparison to other mechanisms of speciation.

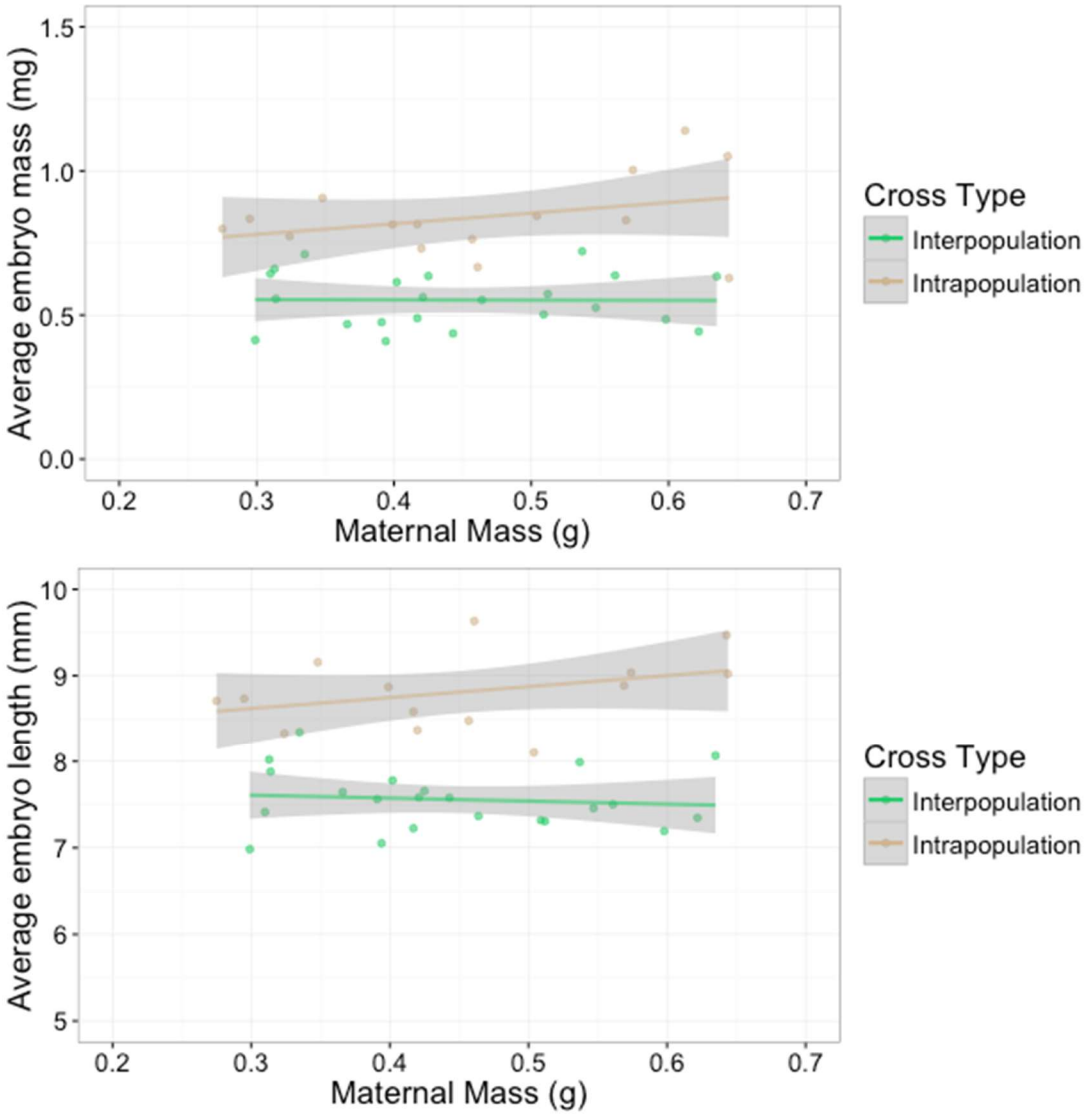

Figure S2.1. Average embryo mass (top) and length (top) of offspring resulting from intrapopulation (green) and interpopulation (tan) crosses performed in the placental species *Poecilopsis prolifica* modeled as a function of the mass of their mother. The shaded region represents the 95% confidence interval.

Table S2.1. Locality information and environmental measures for eleven collection sites used to establish stock tanks in this study.

Species	Site ID	Collection Year	River	Elevation	pH	Temperature	Canopy Cover	Latitude	Longitude	Annual Rainfall
P. prolifica	OA	2014	Baluarte	40m	8-8.5	25^{O}C	0%	N 23^{O}3'45"	W 105^{O}50'37.5"	481.4
	OB		Presidio	19m	7.5-8.0	25^{O}C	5%	N 23^{O}16'31.7"	W 106^{O}14'27.5"	751.6
	OC		Piaxtla	45m	8-8.5	22^{O}C	10%	N 23^{O}53"17.1'	W 106^{O}37'07.0"	751.6
	OD		Acaponeta	20m	7.5-8.0	23^{O}C	0%	N 22^{O}29'24.5"	W 105^{O}21'19.4"	24
P. infans	IA	2014 & 2015	Ameca	1241m	8-8.5	25^{O}C	25%	N 20^{O}33'3.5"	W 103^{O}57'7.5"	546.3
	IB		de la Pasión	1536m	8-8.5	22^{O}C	25%	N 20^{O}9'38.3"	W 103^{O}2'20.0"	1076.4
	IC		Santiago	1191m	8-8.5	27^{O}C	5%	N 21^{O}12'26.4"	W 103^{O}22'4.6"	805
	ID		Juchipala	1371m	9-9.5	20^{O}C	0%	N 21^{O}39'4.8"	W 102^{O}57'56.0"	419.8
P. gracilis	GA	2004	Motagua	120m	N/A	N/A	N/A	N 14^{O}57'51.5"	W 89^{O}34'43.5"	1910.6
	GB	2014	Tampaón	101m	7-7.5	22^{O}C	40%	N 21^{O}58'33"	W 98^{O}57'42"	1594
	GC		Coatzacoalcos	89.7m	8-8.5	21^{O}C	12.5%	N 17^{O}8'59"	W 95^{O}7'6.1"	579

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The effect of reproductive mode on speciation in Cyprinodontiformes

Abstract

The intensity of mother-offspring conflicts are predicted to differ as a function of reproductive mode. Intense parent-offspring conflicts can drive the evolution of post-zygotic reproductive isolation, and thus it has been hypothesized that the rate that post-zygotic reproductive isolation evolves differs among vertebrates that differ in their mode of reproduction. I make use of the diversity of reproductive adaptations present in Cyprinodontiform fishes to test how rates of post-zygotic reproductive isolation and macroevolutionary diversification differ as a function of reproductive mode. I amassed a dataset of all interspecific crosses performed within the group, and calculated an index of post-zygotic isolation for each cross. Post-zygotic reproductive isolation evolved at an accelerated rate in viviparous taxa relative to oviparous taxa. The estimated level of post-zygotic reproductive isolation was higher among matrotrophs than among lecithotrophs at all genetic distances, but the rate that post-zygotic reproductive evolved was not significantly different between the two groups. Speciation and diversification rates estimated from phylogenies were higher for viviparous taxa than for oviparous taxa, but lecithotrophic species had a marginally higher speciation rate than matrotrophs. I find evidence that variation in reproductive mode, particularly between oviparous and viviparous species, has a major impact on the evolution of reproductive barriers and influences patterns of diversification in Cyprinontiformes.

Introduction

Understanding how mechanisms of speciation that operate at the population level influence large-scale patterns of species richness remains an ongoing challenge in evolution (Kisel et al. 2012; Rabosky and Matute 2013). At the microevolutionary scale, numerous mechanisms are known to cause the evolution of reproductive isolation and potentially serve as drivers of speciation (Nosil et al. 2002; Coyne and Orr 2004; Schluter 2009; Sobel et al. 2010). At the macroevolutionary scale, speciation rates estimated from phylogenies are highly variable among taxa often in conjunction with biological differences, such as floral structure or habitat type (Rabosky et al. 2007; Goldberg et al. 2010; Jetz et al. 2012; Bloom et al. 2013; Rabosky et al. 2013). Given that speciation is defined by the evolution of reproductive isolation under a biological species concept, speciation rates are assumed to be limited by how quickly reproductive barriers form (Coyne and Orr 2004; Sobel et al. 2010). However, disconnects between the evolution of reproductive isolation evaluated in the context of microevolutionary studies and the rate of speciation can occur for multiple reasons (Rabosky 2015). Speciation is the product of pre- and/or post-zygotic reproductive isolation, and the relative importance of each of them in driving the speciation process can vary considerably among groups of organisms (Coyne and Orr 2004). Additionally species diversification estimated from molecular phylogenies is a net rate of speciation minus extinction. Factors that influence the likelihood of extinction, the persistence of incipient species, and the opportunities for geographic isolation among groups of organisms can all affect rates of evolutionary diversification at macroevolutionary scales (Wiens 2004; Jablonski 2008; Rosenblum et

al. 2012; Rabosky 2015). Accordingly, the multiplicity of factors that lie between the estimation of the rate of evolution of pre- or post-zygotic isolation and net rate of diversification means that the microevolutionary underpinning of reproductive isolation may not accurately predict speciation rate. Here, I quantify how well differences in the mode of reproduction among taxa can influence rates of post-zygotic reproductive isolation evolution and macroevolutionary diversification.

The Viviparity-driven conflict hypothesis (VDCH) posits that the intensity of parent-offspring conflicts vary among species that differ in reproductive mode, and this variation influences how quickly post-zygotic barriers will evolve among taxa (Zeh and Zeh 2000; Zeh and Zeh 2008). Parent-offspring conflicts are the byproduct of asymmetries in the relatedness of parents and their offspring (Trivers 1974). The evolutionarily optimal strategy for a mother is to distribute her resources evenly among all of her offspring because she is equally related to all of them. In contrast, an individual offspring is most closely related to itself that to its mother or siblings, therefore it stands to gain by obtaining more resources than is optimal for the mother to give. Mothers and offspring are therefore in perpetual conflict over optimal levels of maternal provisioning (Trivers 1972, 1974). This results in an antagonistic coevolution between mothers and offspring within a population, akin to an evolutionary tug-of-war, which in turn can lead to incidental divergence among populations when different adaptations arise in response to the same selection pressures (Dawkins and Krebs 1979; Arnqvist and Rowe 2005; Kolliker et al. 2010). Coevolution in this case occurs over reproductive traits, thereby increasing the likelihood that divergence among populations will lead to the formation of

post-zygotic reproductive barriers (Rice 1997; Rice and Holland 1997; Crespi and Nosil 2013). Parent-offspring conflicts can lead to speciation, but the intensities of conflict are predicted to vary among modes of reproduction (Zeh and Zeh 2000; Zeh and Zeh 2008). Oviparous (egg-laying) species experience minimal conflict, because offspring are isolated from their mother during development and the mother is in full control of her investment since she pre-provisions the yolk prior to fertilization (Zeh and Zeh 2000; Crespi and Semeniuk 2004; Furness et al. 2015a). In contrast, offspring in viviparous (live-bearing) species develop internally and are given a window of opportunity to manipulate the levels of provisioning to their advantage (Zeh and Zeh 2000; Crespi and Semeniuk 2004; Furness et al. 2015a). The subsequent evolution of matrotrophy (mother-feeding), active provisioning of offspring during development (e.g. the placenta), is predicted to provide additional avenues for offspring to manipulate the levels of provisioning they receive and therefore exacerbates conflicts within viviparous species (Crespi and Semeniuk 2004; Furness et al. 2015a). Post-zygotic reproductive isolation is therefore predicted to evolve slowest among viviparous taxa, at intermediate rates among viviparous lecithotrophs (yolk-feeders), fastest among viviparous matrotrophs.

A growing body of literature supports the prediction that reproductive mode influences speciation rates at micro- and macroevolutionary scales. Post-zygotic reproductive isolation evolves more quickly in mammals, most of which are viviparous matrotrophs, than in birds, amphibians, and squamate reptiles, which are largely oviparous (Wilson et al. 1974; Prager and Wilson 1975; Fitzpatrick 2004). Even within fish and reptiles, a transition to livebearing is associated with accelerated rates of

speciation estimated at macroevolutionary scales (Lynch 2009; Lambert and Wiens 2013; Helmstetter et al. 2016). In teleost fish, the age of the common ancestor between successfully hybridizing oviparous species is significantly older than for viviparous species (~35 mya vs. 10 mya; (Coleman et al. 2009)). Post-zygotic reproductive isolation was minimal among species of oviparous Centrarchids (sunfish) diverged for over six million years (Bolnick and Near 2005). In contrast, post-zygotic reproductive barriers were observed among populations of a placental fish species (*Heterandria formosa*) separated for only 10,000 years (Schrader and Travis 2008; Schrader et al. 2011). Lastly, a direct comparison between closely related *Poeciliopsis* revealed that post-zygotic reproductive incompatibilities evolved significantly faster in placental species than in non-placental species (Morrison et al. unpub). Existing evidence suggests that reproductive mode influences the rate of evolution of post-zygotic reproductive isolation, but do they also affect speciation rates? Pre-zygotic barriers generally contribute more to reproductive isolation among species and often evolve more rapidly than post-zygotic barriers (Coyne & Orr 1989; Grant and Grant 1999; Mendelson 2003). If the influence of pre-zygotic barriers dominate speciation, then speciation rates will potentially be disconnected from the variation in rates of post-zygotic reproductive isolation that exists among the different reproductive modes. An explicit test of the VDCH at both scales of evolution is still needed.

Cyprinodontiformes is an Order of ~1254 small freshwater fish species mostly native to Africa and the Americas, and includes the killifish and livebearers (Nelson et al. 2016). Across the Order, species exhibit a wide variety of phenotypes from internal and

external fertilization, oviparity and viviparity, to lecithotrophy and matrotrophy (Wourms 1981; Blackburn et al. 1985; Wourms et al. 1988). A variety of matrotrophic mechanisms analogous to the mammalian placenta are present within the group (Turner 1940a, b; Wourms et al. 1988; Hollenberg and Wourms 1995). Moreover, viviparity and matrotrophy have both evolved multiple times within Cyprinodontiformes (Reznick et al. 2002; Blackburn 2005; Pollux et al. 2009), providing natural replicates for comparisons among taxa that differ in reproductive mode. In this study, I examine the effect of reproductive mode on rates of evolution of reproductive isolation and speciation in Cyprinodontiformes. I predict that rates of post-zygotic reproductive isolation evolution and estimates of speciation rate will be lowest in oviparous species, intermediate in lecithotrophic viviparous species, and highest in matrotrophic viviparous species

Methods

Hybridization Dataset Assembly

All available data on interspecific mating crosses in Cyprinodontiformes was derived from scientific publications, government documents, and aquarium hobbyist literature. The initial dataset was trimmed to only include cases of reciprocal hybrid crosses, and to exclude cases where DNA sequence data was absent from one of the crossed species, making it impossible to estimate time since divergence between the two species hybridized. I also excluded speculative reports of hybrids observed in an uncontrolled setting, and reports in which the results of the cross were not quantified by any metric, making it impossible to assess the degree of post-zygotic reproductive

isolation between the two species. For all of the interspecific crosses in the reduced dataset, pairwise p-distances were estimated in MUSCLE (Edgar 2004) from a concatenated set of mitochondrial genes (see Tree Construction for list of genes). The level of post-zygotic reproductive isolation for each cross was scored on a scale of 0 to 1 following the methods of Yukilevich (2012). For each cross direction, a value of 0 indicates that both sexes of hybrid offspring are viable and fertile, 0.5 indicates one sex of the hybrid offspring is viable or infertile, and 1 indicates that hybrid offspring from both sexes are inviable or sterile (Yukilevich 2012). Initial scores were multiplied by the proportion of the offspring that exhibited the irregular phenotype to correct for partial sterility or inviability. The values for each cross direction were averaged into a single value representative of the interspecific cross. Observed levels of reproductive isolation among species pairs are not independent of their phylogenetic relatedness (Coyne and Orr 1989). To control for the effects of phylogeny on the likelihood of evolving reproductive barriers, I applied phylogenetic corrections to the reproductive isolation scores following the methods of Fitzpatrick et al. (2006). The ultrametric tree for Cyprinodontiformes (see Tree Construction) was used to average non-independent values across phylogenetic nodes.

Tree Construction

All available sequence data (Table S3.1) from five mitochondrial (12S, 16S, COI, CYB, ND2) and three nuclear genes (28S, Rag1, RHO) was downloaded from GenBank (Clark et al. 2016) in January of 2015 for all species of Cyprinodontiformes (~1254 species) and from several outgroups (Atherinomorpha - *Oryzias latipes*, *Atherinops*

affinis, *Menidia beryllina*, *Chirostoma humboldtianum*, *Melanotaenia duboulayi*, *Rheocles wrightae*, *Craterocephalus stercusmuscarum*, *Atherinomorus lacunosus*; Cichlidae - *Craterocephalus stercusmuscarum, Paratilapia polleni*; Perciformes - *Chromis cyanea*; Beloniformes – *Exocoetus volitans*). Sequences from each gene were initially aligned in Geneious (Kearse et al. 2012) using the Geneious alignment algorithm, and then manually adjusted in Geneious. Ambiguous, misidentified, and poorly annotated sequences were removed from the final alignments. Fishbase (Froese and Pauly 2012) and the Catalog of Fishes (Eschmeyer et al. 2014) were used to resolve taxonomic ambiguities and remove single species that were present in the alignments under multiple synonyms. Individual gene alignments were concatenated using Sequence Matrix (Vaidya et al. 2011). The final concatenated alignment was 13916 base pairs in length and included 647 species. Dated fossils and secondary clade age estimates for Actinopterygian fish were taken from the literature to serve as calibration points for time tree construction (Table S3.3).

A maximum likelihood (ML) tree was estimated using RAxML-HPC v.8 (CIPRES platform; (Miller et al. 2010; Stamatakis 2014)), a GTRCAT + G model of molecular evolution for each of the nine partitions, with 500 bootstrap replicates, randomized MP starting trees, and with all free parameters estimated. A fossil-calibrated ultrametric tree was estimated in BEAST v1.8.4 (CIPRES Platform, (Drummond and Rambaut 2007; Suchard and Rambaut 2009; Miller et al. 2010)) using the ML tree as the tree prior. PartitionFinder (Lanfear et al. 2012) was used to determine the most appropriate model of nucleotide substitution for each gene, and the concatenated dataset

was analyzed with a mixed-model partitioning scheme (Table S3.1). The Atherinomorpha, Cichlidae, and Perciformes clades were constrained as monophylies throughout tree construction. A relaxed lognormal molecular clock model of evolution was used, allowing substitution rates to vary among taxa, and a birth-death prior was used for rates of cladogenesis. Six fossil calibrations and three secondary calibrations (Table S3.3), secondary calculations from Betancur-R et al. (2013) were used to date nodes during tree construction. Seven independent BEAST analyses were run for 100 million generations sampling trees every 10,000 generations. Tracer v1.6.0 (Rambaut et al. 2015) was used to examine the convergence and mixing of runs, and to ensure effective sample sizes (ESS) were >200 for all parameters. Trees and logs from all seven runs were combined using LogCombiner v.1.8.3 (Drummond et al. 2012) after discarding the first 10 million generations as burn-in, and TreeAnnotator v.1.8.3 (Drummond et al. 2012) was used to generate the Maximum Clade Credibility (MCC) tree with target node heights. Outgroup taxa were pruned from the tree for all subsequent analyses.

Character State Scoring

The literature was compiled to determine the reproductive mode of every species of Cyprinodontiformes. Species were first categorized as either viviparous (~931 species) or oviparous (~333 species) oviparity. Viviparous species were further categorized as lecithotrophic or matrotrophic, with all oviparous species treated as lecithotrophic by definition. The matrotrophy index (Wourms et al. 1988; Reznick et al. 2002) was as the criteria for determining if an individual species was lecithotrophic (MI is ≤ 1) or matrotrophic (MI > 1). The matrotrophy index (MI) is estimated as the dry weight of the

embryo at birth divided by the dry weight of the embryo at fertilization. When a species MI is ≤ 1, it indicates that developing embryos lose or maintain their weight throughout development and offspring development is likely fueled solely by the energy provided in the pre-provisioned yolk. When a species has an MI value > 1, developing embryos are gaining weight throughout development and are likely receiving active provisioning from their mother. When possible, MI values were taken from the published literature estimated using published dissection data on the size of embryos throughout development. For species where embryo size data was absent, I dissected 6-10 pregnant females from the Ichthyology collections of the University of Michigan Museum of Zoology (UMMZ) and the Smithsonian. For species in the family Goodeidae, dissected embryos were staged used following the 6-stage methods of (Guerrero-Estévez and Moreno-Mendoza 2012). In cases where embryos from the first and last stage of development were not obtained, a conservative estimate of MI was calculated by using the youngest and oldest available stage of development as end points.

Hybridization Analysis

I used generalized linear models to examine the effect of reproductive mode on rates of reproductive barrier formation in the R environment (Team 2000). Levels of interspecific reproductive isolation were analyzed as a function of genetic distance (p-distance), reproductive mode, and the interaction between the two. In addition to analyzing all reproductive modes simultaneously, I made pairwise comparisons of viviparity/oviparity and lecithotrophy/matrotrophy. Three of the 108 crosses in the final

dataset occurred between species with different reproductive modes; in these cases the cross was scored as the character state of the more derived phenotype.

Lineage Diversification Analysis

Character state specific rates of speciation were estimated using both Diversitree (FitzJohn 2012) and RevBayes (Höhna et al. 2016). In both cases species were subdivided into one of three character states, 1. Oviparous, 2. Viviparous lecithotrophy, or 3. Viviparous matrotrophy. Ancestral character states, state-specific speciation and extinction rates, and transition rates among each character state were jointly estimated under a Multiple State Speciation Extinction (MuSSE) model in Diversitree (FitzJohn 2012). I compared the fit of the full unconstrained model, where speciation, extinction, and transition rate parameters were allowed to vary, to models with these parameters constrained by performing likelihood ratio tests in a maximum likelihood framework. The full model provided a significantly better fit than any of the constrained model and was used for subsequent analyses. To account for missing taxa, sampling frequencies were adjusted based on the proportion of taxa from each character state present in the tree. There were 118 viviparous species (out of ~333) for which matrotrophy indices were missing. I accounted for these missing data by adjusting the sampling frequencies of viviparous lecithotrophs and viviparous matrotrophs to match three scenarios – 1. The frequencies of the unsampled taxa were consistent with the sampled taxa, 2. The unsampled taxa were assigned whichever state was more common in their genus, 3. The unsampled taxa were assigned the same character state as their closest relative for which there was data. The three scenarios yielded nearly identical results (~50% matrotrophs

and ~50% lecithotrophs), thus I retained the sampling frequencies from scenario 1 in the final analysis. Maximum likelihood parameter estimates were calculated and used as starting values for MCMC estimates of optimal parameter values. The MCMC chain was run for 100,000 generations using the full model and an exponential prior 1/(2r), where r is the character independent diversification rate, with 10% removed as burn-in. MCMC samples were summarized to assess variation in state-dependent speciation, extinction and net diversification rates. Statistical significance of differences in state-dependent speciation, extinction and net diversification rates was determined by comparing the credible intervals of differences among posterior distributions.

Character state-specific rates of speciation, extinction, and evolutionary transitions were independently estimated in RevBayes v1.0.3 (Höhna et al. 2016). Missing taxa were accounted for by defining rho as the proportion of included taxa, and species without matrotrophy indices were scored as missing in the character state file. A birth death prior was used with the mean diversification prior defined as one half of total the number of species in Cyprinodontiformes. Default priors were used for all other estimated parameters. The MCMC chain was run for 100,000 generations sampling every 10 generations, with a burn-in of 5,000 generations and a tuning interval of 200 generations. Statistically significant differences were defined as a lack of overlap in 95% credibility intervals of the posterior probabilities of state-specific parameter estimates.

Results

Hybridization Analysis

There were significant differences in the rate of post-zygotic reproductive isolation evolution among the three reproductive modes tested (genetic distance x reproductive mode interaction - Table 3.1 (top); Figure 3.1). Post-zygotic reproductive isolation evolved significantly faster among viviparous species, either with or without matrotrophy, than oviparous species (genetic distance x reproductive mode interaction – Table 3.1 (middle); Figure S3.1). The slopes of the genetic distance x reproductive mode regressions do not differ between the two forms of viviparous reproduction (genetic distance x reproductive mode interaction – Table 3.1 (bottom)), making it meaningful to compare their intercepts. Independent of genetic distance, the level of interspecific post-zygotic isolation between matrotrophic species was not significantly higher than between lecithotrophs (reproductive mode – Figure 3.1, Table 1 (bottom)).

Table 3.1. Results of generalized linear models testing the effects of genetic distance, mode of reproduction, and the interaction term on the level of post-zygotic reproductive isolation estimated from interspecific reciprocal hybrid crosses.

Oviparity, Lecithotrophic Viviparity, Matrotrophic Viviparity			
	df	χ^2	P
Genetic Distance	1	0.626	0.429
Reproductive Mode	1	3.558	0.059
Genetic Distance x Reproductive Mode	**1**	**7.247**	**0.007**
Oviparity vs. Viviparity			
	df	χ^2	P
Genetic Distance	1	2.8	0.094
Reproductive Mode	**1**	**5.932**	**0.015**
Genetic Distance x Reproductive Mode	**1**	**8.474**	**0.0036**
Lecithotrophy vs. Matrotrophy			
	df	χ^2	P
Genetic Distance	**1**	**12.445**	**<0.0004**
Reproductive Mode	1	0.432	0.511
Genetic Distance x Reproductive Mode	1	2.949	0.086

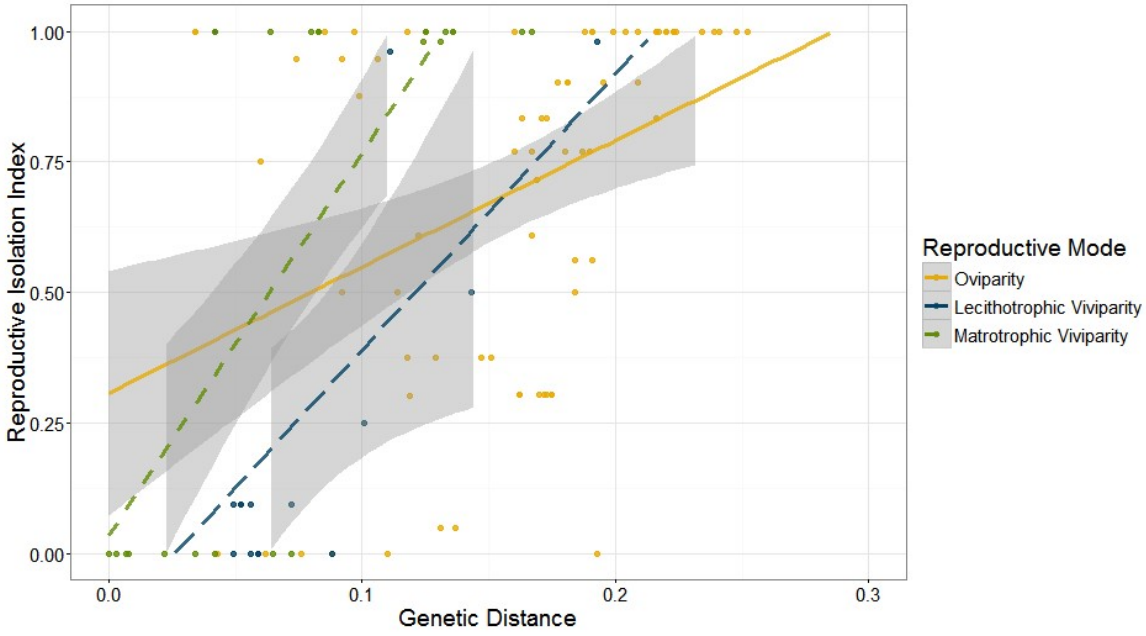

Figure 3.1. Levels of post-zygotic reproductive isolation from interspecific crosses modeled as a function of genetic distance and reproductive mode of the species in Cyprinodontiformes. Lines and points are based on reproductive mode.

Diversification Analysis

The tree was generally consistent with recently published phylogenies of Cyprinodontiformes and the clades contained within (Pollux et al. 2014; Furness et al. 2015b; Helmstetter et al. 2016). All of the currently accepted families of Cyprinodontiformes were monophyletic with exception of the Poeciliidae and Cyprinodontidae (Figure 3.2). Oviparity was reconstructed as the ancestral state for the entire Order, with lecithotrophy preceding the evolution of matrotrophy (Figure 3.3). Viviparity and matrotrophy both have multiple independent origins within the Order (Figure 3.3).

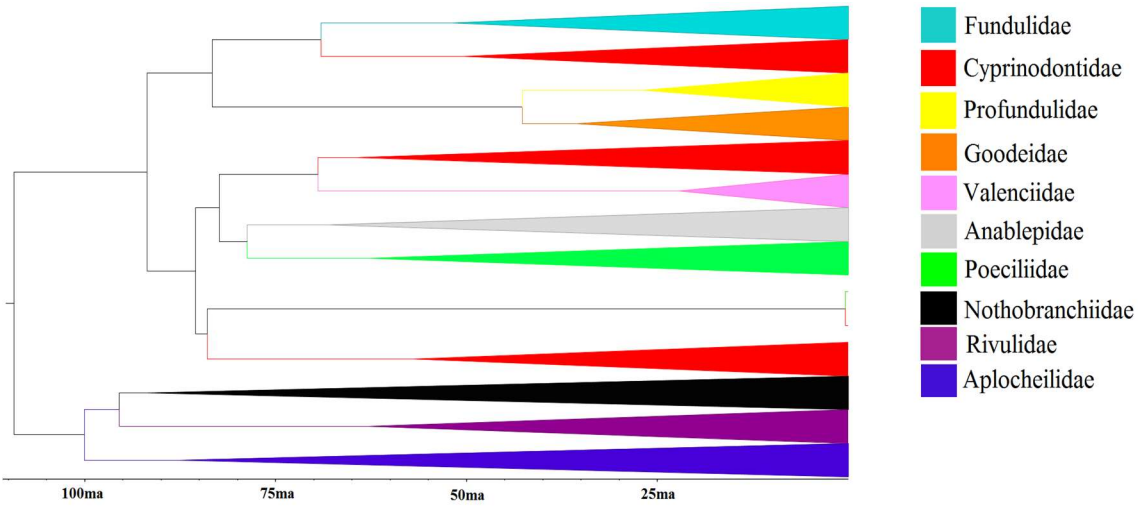

Figure 3.2. A Bayesian maximum clade credibility tree with clades collapsed to show the relationships among the currently accepted Families of Cyprinodontiformes (the width of the clades is standardized and independent of the number of species within that group).

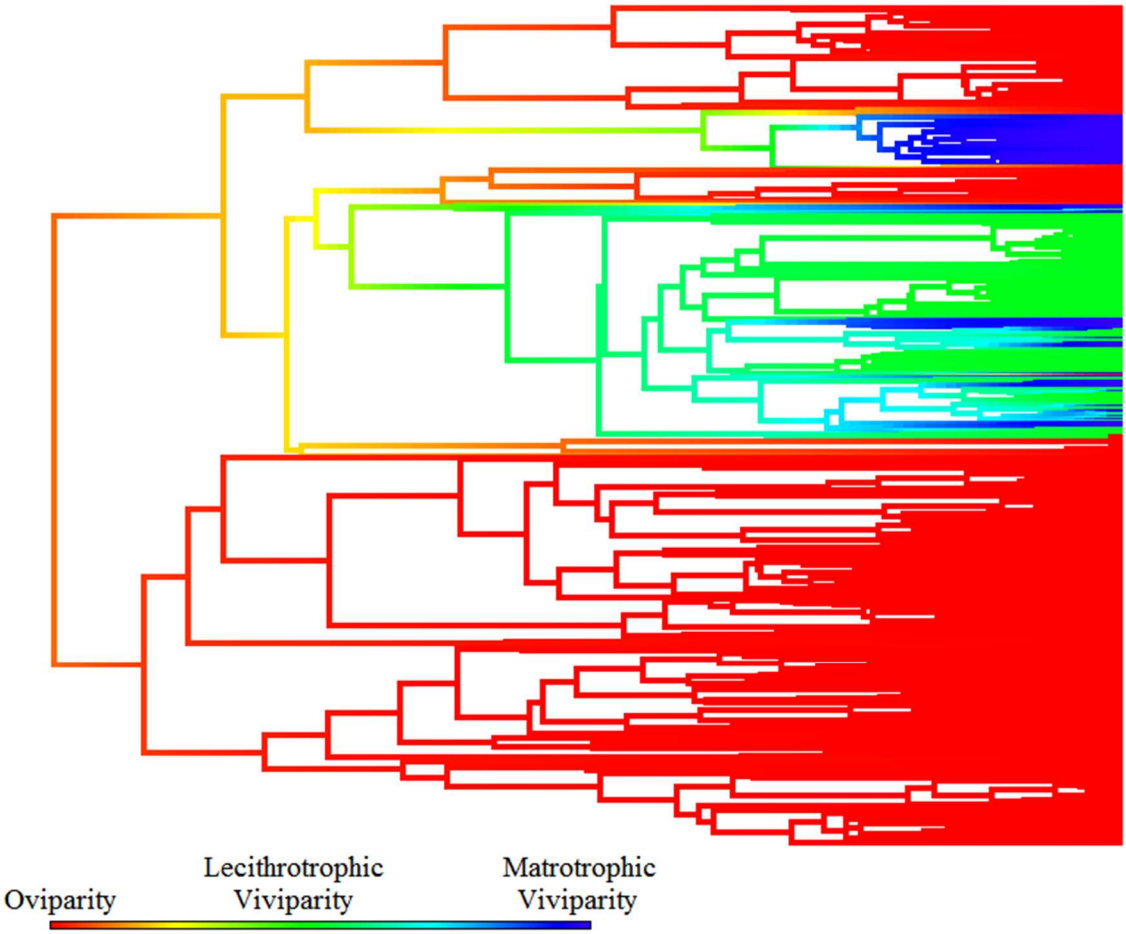

Figure 3.3. Ancestral state reconstruction performed on a Bayesian maximum clade credibility tree of 647 species of Cyprinodontiformes. The killifish are shown in red, with the livebearing groups represented as both blue and green.

The effect of reproductive mode on speciation, extinction, and diversification rates varied depending on the method used to analyze the data. Under the MuSSE model, speciation, extinction, and net diversification did not significantly differ among the three character states (Figure 3.4a). When estimated in RevBayes, speciation and extinction rates were significantly higher in for lecithotrophic viviparity than for oviparity, but did not significantly differ between lecithotrophic viviparity and matrotrophic viviparity or between matrotrophic viviparity and oviparity (Figure 3.4b). Diversification rates estimated in Revbayes were not significantly different among any of the character states (Figure 3.4b). When lecithotrophic and matrotrophic species were pooled as viviparous taxa, then viviparous taxa had a significantly higher rate of diversification than oviparous taxa under a BiSSE model of evolution (Figure S3.1a). Under the same BiSSE model, speciation and extinction rates did not significantly differ between oviparous and viviparous taxa (Figure S3.1a). In RevBayes, speciation and extinction rates were significantly higher for viviparous taxa than oviparous taxa, but the rate of diversification was only marginally higher for viviparity (Figure S3.1b). Lecithotrophy was not significantly different from matrotrophy in any comparison by either statistical model, however, they came closest to differing in speciation rate under the Revbayes model. In this case, Lecithotrophic lineages tend to have higher speciation rates that matrotrophic lineages.

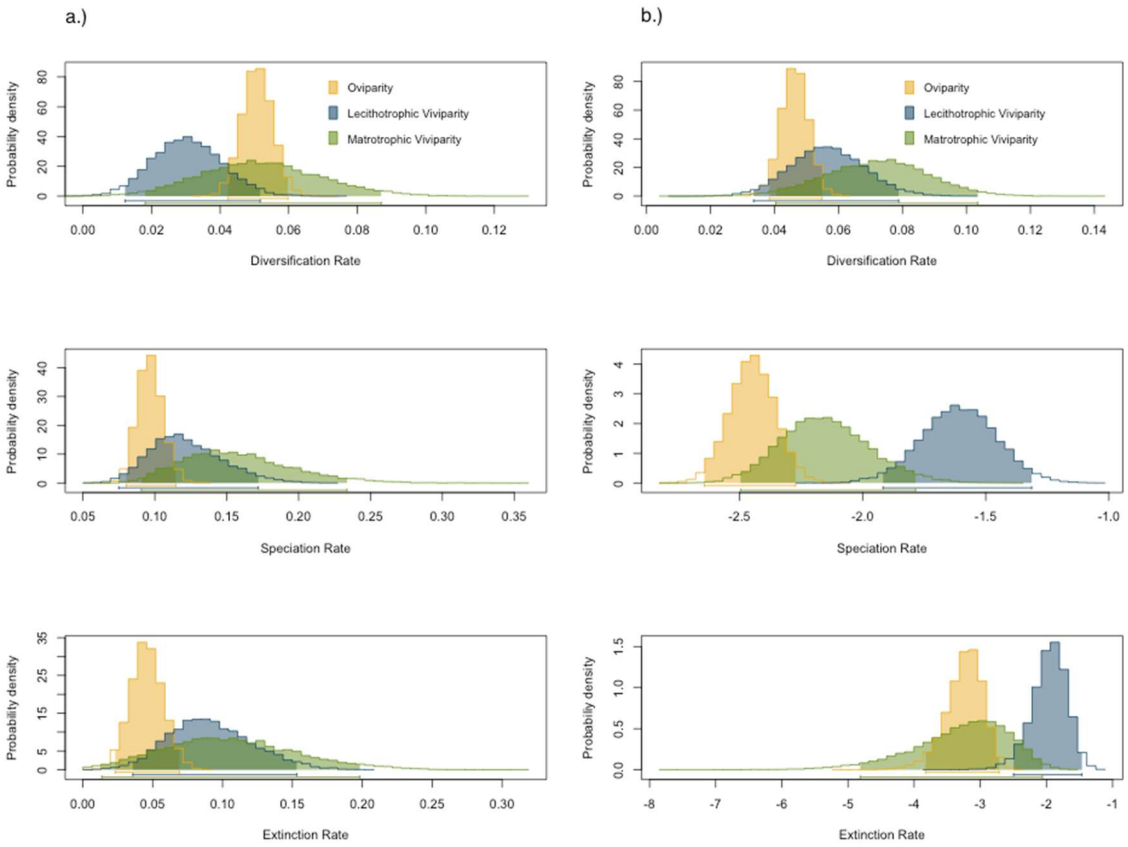

Figure 3.4. Posterior distributions for rates of speciation diversification (top), speciation (middle), and extinction (bottom) estimated with (a) MuSSE and (b) RevBayes, colored by character. 95% credibility intervals for state-specific parameter estimates are shown below each distribution.

Discussion

Theory suggests that intergenomic conflicts between mothers and offspring are driving the evolution of reproductive mode (Crespi and Semeniuk 2004), and that these conflicts will lead to differences in how likely post-zygotic reproductive isolation evolves among taxa that differ in their mode of reproduction (Zeh and Zeh 2000; Zeh and Zeh 2008). Consistent with these predictions I found post-zygotic reproductive isolation evolved significantly faster among viviparous species relative to oviparous species. The

rate that post-zygotic reproductive isolation evolves did not differ between matrotrophs and lecithotrophs, however the level of post-zygotic isolation was higher for matrotrophs at all levels of genetic divergence. My results support the viviparity-driven-conflict hypothesis, as the evolution of viviparity in Cyprinodontiformes is associated with an acceleration in the rate that post-zygotic reproductive barriers evolve among lineages. My result also corroborates previous work that found the age of common ancestors was much older among successfully hybridizing oviparous species than among successfully hybridizing viviparous species (Coleman et al. 2009). It thus appears that the conflict that arises from the evolution of viviparity has an impact on the process of speciation.

The effect matrotrophy has on reproductive isolation is less clear. Against predictions, matrotrophy did not accelerate the evolution of post-zygotic reproductive barriers relative to lecithotrophy. The levels of post-zygotic isolation predicted for matrotrophs is higher than the levels predicted among lecithotrophs, but these differences were not statistically significant (Table 3.1). The direction of the observed differences between lecithotrophs and matrotrophs were consistent with predictions, but reproductive mode does not appear to have a significant impact on the evolution of post-zygotic isolation in viviparous species. The comparison between the two livebearing groups is based on a smaller dataset than the comparison between egg-layers and livebearers, and the inclusion of more cross data from livebearing species would help to clarify if the directional trends consistent with our hypothesis are due to chance sampling or represent a biological differences in the evolution of reproductive barriers among the two groups.

Speciation is the product of both pre- and post-zygotic isolating mechanisms. My hybridization analysis was restricted to measures of post-zygotic isolation. Therefore the impact that reproductive mode has on speciation is incumbent upon the relative importance of pre- and post-zygotic barriers on speciation in Cyprinodontiformes. If speciation is largely driven by the evolution of post-zygotic isolation, I would expect speciation rates to be significantly higher in viviparous taxa relative to oviparous taxa. Here I can test these predictions by comparing the results of the hybridization analysis with the findings of my comparative phylogenetic work.

Evolutionary Diversification

The variation in speciation and diversification rates I observed among the different modes of reproduction matched the results of my hybridization analysis in some cases, but not in others. The MuSSE model produced similar parameter values for all three modes of reproduction, and fails to support my predictions. When species were pooled into oviparous and viviparous taxa, however, the BiSSE model found diversification rates to be higher for viviparous taxa. A higher diversification rate for viviparous taxa could potentially corroborate the predictions of the viviparity-driven-conflict hypothesis since the net result is a higher rate of increasing species richness per unit time, however this effect was not driven by differences in the estimated speciation rates (Figure S3.2a). Moreover, the VDCH explicitly predicts that speciation rates will be elevated in viviparous taxa, and makes no predictions with regard to extinction rate variation in association with reproductive mode (Zeh and Zeh 2000; Zeh and Zeh 2008).

The results of the RevBayes analysis fit the predictions of the VDCH, and fit my findings from the hybridization study. Speciation rates were significantly higher for viviparous lecithotrophs than for oviparous taxa, but did not significantly differ among the other comparisons (Figure 3.4b). When species were pooled into oviparous and viviparous taxa, viviparous species had significantly higher speciation rates than oviparous species (Figure S3.2b). The RevBayes analysis strongly suggests that viviparity causes an increase in speciation rate that is potentially slowed by the subsequent evolution of matrotrophy. Increased rates of speciation in viviparous taxa has been documented in multiple groups, including lizards (Lambert and Wiens 2013), Vipers (Lynch 2009), and Cyprinodontiform fishes (Helmstetter et al. 2016). A decrease in speciation rates associated placentation, a form of matrotrophy, has been observed in the fish family Poeciliidae (Meredith et al. unpub). Moreover, there is strong evidence that the evolution of matrotrophy causes a reduction in pre-mating sexual selection (Pollux et al. 2014). Given that pre-zygotic barriers dominate speciation in other systems (Coyne and Orr 2004), it is possible that the decrease in speciation rate observed here is driven by the shift to matrotrophy and loss of pre-mating sexually selected traits. Nonetheless, the differences I observed in speciation rate among lecithotrophs and matrotrophs were not significantly different from one another, and further sampling is required to determine if the marginal differences were due to a real effect or lack of statistical power.

Given that reproductive isolation is a key component in the process of speciation, I predicted that speciation rates estimated from phylogenies would mirror the variation I

observed in rates of post-zygotic reproductive isolation evolution. However, this was only partially the case. In Cyprinodontiformes, viviparity leads to accelerated rates of post-zygotic isolation evolution and increased rates of speciation or diversification at macroevolutionary scales. Both patterns provide strong support for the predictions of the viviparity-driven-conflict hypothesis. Matrotrophy was associated with a positive but non-significant effect in the rate of post-zygotic isolation evolution, but was associated with a marginal decrease in speciation rates. Matrotrophy may decrease speciation rates as a consequence of weakened pre-zygotic reproductive isolation, but further work is required to determine the reality of this pattern.

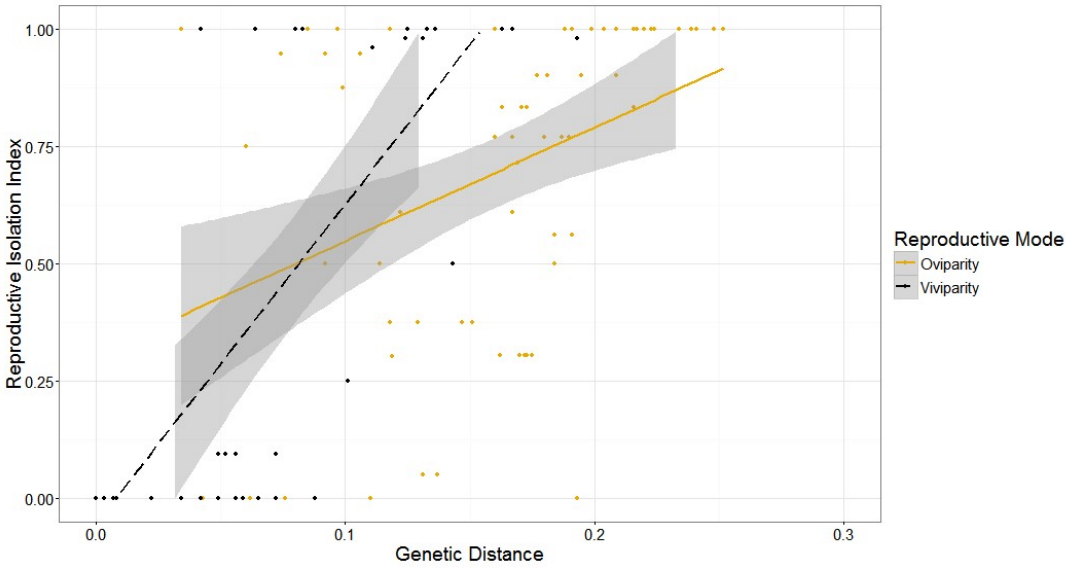

Figure S3.1. Levels of post-zygotic reproductive isolation from interspecific crosses modeled as a function of genetic distance and reproductive mode of the species. Cyprinodontiformes. Lines and points are based on reproductive mode.

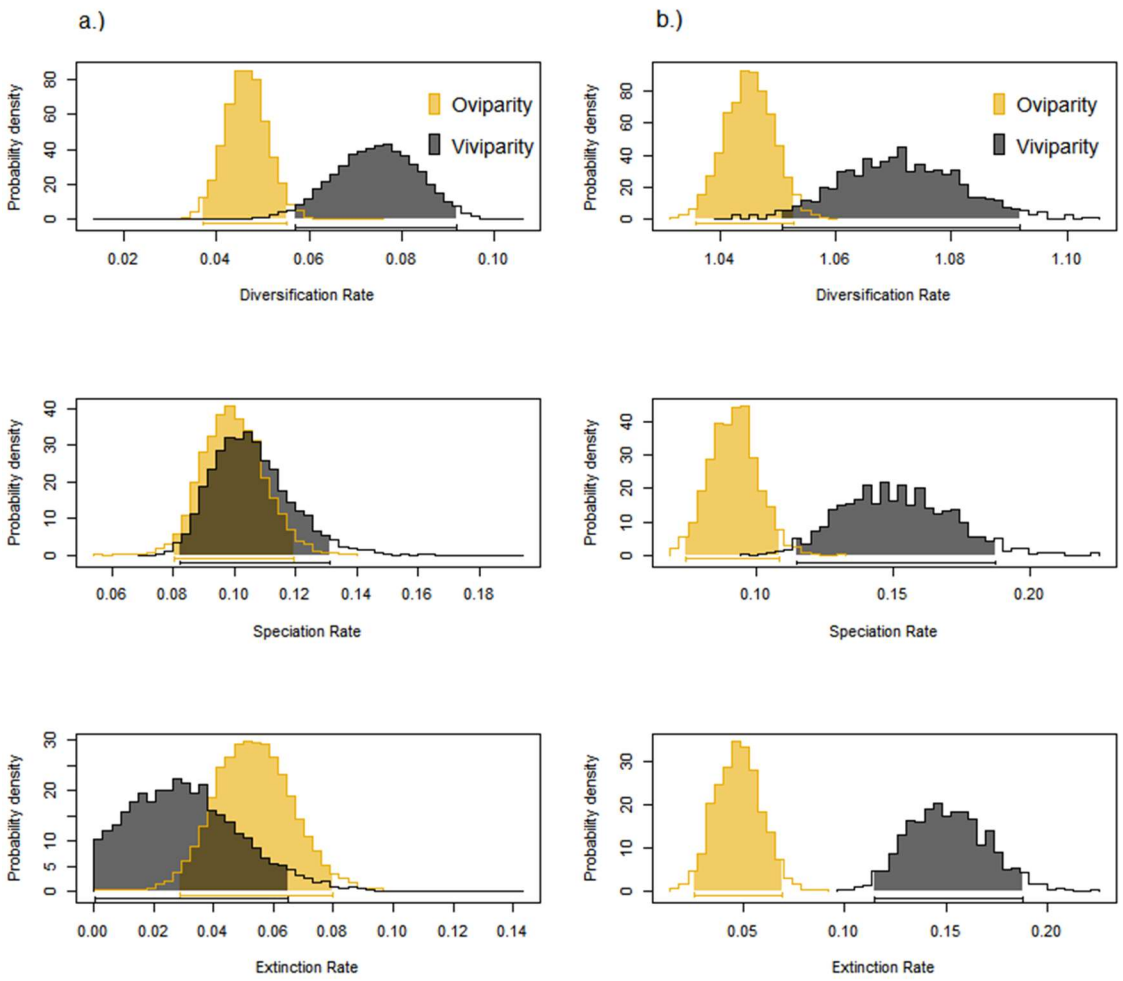

Figure S3.2. Posterior distributions for rates of speciation diversification (top), speciation (middle), and extinction (bottom) estimated with (a) BiSSE and (b) RevBayes, colored by character. 95% credibility intervals for state-specific parameter estimates are shown below each distribution.

Table S3.1. Table of accession numbers for genes used to construct tree

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Adinia xenica	O		KF929573.1				GQ119680	GQ119858	
Alfaro cultratus	L				EF017480	U80048	EF017531	EF017429	
Alfaro huberi	?								
Allodontichthys hubbsi	M		AY356553.1				AF510836		
Allodontichthys polylepis	M		AY356555.1				AF510839		
Allodontichthys tamazulae	M		AY356556.1				AF510838		
Allodontichthys zonistius	M		AY356558.1				AF510840		
Alloophorus robustus	M		AY356561.1				AF510813		
Allotoca catarinae	M		AY356562.1				AF510793		
Allotoca diazi	M		AY356554.1				AF510790		
Allotoca dugesii	?		AY356557.1				AF510801		
Allotoca goslinei	M		AY356559.1				AF510800		
Allotoca maculata	M		AY356560.1				AF510797		
Allotoca meeki	M						AF510791		
Allotoca regalis	?		AY356563.1				AF510799		
Allotoca zacapuensis	?						AF510789		
Ameca splendens	M		AY356564.1				AF510818		
Anableps anableps	M				EF017456		EF017508	EF017405	EU637935.1
Anableps dowei	M								
Anableps microlepis	?								
Anablepsoides amanan	O								
Anablepsoides amphoreus	O		AF002618.1		U41795.1	AF002550	U41777.1		
Anablepsoides atratus	O		AF002600.1		AF002431.1	AF002535	AF002481		
Anablepsoides bahianus	O								
Anablepsoides beniensis	O								
Anablepsoides bondi	O								
Anablepsoides cajariensis	O								
Anablepsoides caurae	O								
Anablepsoides cearensis	O								
Anablepsoides christinae	O								
Anablepsoides cryptocallus	O	AF092394.1			U41794.1	AF092327.1	U41776.1		
Anablepsoides deltaphilus	O	AF092395.1	AF002616.1		AF002444.1	AF002548	AF002494.1		
Anablepsoides derhami	O								
Anablepsoides elongatus	O								
Anablepsoides erberi	O								
Anablepsoides gaucheri	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Anablepsoides hartii	O		HQ405516.1		U41796.1	AF002551	HQ612227		KC702079.1
Anablepsoides holmiae	O								
Anablepsoides igneus	O								
Anablepsoides immaculatus	O		AF002620.1		U41797.1	AF002552	U41779		
Anablepsoides intermittens	O								
Anablepsoides iridescens	O	AF092391.1			AF092324.1	AF092324.1			KC702080.1
Anablepsoides jucundus	O	AF092392.1	AF002612.1		AF002441.1	AF002545	AF002491		
Anablepsoides lanceolatus	O								
Anablepsoides limoncochae	O								
Anablepsoides lungi	O								
Anablepsoides mazaruni	O								
Anablepsoides micropus	O								
Anablepsoides monticola	O								
Anablepsoides ophiomimus	O	AF092399.1	AF002613.1		AF002442.1	AF002546.1	AF002492		KC702085.1
Anablepsoides ornatus	O								
Anablepsoides parlettei	O								
Anablepsoides peruanus	O								
Anablepsoides rubrolineatus	O		AF002614.1		AF002443.1	AF002547	AF002493		
Anablepsoides speciosus	O								
Anablepsoides stagnatus	O	AF092398.1	AF002615.1		U41793.1	U73255	U41774.1		
Anablepsoides taeniatus	O								
Anablepsoides tessellatus	O								
Anablepsoides tocantinensis	O								
Anablepsoides urophthalmus	O	AY946273.1	AY946273.1		AY946278.1	AY946278.1			
Anablepsoides waimacui	O	AF092397.1			AF092330.1	AF092330.1			
Anablepsoides xanthonotus	O								
Anablepsoides xinguensis	O								
Aphanius almiriensis	O		KJ552735.1						
Aphanius anatoliae	O	AF451648.1	KJ552704.1		AF451681	AF451648.1			
Aphanius apodus	O	AF449323.1			AF449385.1	AF449323.1			
Aphanius arakensis	O								
Aphanius asquamatus	O	AF449306.1			AF449368	U05976	U06190		
Aphanius baeticus	O								
Aphanius burdurensis	O								
Aphanius chantrei	O					U05979	U06193		
Aphanius danfordii	O	AF449302.1			AF451693	AF449302.1			
Aphanius desioi	O								
Aphanius dispar	O	AF449334.1			KF983853.1	U05964.1			

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Aphanius dispar richardsoni	O	AF449329.1			AF449391	AF449329.1			
Aphanius farsicus	O	AY593484.1			AY593493.1	AY593484.1	KF559225.1		
Aphanius fasciatus	O	AF449313.1			AF449371	AF299273.1	AF299273		
Aphanius ginaonis	O	AF449335.1			AF449397	AF449335.1			
Aphanius iberus	O	AF449322.1	KJ552729.1		AF449380	AF449322.1	DQ367529.1		
Aphanius isfahanensis	O	AY593489.1	AY593489.1		AY593498	AY593489.1	JN565969.1		
Aphanius mento	O	AF449327.1			AF449388	U05967.1	U06181.1		
Aphanius mesopotamicus	O						JN565968.1		
Aphanius pluristriatus	O						KJ196330.1		
Aphanius punctatus	O								
Aphanius saourensis	O						DQ367527		
Aphanius sirhani	O	AF449328.1			AF449390	AF449328.1			
Aphanius sophiae	O	AF449314.1			AY593491	AF449314.1	KF559221.1		
Aphanius splendens	O	AF449287.1			AF449349.1	AF449287.1			
Aphanius stiassnyae	O								
Aphanius sureyanus	O	AF449288.1	KJ552758.1		AF449350.1	AF449288.1			
Aphanius transgrediens	O	AF449290.1	KJ552710.1		AF449351.1	AF449290.1			
Aphanius villwocki	O	AF449305.1			AF449367.1	AF449305.1			
Aphanius vladykovi	O	AF449315.1			AY593496.1	AF449315.1	DQ367526.1		
Aphyolebias boticarioi	O								
Aphyolebias claudiae	O								
Aphyolebias manuensis	O								
Aphyolebias obliquus	O								
Aphyolebias peruensis	O	AF092407.1	AF002638.1		AF002456.1	AF002569	AF002506	EF455718	
Aphyolebias rubrocaudatus	O								
Aphyolebias schleseri	O								
Aphyolebias wischmanni	O								
Aphyosemion abacinum	O								
Aphyosemion ahli	O		EF417014.1		AF002366.1		KC893931.1		KC702032.1
Aphyosemion alpha	O		DQ267387.1		DQ278418.1		KC893920.1		
Aphyosemion amoenum	O						KC893932.1		
Aphyosemion aureum	O				AF002385.1		AF002317		
Aphyosemion australe	O		EF417019.1		AF002367.1	U73245.1	EU272816.1		KC702035.1
Aphyosemion bamilekorum	O						DQ981778		
Aphyosemion batesii	O				AF002350.1		AF002286		
Aphyosemion bitaeniatum	O		DQ267390.1		DQ278280.1		KC893922.1		
Aphyosemion bivittatum	O		AF002576.1		AF002373.1	U73246	DQ522260		KC702033.1
Aphyosemion bualanum	O				AF002370.1		AF002303.1		

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Aphyosemion buytaerti	O								
Aphyosemion callipteron	O								
Aphyosemion calliurum	O		EF417033.1		AF002368.1		EU272797		
Aphyosemion cameronense	O				AF002382.1		KC893933.1		
Aphyosemion campomaanense	O						EU272811.1		
Aphyosemion castaneum	O						JF307803.1		
Aphyosemion caudofasciatum	O								
Aphyosemion celiae	O		EF417037.1		AF002369.1		EU885234		
Aphyosemion chauchei	O						JF307796.1		
Aphyosemion christyi	O				AF002390.1		AF002322		
Aphyosemion citrineipinnis	O				AF002386.1		AF002318		
Aphyosemion coeleste	O				AF002387.1		AF002319		
Aphyosemion cognatum	O				AF002392.1		JF307794.1		
Aphyosemion congicum	O						JF307798.1		
Aphyosemion cyanostictum	O				AF002372.1		AF002305.1		
Aphyosemion dargei	O								
Aphyosemion decorsei	O				AF002393.1		AF002325		
Aphyosemion ecucuense	O						KC893925.1		
Aphyosemion edeanum	O		EF417039.1		EU282850.1		EU272815.1		
Aphyosemion elberti	O	AF092355.1			AF092288	AF092288.1	KC893929.1		KC702036.1
Aphyosemion elegans	O				AF002396.1		JF307792.1		
Aphyosemion erythron	O						KC893926.1		
Aphyosemion escherichi	O								
Aphyosemion etsamense	O						AY748295		
Aphyosemion exigoideum	O				AF002376.1		AF002308		KC702037.1
Aphyosemion exiguum	O				AF002371.1		KC893928.1		
Aphyosemion ferranti	O								
Aphyosemion franzwerneri	O		EF417044.1				KC893930.1		
Aphyosemion fulgens	O								
Aphyosemion gabunense	O								
Aphyosemion gabunense boehmi	O				AF002377.1		AF002309.1		
Aphyosemion gabunense gabunense	O								
Aphyosemion gabunense marginatum	O								
Aphyosemion georgiae	O								
Aphyosemion grelli	O								
Aphyosemion hanneloreae	O								
Aphyosemion heinemanni	O		EF417045.1		EF417248.1		EU885236		
Aphyosemion hera	O				DQ286834.1				

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Aphyosemion herzogi	O						EU885235		
Aphyosemion hofmanni	O								KC702038.1
Aphyosemion joergenscheeli	O								
Aphyosemion kouamense	O		EF063366.1		DQ278417.1		KC893921.1		
Aphyosemion koungueense	O						KC893919.1		
Aphyosemion labarrei	O				AF002389.1		AF002321.1		
Aphyosemion lamberti	O				AF002397.1		JF307781.1		
Aphyosemion lefiniense	O								
Aphyosemion lividum	O		EU282845.1		EU282847.1				
Aphyosemion loennbergii	O		DQ267417.1		DQ278363.1		KC893884.1		
Aphyosemion louessense	O				AF002378.1		AF002310		KC702039.1
Aphyosemion lugens	O		DQ267401.1		DQ278413.1		KC893894.1		
Aphyosemion lujae	O								
Aphyosemion maculatum	O				AF002383.1		AF002315		
Aphyosemion malumbresi	O		EF063372.1		EF063381.1		KC893927.1		
Aphyosemion melanogaster	O		DQ267367.1		DQ278379.1		KC893892.1		
Aphyosemion melinoeides	O						KC893910.1		
Aphyosemion mimbon	O				AF002384.1		AY748288.1		
Aphyosemion musafirii	O						JF307804.1		
Aphyosemion ocellatum	O				AF002388.1		AF002320.1		KC702034.1
Aphyosemion ogoense	O				AF002379.1		AF002311		KC702040.1
Aphyosemion omega	O						KC893898.1		
Aphyosemion pamaense	O								
Aphyosemion pascheni	O		EF417046.1						
Aphyosemion pascheni festivum	O		EF417041.1		EU282841.1				
Aphyosemion passaroi	O								
Aphyosemion plagitaenium	O								
Aphyosemion poliaki	O		DQ267406.1		DQ278277.1		KC893913.1		
Aphyosemion polli	O						JF307801.1		
Aphyosemion primigenium	O				AF002380.1		AF002312		KC702041.1
Aphyosemion pseudoelegans	O								
Aphyosemion punctatum	O				AF002400.1		AF002332		
Aphyosemion punctulatum	O		DQ267363.1		DQ278400.1		KC893907.1		
Aphyosemion raddai	O								
Aphyosemion rectogoense	O				AF002399.1		JF307799.1		
Aphyosemion riggenbachi	O		DQ267412.1		DQ278291.1		KC893889.1		
Aphyosemion schioetzi	O								
Aphyosemion schluppi	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Aphyosemion schoutedeni	O								
Aphyosemion seegersi	O								
Aphyosemion splendopleure	O		DQ267416.1		DQ278388.1		KC893924.1		
Aphyosemion striatum	O				AF002381.1		AF002313		
Aphyosemion teugelsi	O								
Aphyosemion thysi	O								
Aphyosemion tirbaki	O								
Aphyosemion trilineatus	O								
Aphyosemion volcanum	O		DQ267415.1		DQ278416.1		KC893879.1		
Aphyosemion wachtersi	O								
Aphyosemion wildekampi	O				AF002401.1		AF002333.1		
Aphyosemion wuendschi	O								
Aphyosemion zygaima	O								
Aplocheilichthys antinorii	O								
Aplocheilichthys atripinna	O								
Aplocheilichthys brichardi	O								
Aplocheilichthys bukobanus	O								
Aplocheilichthys centralis	O								
Aplocheilichthys fuelleborni	O								
Aplocheilichthys hutereaui	O		AY356594.1						
Aplocheilichthys jeanneli	O								
Aplocheilichthys johnstoni	O								
Aplocheilichthys katangae	O								
Aplocheilichthys kingii	O								
Aplocheilichthys kongoranensis	O								
Aplocheilichthys lacustris	O								
Aplocheilichthys lualabaensis	O								
Aplocheilichthys macrurus	O								
Aplocheilichthys mahagiensis	O								
Aplocheilichthys meyburghi	O								
Aplocheilichthys moeruensis	O								
Aplocheilichthys myaposae	O								
Aplocheilichthys myersi	O								
Aplocheilichthys pumilus	O								
Aplocheilichthys rudolfianus	O								
Aplocheilichthys spilauchen	O								
Aplocheilichthys vitschumbaensis	O								
Aplocheilus blockii	O					EF591764			

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Aplocheilus dayi	O								
Aplocheilus kirchmayeri	O								
Aplocheilus lineatus	O				U73260.1	U73235	U73282		KC702042.1
Aplocheilus panchax	O	EF455702.1			EF455699	U73236	U73283	EF455705	
Aplocheilus parvus	O					EF591765			
Aplocheilus werneri	O								
Archiaphyosemion guineense	O					FJ872031	AF000711		
Ataeniobius toweri	M		JQ935854.1				AF510779		
Atherinomorus lacunosus	O	KJ667868.1	JF492911.1	AY655680.1		GQ485307	GU932753	JX189786.1	
Atherinops affinis	O					EF458402	FJ264404		
Atlantirivulus depressus	O								
Atlantirivulus haraldsiolii	O	AF092379.1			AF092312.1	AF092312.1			KC702078.1
Atlantirivulus janeiroensis	O		AF002632.1		AF002450.1	AF002563	AF002500		
Atlantirivulus jurubatibensis	O								
Atlantirivulus lazzarotoi	O								
Atlantirivulus luelingi	O	AF092381.1	AF002633.1		AF002451.1	AF002564	AF002501		
Atlantirivulus nudiventris	O								
Atlantirivulus riograndensis	O								
Atlantirivulus santensis	O	AF092380.1	GU701925.1		AF002452.1	AF002565	AF002502	EF455708	KC702088.1
Atlantirivulus simplicis	O								
Atlantirivulus unaensis	O								
Austrofundulus guajira	O	AY850649.1	AY850649.1		AY850672.1	AY850672.1			
Austrofundulus leohoignei	O	AY850656.1	AY850656.1		AY850679.1	AY850679.1			KC702044.1
Austrofundulus leoni	O	AY850654.1	AY850654.1		AY850674.1	AY850674.1			
Austrofundulus limnaeus	O	AY850648.1	AF002589.1		U73278.1	U73254.1	U73300.1		KC702045.1
Austrofundulus myersi	O	AY850643.1							
Austrofundulus rupununi	O	AY850657.1	AY850657.1		AY850681.1	AY850681.1			
Austrofundulus transilis	O	AY850663.1	AY850663.1		AF002419.1	AF002521.1	AF002469.1	EF455715.1	KC702046.1
Austrolebias adloffi	O				AF244413.1	AF244443.1	AY724376.1		KC702048.1
Austrolebias affinis	O		AF002579.1		AF244416.1	AF244437.1	AF245464.1		
Austrolebias alexandri	O	AF092369.1			AF092302.1	AF092302.1	AF245011.1		KC702049.1
Austrolebias apaii	O						HQ149647.1		
Austrolebias arachan	O								
Austrolebias bellottii	O	AF092370.1			AF243421.1	AF244442.1	AF245006.1		KC702050.1
Austrolebias carvalhoi	O								
Austrolebias charrua	O						AY724377.1		
Austrolebias cheradophilus	O				AF244424.1	AF244431.1	AF245467.1		
Austrolebias cinereus	O					AF244439.1	KF027479.1		

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Austrolebias cyaneus	O				AF244426.1	AF244445.1	AF245461.1		
Austrolebias duraznensis	O				AF244414.1	AF244433.1	KF192624.1		
Austrolebias elongatus	O		JX111789.1				FJ826892.1		
Austrolebias gymnoventris	O				AF244418.1	AF244438.1	KF027480.1		
Austrolebias ibicuiensis	O								
Austrolebias jaegari	O								
Austrolebias juanlangi	O								
Austrolebias litzi	O								
Austrolebias luteoflammulatus	O					AF244444.1	KF027482.1		
Austrolebias melanoorus	O				AF244422.1		AY724373.1		
Austrolebias minuano	O								
Austrolebias monstrosus	O						FJ826895.1		
Austrolebias nachtigalli	O								
Austrolebias nigripinnis	O				AF244415.1	AF244432.1	AF245013.1		
Austrolebias nigrofasciatus	O						AY724407.1		
Austrolebias nioni	O				AF244421.1	AF244435.1	AF245457.1		
Austrolebias nonoiuliensis	O						FJ826896.1		
Austrolebias paranaensis	O								
Austrolebias patriciae	O						FJ826897.1		
Austrolebias paucisquama	O								
Austrolebias periodicus	O						FJ826898.1		
Austrolebias prognathus	O				AF244425.1		KF027486.1		
Austrolebias quirogai	O						KF027488.1		
Austrolebias reicherti	O						AY724392.1		
Austrolebias robustus	O						FJ826900.1		
Austrolebias toba	O								
Austrolebias univentripinnis	O								
Austrolebias vandenbergi	O						FJ826901.1		
Austrolebias varzeae	O								
Austrolebias vazferreirai	O						AF245015.1		
Austrolebias viarius	O				AF243423.1	AF244441.1	AY724386.1		
Austrolebias wolterstorffi	O				AF244419.1	AF244434.1	AF245014.1		
Belonesox belizanus	L	HM443919.1	JQ840428.1		EF017467	JQ612957.1	JX556410.1	EF017416	
Brachyrhaphis cascajalensis	L						FJ178767		
Brachyrhaphis episcopi	L						U68306		
Brachyrhaphis hartwegi	?	EF017571.1			EF017469	EF017571.1	EF017521	EF017418	
Brachyrhaphis hessfeldi	?								
Brachyrhaphis holdridgei	L						U68308		

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Brachyrhaphis olomina	?								
Brachyrhaphis parismina	L						FJ178768		
Brachyrhaphis punctifer	?						U68312		
Brachyrhaphis rhabdophora	L	EF017572.1			EF017470	EF017572.1	KJ081609.1	EF017419	
Brachyrhaphis roseni	L						KJ081590.1		
Brachyrhaphis roswithae	?								
Brachyrhaphis terrabensis	L	EF017570.1			EF017468	EF017570.1	KJ081607.1	EF017417	
Callopanchax huwaldi	O				AF000676.1		AF000698.1		KC702092.1
Callopanchax monroviae	O					GU553013			
Callopanchax occidentalis	O			FJ872048.1	AF092293	FJ872034.1	AF000695		KC702092.1
Callopanchax sidibei	O					GU553012			
Callopanchax toddi	O				AF000677.1	GU553010	AF000699.1		
Campellolebias brucei	O								KC702047.1
Campellolebias chrysolineatus	O				AF002413.1	AF002515.1	AF002464.1		
Campellolebias dorsimaculatus	O	AF092362.1	AF002584.1		AF002414.1	AF002516.1	AF002465.1		
Campellolebias intermedius	O								
Carlhubbsia kidderi	?						FJ178778	FJ185091	
Carlhubbsia stuarti	L	EF017581.1			EF017481	EF017581.1	EF017532.1	EF017430.1	
Chapalichthys encaustus	M		AY356570.1				AF510816		
Chapalichthys pardalis	M		AY356567.1						
Chapalichthys peraticus	?								
Characodon audax	M		AY356568.1				AF510824		
Characodon garmani	?								
Characodon lateralis	M		AY356569.1				AF510820		
Chirostoma humboldtianum	O	EF602074.1				DQ370026	KC736402.1	JQ282071.1	
Chromis cyanea	O		JQ707136.1		AF285925	AF285947	AY208529	AY208639	
Cnesterodon brevirostratus	?								
Cnesterodon carnegiei	?								
Cnesterodon decemmaculatus	L	EF017579.1	JX111729.1		EF017478	EF017579.1	EF017529	EF017427	GU179271
Cnesterodon holopteros	?								
Cnesterodon hypselurus	?	GU179231.1					GU179185	GU179260	GU179272
Cnesterodon iguape	?								
Cnesterodon omorgmatos	?								
Cnesterodon pirai	?								
Cnesterodon raddai	?								
Cnesterodon septentrionalis	?								
Craterocephalus stercusmuscarum	O		HM006954.1		HQ615468.1		GU932877		
Crenichthys baileyi	O		AY356571.1				AF510819	FJ185089	

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Crenichthys nevadae	O						U09105.1		
Cualac tessellatus	O	AY902109.1				U05968.1	AY902051.1		
Cubanichthys cubensis	O								
Cubanichthys pengelleyi	O					U05971.1			
Cynodonichthys birkhahni	O		AF002625.1		AF002448.1	AF002557	AF002498		
Cynodonichthys boehlkei	O								
Cynodonichthys brunneus	O	AF092383.1			AF092316.1	AF092316.1			
Cynodonichthys chucunaque	O		AF002624.1		AF002447.1	AF002556.1	AF002497.1		
Cynodonichthys elegans	O								
Cynodonichthys frommi	O	AF092384.1			AF092317.1	AF092317.1			
Cynodonichthys fuscolineatus	O		AF002631.1		U41786.1	AF002562	U41770		
Cynodonichthys glaucus	O								
Cynodonichthys godmani	O								
Cynodonichthys hendrichsi	O								
Cynodonichthys hildebrandi	O		AF002621.1		U41791.1	AF002553.1	U44746.1		
Cynodonichthys isthmensis	O		AF002630.1		U41785.1	AF002561	U41769.1		
Cynodonichthys kuelpmanni	O								
Cynodonichthys leucurus	O								
Cynodonichthys magdalenae	O	AF092382.1	AF002623.1		U41790.1	AF002555	U41773.1		KC702081.1
Cynodonichthys monikae	O								
Cynodonichthys montium	O								
Cynodonichthys myersi	O								
Cynodonichthys pacificus	O								
Cynodonichthys rubripunctatus	O								
Cynodonichthys siegfriedi	O								
Cynodonichthys sucubti	O								
Cynodonichthys tenuis	O	AF092386.1	EU751964.1		U41789.1	AF002558	U41772.1		KC702089.1
Cynodonichthys uroflammeus	O		AF002622.1		U41792.1	AF002554	U41775		
Cynodonichthys villwocki	O								
Cynodonichthys wassmanni	O								
Cynodonichthys weberi	O	AF092385.1	AF002629.1		U41787.1	AF002560	U41768		
Cynolebias albipunctatus	O								
Cynolebias altus	O								
Cynolebias attenuatus	O								
Cynolebias gibbus	O								
Cynolebias gilbertoi	O								
Cynolebias griseus	O								
Cynolebias itapicuruensis	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Cynolebias leptocephalus	O								
Cynolebias microphthalmus	O								
Cynolebias paraguassuensis	O								
Cynolebias parnaibensis	O								
Cynolebias perforatus	O								
Cynolebias porosus	O								
Cynolebias vazabarrisensis	O								
Cynopoecilus fulgens	O								
Cynopoecilus intimus	O								
Cynopoecilus melanotaenia	O	AF092363.1			AF244427.1	AF092296.1	AF245465.1		
Cynopoecilus multipapillatus	O								
Cynopoecilus nigrovittatus	O								
Cyprinodon albivelis	O	AY902164.1					AY902106		
Cyprinodon alvarezi	O	AY902139.1					AY902081		
Cyprinodon arcuatus	O								
Cyprinodon artifrons	O	GQ181012.1	EU751788.1				AY902054	GQ180996.1	
Cyprinodon atrorus	O	AY902157.1					AY902099	EU086367.1	
Cyprinodon beltrani	O					AF215477.1			
Cyprinodon bifasciatus	O	AY902156.1					AY902098.1	EU086368.1	
Cyprinodon bobmilleri	O								
Cyprinodon bondi	O	DQ218103.1					AY902066.1		
Cyprinodon bovinus	O	AY902133.1					AY902075.1		
Cyprinodon brontotheroides	O								
Cyprinodon ceciliae	O								
Cyprinodon dearborni	O	GQ181018.1					AY902064	GQ180995.1	
Cyprinodon desquamator	O								
Cyprinodon diabolis	O	AF028309.1					U06184		
Cyprinodon elegans	O	AY902135.1					AY902077		
Cyprinodon eremus	O	AF198984.1							
Cyprinodon esconditus	O								
Cyprinodon eximius	O	AY902145.1					AY902084		
Cyprinodon fontinalis	O	AY902160.1					AY902101		
Cyprinodon higuey	O	DQ218122.1							
Cyprinodon hubbsi	O	JX856161.1							
Cyprinodon inmemoriam	O								
Cyprinodon julimes	O						KF537339.1		
Cyprinodon labiosus	O								
Cyprinodon laciniatus	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Cyprinodon latifasciatus	O								
Cyprinodon longidorsalis	O								
Cyprinodon macrolepis	O	AY902140.1					AY902082		
Cyprinodon macularius	O	AY902161.1					AY902103		
Cyprinodon maya	O					AF215478			
Cyprinodon meeki	O	AY902153.1					AY902094		
Cyprinodon nazas	O	AY902151.1					AY902091		
Cyprinodon nevadensis	O	GU936286.1					AY902100.1		
Cyprinodon nevadensis nevadensis	O	AF028301.1							
Cyprinodon nichollsi	O	DQ218118.1							
Cyprinodon pachycephalus	O	AY902147.1					AY902089.1		
Cyprinodon pecosensis	O	AY902131.1					AY902072		
Cyprinodon pisteri	O	AY902163.1					AY902105		
Cyprinodon radiosus	O	AY902165.1					AY902107		
Cyprinodon riverendi	O	DQ218132.1							
Cyprinodon rubrofluviatilis	O	AY902129.1	EF442803.2		EF442803.2	EF442803.2	AY902071.1		
Cyprinodon salinus	O	AF028300.1							
Cyprinodon salinus milleri	O								
Cyprinodon salvadori	O								
Cyprinodon simus	O								
Cyprinodon suavium	O								
Cyprinodon tularosa	O	AY902123.1					AY902065		
Cyprinodon variegatus	O	EF050727.1	KF929810.1		AF449406	U05969.1	AY902058.1	KF141215.1	
Cyprinodon variegatus ovinus	O	JN651665.1						JN651549.1	
Cyprinodon variegatus variegatus	O	JX856168.1						JN651593.1	
Cyprinodon verecundus	O								
Cyprinodon veronicae	O	AY902137.1					AY902078		
Empetrichthys latos	O		AY356573.1				U09108.1		
Empetrichthys merriami	O								
Epiplatys annulatus	O								
Epiplatys ansorgii	O								
Epiplatys atratus	O								
Epiplatys barmoiensis	O								
Epiplatys biafranus	O								
Epiplatys bifasciatus	O								KC702059.1
Epiplatys chaperi	O				U73265.1	U73240.1	AF000693		
Epiplatys chevalieri	O								
Epiplatys coccinatus	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Epiplatys dageti	O								
Epiplatys dageti monroviae	O								
Epiplatys duboisi	O								
Epiplatys esekanus	O								
Epiplatys etzeli	O								
Epiplatys fasciolatus	O								
Epiplatys grahami	O								
Epiplatys guineensis	O								
Epiplatys hildegardae	O								
Epiplatys huberi	O								
Epiplatys infrafasciatus	O	FJ872061.1		FJ872049.1		FJ872035.1	DQ981783.1		
Epiplatys josianae	O								
Epiplatys lamottei	O								KC702058.1
Epiplatys longiventralis	O								
Epiplatys maeseni	O				U73268.1	U73243.1	AF000712		
Epiplatys mesogramma	O								
Epiplatys multifasciatus	O				U73264.1	U73239	AF000692		
Epiplatys neumanni	O								
Epiplatys njalaensis	O								
Epiplatys olbrechtsi	O								
Epiplatys phoeniceps	O								
Epiplatys roloffi	O				U73266.1	U73241	AF000694		
Epiplatys ruhkopfi	O								
Epiplatys sangmelinensis	O								
Epiplatys sexfasciatus	O	FJ872061.1		FJ872049.1		FJ872035	DQ981783		
Epiplatys sexfasciatus rathkei	O								
Epiplatys sexfasciatus togolensis	O								
Epiplatys singa	O	AF092358.1			AF092291				KC702060.1
Epiplatys spilargyreius	O								
Epiplatys zenkeri	O								
Episemion krystallinoron	O						DQ981771.1		
Exocoetus volitans	O								
Fenerbahce devosi	O								
Fenerbahce formosus	O					JF307818	JF307808		
Floridichthys carpio	O		JQ842471.1		AF449407	U05970	U06189		
Floridichthys polyommus	O		JQ840506.1						
Fluviphylax obscurus	O								
Fluviphylax palikur	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Fluviphylax pygmaeus	O	EF017561.1			EF017459.1	EF017561.1	EF017511.1	EF017408.1	
Fluviphylax simplex	O								
Fluviphylax zonatus	O								
Foerschichthys flavipinnis	O					AF002407	AF002409		
Fundulopanchax amieti	O				AF002359.1	AF002341	AF002294		
Fundulopanchax arnoldi	O								KC702062.1
Fundulopanchax avichang	O								
Fundulopanchax cinnamomeus	O				AF002361.1	AF002343	AF002296		
Fundulopanchax fallax	O				AF002355.1	AF002338	AF002291		
Fundulopanchax filamentosus	O			DQ533033.1	DQ533202.1	DQ532876	AF002287		
Fundulopanchax gardneri gardneri	O	AF092356.1	JN021666.1		AF092289	AF002344	AF002297		KC702061.1
Fundulopanchax gardneri lacustris	O								
Fundulopanchax gardneri mamfensis	O								
Fundulopanchax gardneri nigerianus	O								
Fundulopanchax gresensi	O								
Fundulopanchax gularis	O				AF002356.1	AF002339	AF002292		
Fundulopanchax intermittens	O								
Fundulopanchax kamdemi	O								
Fundulopanchax marmoratus	O								
Fundulopanchax mirabilis	O				U73272.1	U73247	U73294		KC702063.1
Fundulopanchax moensis	O								
Fundulopanchax ndianus	O				AF002360.1	AF002342	AF002295		
Fundulopanchax oeseri	O					AF002345	AF002298		
Fundulopanchax powelli	O								
Fundulopanchax puerzli	O								
Fundulopanchax robertsoni	O				AF002352.1	AF002335	AF002288		
Fundulopanchax rubrolabialis	O								
Fundulopanchax scheeli	O				AF002365.1	AF002346	AF002299		
Fundulopanchax sjoestedti	O				U73273.1	U73248	DQ981782.1		
Fundulopanchax spoorenbergi	O								
Fundulopanchax traudeae	O								
Fundulopanchax walkeri	O				AF002353.1	AF002336	AF002289		
Fundulopanchax deltaensis	O				AF002354.1	AF002337	AF002290		
Fundulus albolineatus	O								
Fundulus bermudae	O						GQ119682.1	GQ119859	
Fundulus bifax	O						KC204758.1		
Fundulus blairae	O		KF929893.1				GQ119686	GQ119862	
Fundulus catenatus	O		JN026631.1				GQ119692	GQ119863	

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Fundulus chrysotus	O		KF929894.1				GQ119698	GQ119867	
Fundulus cingulatus	O		HQ557444.1				GQ119699		
Fundulus confluentus	O		JQ842472.1				AF312851	GQ119873	
Fundulus diaphanus	O	NC_012361.1	JQ354092.1		NC_012361.1	EF458413	FJ264414	GQ119878	
Fundulus diaphanus menona	O								
Fundulus dispar	O		KF929895.1				GQ119707	GQ119881	
Fundulus escambiae	O		HQ557447.1				GQ119710	GQ119883	
Fundulus euryzonus	O		JN026647.1				KF245879.1		
Fundulus grandis	O	NC_012377.1	NC_012377.1		NC_012377.1	NC_012377.1	EU482164	GQ119884	
Fundulus grandissimus	O								
Fundulus heteroclitus	O	NC_012312.1	NC_012312.1		NC_012312.1	NC_012312.1	NC_012312.1		
Fundulus jenkinsi	O		HQ557160.1				GQ119721		
Fundulus julisia	O						GQ119722	GQ119891	
Fundulus kansae	O		JN026653.1				GQ119724	GQ119894	
Fundulus lima	O		KF929897.1			AY860527	L31593.1	GQ119899	
Fundulus lineolatus	O		KF929898.1				GQ119729	GQ119897	
Fundulus luciae	O						GQ119732	GQ119900	
Fundulus majalis	O						GQ119733	GQ119903	
Fundulus notatus	O		JN026665.1				GQ119741	GQ119909	
Fundulus nottii	O						GQ119736	GQ119905	
Fundulus olivaceus	O	AP006776.1	AP006776.1		AP006776.1	AP006776.1	DQ179625	GQ119913	
Fundulus parvipinnis	O		GU440324.1			AY860527.1	GQ119747.1	GQ119915.1	
Fundulus persimilis	O								
Fundulus philpisteri	O								
Fundulus pulvereus	O						AF312853	GQ119917	
Fundulus rathbuni	O						GQ119751	GQ119919	
Fundulus relictus	O						AF312831		
Fundulus rubrifrons	O		HQ937017.1				GQ119755	GQ119921	
Fundulus saguanus	O								
Fundulus sciadicus	O		JN026679.1				GQ119756	GQ119922	
Fundulus seminolis	O						GQ119760	GQ119927	
Fundulus similis	O		KF929900.1				GQ119759	GQ119924	
Fundulus stellifer	O		JN026687.1				GQ119764	GQ119929	
Fundulus waccamensis	O		JN026688.1						
Fundulus zebrinus	O		AF208284.1		AF221749	AF221755	AF220445	GQ119932	
Gambusia affinis	L	AP004422.1	AP004422.1	AF152163.1	AP004422.1	AP004422.1	AP004422.1	EF017411	
Gambusia alvarezi	L								
Gambusia amistadensis	L								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Gambusia atrora	L	EF017565.1			EF017463	EF017565.1	EF017515	EF017412	
Gambusia aurata	L	JX275468.1	JQ935863.1				JF437630.1		
Gambusia baracoana	L								
Gambusia beebei	L								
Gambusia bucheri	L								
Gambusia clarkhubbsi	L	JX275469.1					JX275483.1		
Gambusia dominicensis	L								
Gambusia echeagarayi	L								
Gambusia eurystoma	L						U18206.1		
Gambusia gaigei	L	JX275470.1							
Gambusia geiseri	L	JX275471.1					U18207		
Gambusia georgei	L								
Gambusia heterochir	L	JX275472.1					DQ075682.1		
Gambusia hispaniolae	L						U18209		
Gambusia holbrooki	L	HM443937.1	GU183103.1		HQ615475.1	U80050.1	GU183104.1		
Gambusia hurtadoi	L	JX275473.1					JX275485.1		
Gambusia krumholzi	L						JX679668.1		
Gambusia lemaitrei	L	JF437626.1					JF437629.1		
Gambusia longispinis	L								
Gambusia luma	L						U18213.1		
Gambusia manni	L						U18214		
Gambusia marshi	L						U18215		
Gambusia melapleura	L						U18216		
Gambusia monticola	L								
Gambusia myersi	L								
Gambusia nicaraguensis	L						U18217		
Gambusia nobilis	L								
Gambusia panuco	L		JQ935865.1				U18219		
Gambusia pseudopunctata	L								
Gambusia punctata	L		FN545685.1				U18220.1		
Gambusia puncticulata	L		FN545652.1				U18221.1		
Gambusia quadruncus	L								
Gambusia regani	L		JQ935866.1						
Gambusia rhizophorae	L		FN545633.1				U18223.1		
Gambusia senilis	L								
Gambusia sexradiata	L		EU751809.1				U18224.1		
Gambusia speciosa	L	JF437628.1					JF437631.1		
Gambusia vittata	L	EF017568.1	JQ935874.1		EF017466.1	EF017568.1	EF017518	EF017415.1	

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Gambusia wrayi	L	EF017566.1			EF017464	EF017566.1	EF017516	EF017413	
Gambusia xanthosoma	L								
Gambusia yucatana	L		EU751811.1				U18226		
Gambusia zarskei	L								
Garmanella pulchra	O		EU751823.1			U05975	U06188		
Girardinichthys ireneae	?								
Girardinichthys multiradiatus	M		AY356576.1				AF510786		
Girardinichthys viviparus	M		AY356575.1				AF510788		
Girardinus creolus	L	EF017594.1	FN545611.1		EF017494.1	EF017594.1	EF017545.1	EF017442.1	
Girardinus cubensis	L								
Girardinus denticulatus	L		FN545609.1				FJ178729.1	FJ185102.1	
Girardinus falcatus	L		FN545684.1				FJ178763.1	FJ185098.1	
Girardinus metallicus	L	EF017593.1	FN545683.1		EF017493.1	U80052.1	FJ178674.1	FJ185103.1	
Girardinus microdactylus	L		FN545617.1				FJ178690.1	FJ185097.1	
Girardinus uninotatus	L		FN545605.1				FJ178722.1	FJ185093.1	
Gnatholebias zonatus	O		AF002591.1		AF002422.1	AF002524.1	AF002472.1	EF455711.1	KC702073.1
Goodea atripinnis	M		AY356577.1				AF510777		
Goodea gracilis	M						AF510770		
Goodea luitpoldii	?								
Heterandria anzuetoi	L					JQ612955.1	JQ612874.1		
Heterandria attenuata	L								
Heterandria bimaculata	L	EF017573.1	EU751838.1		EF017471	EF017573.1	EF017523	EF017420	
Heterandria cataractae	L					JQ612951.1	JQ612898.1		
Heterandria dirempta	L					JQ612945.1	JQ612902.1		
Heterandria formosa	M	AF084973.1	KF929965.1		EF017473	JQ612956.1	KF633111.1	EF017422	
Heterandria jonesii	L	EF017574.1	JQ935925.1		EF017472	JQ612931.1	EF017524.1	EF017421	
Heterandria litoperas	L					JQ612942.1	JQ612877.1		
Heterandria tuxtlaensis	L								
Heterophallus milleri	?	EF017567.1			EF017465.1	EF017567.1	EF017517.1	EF017414.1	
Heterophallus rachovii	?	HM443920.1					HM443901.1		
Hubbsina turneri	M		AY356578.1				AF510841		
Hylopanchax leki	O								
Hylopanchax moke	O								
Hylopanchax ndeko	O								
Hylopanchax silvestris	O								
Hylopanchax stictopleuron	O								
Hypsolebias adornatus	O								
Hypsolebias antenori	O		AF002580.1		U73276.1	U73252.1	KF311233.1		

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Hypsolebias flagellatus	O		HQ833481.1				JQ612743.1		
Hypsolebias flammeus	O								KC702054.1
Hypsolebias flavicaudatus	O						JQ612776.1		KC702055.1
Hypsolebias ghisolfii	O								
Hypsolebias gilbertobrasili	O						JQ612771.1		
Hypsolebias guanambi	O		HQ833484.1				JQ612767.1		
Hypsolebias harmonicus	O						JQ612736.1		
Hypsolebias hellneri	O						JQ612735.1		
Hypsolebias igneus	O		HQ833482.1				JQ612740.1		
Hypsolebias janaubensis	O		HQ833489.1				JQ612774.1		
Hypsolebias longignatus	O								
Hypsolebias lopesi	O								
Hypsolebias macaubensis	O								
Hypsolebias magnificus	O	AF092368.1			AF092301.1	AF092301.1			KC702056.1
Hypsolebias mediopapillatus	O		HQ833478.1				JQ612737.1		
Hypsolebias nitens	O						JQ612778.1		
Hypsolebias nudiorbitatus	O						JQ612742.1		
Hypsolebias pterophyllus	O						JQ612748.1		
Hypsolebias radiseriatus	O						JQ612751.1		
Hypsolebias sertanejo	O						JQ612753.1		
Hypsolebias tocantinensis	O								
Hypsopanchax catenatus	O								
Hypsopanchax deprimozi	O								
Hypsopanchax jobaerti	O								
Hypsopanchax jubbi	O								
Hypsopanchax platysternus	O								
Hypsopanchax zebra	O								
Ilyodon cortesae	?								
Ilyodon furcidens	M		AY356579.1				AF510831		
Ilyodon lennoni	?								
Ilyodon whitei	M		AY356580.1				AF510834		
Ilyodon xantusi	M						AF510830		
Jenynsia alternimaculata	?								
Jenynsia diphyes	?								
Jenynsia eigenmanni	?								
Jenynsia eirmostigma	?								
Jenynsia lineata	M	EF017559			EF017457		EF017509	EF017406	
Jenynsia maculata	?				AF449404				

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Jenynsia multidentata	?		JX111779.1						
Jenynsia obscura	?								
Jenynsia onca	?								GQ221670.1
Jenynsia sanctaecatarinae	?								
Jenynsia tucumana	?								
Jenynsia unitaenia	?								
Jenynsia weitzmani	?								
Jordanella floridae	O	AY902108.1	JN026924.1	DQ533046.1	U73258.1	DQ532888	AY902050	KF141266.1	
Kryptolebias brasiliensis	O	AY946276.1	AY946276.1		AY946281.1	AY946281.1		EF455707	
Kryptolebias campelloi	O								
Kryptolebias caudomarginatus	O	AF092361.1	AF002597.1		AF002428.1	AF002530	AF002478.1		KC702076.1
Kryptolebias gracilis	O								
Kryptolebias hermaphroditus	O								
Kryptolebias marmoratus	O	NC_003290.1	NC_003290.1		NC_003290.1	NC_003290.1	NC_003290.1		KC702083.1
Kryptolebias ocellatus	O		AF002599.1		AF002430.1	AF002532	AF002480.1		
Kryptolebias sepia	O	AY946272.1	AY946272.1		AY946277.1	AY946277.1			
Lacustricola maculatus	O								
Lacustricola matthesi	O								
Lacustricola mediolateralis	O								
Lacustricola nigrolateralis	O								
Lacustricola omoculatus	O								
Lacustricola usanguensis	O								
Laimosemion agilae	O	AF092377.1	AF002603.1		AF002432.1	AF002536	AF002482		
Laimosemion altivelis	O								
Laimosemion amanapira	O								
Laimosemion breviceps	O	AF092376.1			JX885658.1	JX885659.1	JX885660.1		
Laimosemion cladophorus	O								
Laimosemion corpulentus	O								
Laimosemion dibaphus	O								
Laimosemion frenatus	O	AF092378.1	AF002606.1		AF002435.1	AF002539	AF002485		
Laimosemion geayi	O	AY946274.1	AF002604.1		AF002433.1	AF002537	AF002483		
Laimosemion gransabanae	O	AF092375.1			AF092308.1	AF092308.1			
Laimosemion kirovskyi	O	AY578711.1			AY578719.1	AY578719.1			
Laimosemion lyricauda	O	AY578717.1	AF002610.1		AF002439.1	AF002543	AF002489		
Laimosemion mahdiaensis	O				DQ501248.1	DQ501249	DQ501250.1		KC702082.1
Laimosemion nicoi	O								
Laimosemion paryagi	O				JX885661.1	JX885662.1	JX885663.1		
Laimosemion rectocaudatus	O	AY578716.1	AF002611.1		AF002440.1	AF002544	AF002490.1		

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Laimosemion romeri	O								
Laimosemion sape	O								
Laimosemion strigatus	O		AF002605.1		AF002434.1	AF002538	AF002484		
Laimosemion tecminae	O	AF092374.1			AF092307.1	AF092307.1			
Laimosemion torrenticola	O								
Laimosemion uakti	O								
Laimosemion uatuman	O								
Laimosemion xiphidius	O		AF002607.1		AF002436.1	AF002540	AF002486.1		
Lamprichthys tanganicanus	O								
Leptolebias aureoguttatus	O		AF002581.1		AF002411.1	AF002513	AF002462.1		
Leptolebias citrinipinnis	O		AF002582.1		U73277.1	U73253.1	U73299.1		
Leptolebias itanhaensis	O								
Leptolebias leitaoi	O								
Leptolebias marmoratus	O								
Leptolebias opalescens	O								
Leptolebias splendens	O								
Leptolucania ommata	O		HQ557457.1						
Limia caymanensis	L	AF353192.1							
Limia dominicensis	L	EF017582.1			EF017482	EF017582.1	EF017533	EF017431	GU179273
Limia fuscomaculata	L								
Limia garnieri	L								
Limia grossidens	L								
Limia heterandria	L								
Limia immaculata	L								
Limia melanogaster	L	EF017583.1			EF017483	EF017583.1	EF017534	EF017432	GU179274
Limia melanonotata	L	AF353197.1	JX968692.1						
Limia miragoanensis	L								
Limia nigrofasciata	L	AF031391.1							
Limia ornata	L								
Limia pauciradiata	L	AF353196.1							
Limia perugiae	L	AF031392.1							
Limia rivasi	L								
Limia sulphurophila	L								
Limia tridens	L	EF017584.1			EF017484	EF017584.1	EF017535	EF017433	
Limia versicolor	L	AF353193.1							
Limia vittata	L	AF353201.1	JX968689.1			JQ612960.1	FJ178765		
Limia yaguajali	L								
Limia zonata	L	AF353194.1							

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Llanolebias stellifer	O	AF092420.1			AF092353.1	AF092353.1		EF455713.1	KC702071.1
Lucania goodei	O		HQ557450.1				GQ119768	GQ119933	
Lucania interioris	O								
Lucania parva	O		HQ579046.1				GQ119769	GQ119934	
Maratecoara formosa	O	AF092411.1			AF092344.1	AF092344.1			KC702064.1
Maratecoara lacortei	O	AF092410.1	AF002585.1		AF002415.1	AF002517.1	AF002466.1		
Maratecoara splendida	O								
Megupsilon aporus	O	AY902110.1				U05978.1	AY902052.1		
Melanorivulus apiamici	O		GU701519.1		AF002453.1	AF002566.1	AF002503		
Melanorivulus bororo	O								
Melanorivulus crixas	O								
Melanorivulus cyanopterus	O								
Melanorivulus dapazi	O								
Melanorivulus decoratus	O								
Melanorivulus egens	O								
Melanorivulus faucireticulatus	O								
Melanorivulus giarettai	O								
Melanorivulus illuminatus	O								
Melanorivulus jalapensis	O								
Melanorivulus javahe	O								
Melanorivulus karaja	O								
Melanorivulus kayabi	O								
Melanorivulus kayapo	O								
Melanorivulus kunzei	O								
Melanorivulus litteratus	O								
Melanorivulus megaroni	O								
Melanorivulus modestus	O								
Melanorivulus paracatuensis	O								
Melanorivulus paresi	O								
Melanorivulus parnaibensis	O								
Melanorivulus pictus	O	AF092388.1			AF092321.1	AF092321.1			KC702086.1
Melanorivulus pindorama	O								
Melanorivulus pinima	O								
Melanorivulus planaltinus	O								
Melanorivulus punctatus	O	AF092389.1	AF002636.1		AF002454.1	AF002567	AF002504		KC702087.1
Melanorivulus rossoi	O								
Melanorivulus rubromarginatus	O								
Melanorivulus rutilicaudus	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Melanorivulus salmonicaudus	O								
Melanorivulus scalaris	O								
Melanorivulus schuncki	O								
Melanorivulus ubirajarai	O								
Melanorivulus violaceus	O	AF092387.1	AF002637.1		AF002455.1	AF002568	AF002505		KC702090.1
Melanorivulus vittatus	O								
Melanorivulus zygonectes	O								
Melanotaenia duboulayi	O	KJ667895.1	KF491262.1			AY461521	HM007047		
Menidia beryllina	O	KC736464.1	KF930119.1				HQ691351.1	KF141280.1	
Micromoema xiphophora	O	AF092418.1	AF002592.1		AF002423.1	AF002525.1	AF002473.1	EF455720.1	
Micropanchax bracheti	O								
Micropanchax camerunensis	O								
Micropanchax ehrichi	O								
Micropanchax keilhacki	O								
Micropanchax loati	O								
Micropanchax macrophthalmus	O								
Micropanchax pelagicus	O								
Micropanchax pfaffi	O								
Micropanchax scheeli	O								
Micropoecilia bifurca	M								
Micropoecilia branneri	M	GU179233.1					GU179187	GU179262	GU179276
Micropoecilia minima	?								
Micropoecilia picta	M	GU179237.1			EF017486	EF017586.1	GQ855725.1	GU179266	GU179280
Millerichthys robustus	O								
Moema apurinan	O								
Moema hellneri	O								
Moema heterostigma	O								
Moema nudifrontata	O								
Moema pepotei	O								
Moema piriana	O		AF002639.1		AF002457.1	AF002570	AF002507.1		
Moema portugali	O								
Moema quiii	O								
Moema staecki	O	AF092406.1	AF002640.1		AF002458.1	AF002571.1	AF002508.1	EF455719	
Nematolebias papilliferus	O								
Nematolebias whitei	O	AF092365.1	AF002577.1		U41802.1	AF002511	U41784.1		KC702057.1
Neofundulus acutirostratus	O								
Neofundulus guaporensis	O								
Neofundulus ornatipinnis	O	AF092403.1			AF092336.1	AF092336.1	FJ826902.1		

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Neofundulus paraguayensis	O	AF092405.1	AF002643.1		AF002460.1	AF002573	AF002510.1	EF455722	KC702065.1
Neofundulus parvipinnis	O								
Neoheterandria cana	L								
Neoheterandria elegans	L				EF017476	EF017476.1	EF017528.1	EF017425	
Neoheterandria tridentiger	L	EF017576.1			EF017474	EF017576.1	EF017526	EF017423	
Nimbapanchax jeanpoli	O	FJ872057.1		FJ872043.1		FJ872029.1			
Nimbapanchax leucopterygius	O	FJ872054.1		FJ872040.1		FJ872026.1			
Nimbapanchax melanopterygius	O	FJ872052.1		FJ872038.1		FJ872024.1			
Nimbapanchax petersi	O			FJ872044.1	AF000690.1	FJ872030.1	AF000714.1		
Nimbapanchax viridis	O	FJ872055.1		FJ872041.1	AF000691.1	FJ872027.1	AF000715.1		
Nothobranchius albimarginatus	O		JQ310161.1						
Nothobranchius annectens	O								
Nothobranchius bojiensis	O								
Nothobranchius boklundi	O								
Nothobranchius brieni	O								
Nothobranchius cardinalis	O		JQ310162.1						
Nothobranchius eggersi	O	GU138045.1	EF464686.1			GU138042.1			
Nothobranchius elongatus	O	EU182591.1				EU401647.1			
Nothobranchius fasciatus	O								
Nothobranchius flammicomantis	O		JQ310163.1						
Nothobranchius foerschi	O		JQ310164.1						
Nothobranchius furzeri	O	GU138046.1	NC_011814.1	EU780557.1	NC_011814.1	NC_011814.1	KC777187.1		
Nothobranchius fuscotaeniatus	O								
Nothobranchius geminus	O								
Nothobranchius guentheri	O		EF464692.1						
Nothobranchius hassoni	O								
Nothobranchius hengstleri	O	EU401646.1	EF464709.1			EU401666.1			
Nothobranchius interruptus	O		JQ310166.1						
Nothobranchius ivanovae	O								
Nothobranchius janpapi	O								
Nothobranchius jubbi	O	EU401628.1	JQ310169.1			EU401651.1			
Nothobranchius kadleci	O		JN021662.1						
Nothobranchius kafuensis	O	GU138059.1			U73274.1	GU138041.1	U73296.1		
Nothobranchius kardashevi	O								
Nothobranchius kilomberoensis	O		JQ310170.1						
Nothobranchius kirki	O		JF444863.1		AF002349.1	U73250			
Nothobranchius korthausae	O		JQ310173.1						
Nothobranchius krammeri	O		JQ310174.1						

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Nothobranchius krysanovi	O								
Nothobranchius kuhntae	O		JN021627.1						
Nothobranchius lourensi	O								
Nothobranchius lucius	O	EU401631.1	JQ310175.1			EU401650.1			
Nothobranchius luekei	O								
Nothobranchius makondorum	O	EU401633.1	JQ310184.1			EU401654.1			
Nothobranchius malaissei	O								
Nothobranchius melanospilus	O	EU401642.1	EU401662.1						
Nothobranchius microlepis	O								
Nothobranchius neumanni	O								
Nothobranchius nubaensis	O								
Nothobranchius ocellatus	O	EU401643.1				EU401663.1			
Nothobranchius oestergaardi	O								
Nothobranchius orthonotus	O	EU401644.1	JN021649.1			EU401664.1			KC702066.1
Nothobranchius palmqvisti	O								
Nothobranchius patrizii	O								
Nothobranchius pienaari	O		JN021659.1						
Nothobranchius polli	O	GU138051.1				GU138033.1			
Nothobranchius rachovii	O	GU138050.1	JN021660.1			GU138031.1			
Nothobranchius robustus	O								
Nothobranchius rosenstocki	O								
Nothobranchius rubripinnis	O								
Nothobranchius rubroreticulatus	O	GU138047.1				GU138032.1			
Nothobranchius ruudwildekampi	O								
Nothobranchius seegersi	O								
Nothobranchius steinforti	O								
Nothobranchius symoensi	O								
Nothobranchius taeniopygus	O								
Nothobranchius thierryi	O		JN021562.1		AF002347.1	AF002405	AF002284		
Nothobranchius ugandensis	O								
Nothobranchius virgatus	O								
Nothobranchius vosseleri	O								
Nothobranchius wattersi	O		JF444871.1						
Nothobranchius willerti	O								
Notholebias cruzi	O								
Notholebias fractifasciatus	O								
Notholebias minimus	O	AF092364.1	AF002583.1		AF002412.1	AF002514.1	AF002463.1		
Ophthalmolebias ilheusensis	O						JQ612734.1		

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Orestias agassizii	O	JX155710.1			AF449408	U05966	JX092172		
Orestias albus	O								
Orestias ascotanensis	O	JX155680.1					JX092143.2		
Orestias chungarensis	O	JX155690.1					JX092158.2		
Orestias crawfordi	O								
Orestias ctenolepis	O								
Orestias cuvieri	O								
Orestias elegans	O								
Orestias empyraeus	O								
Orestias forgeti	O								
Orestias frontosus	O								
Orestias gilsoni	O	AF449347.1			AF449409.1	AF449347.1			
Orestias gloriae	O	JX155685.1					JX092148.2		
Orestias gracilis	O								
Orestias gymnotus	O								
Orestias hardini	O								
Orestias imarpe	O	AF449348.1			AF449410	AF449348.1			
Orestias incae	O								
Orestias ispi	O					U05972	U06186		
Orestias jussiei	O								
Orestias lastarriae	O								
Orestias laucaensis	O	JX155714.1					JX092177.2		
Orestias luteus	O					U05977	U06191		
Orestias minimus	O								
Orestias minutus	O								
Orestias mooni	O								
Orestias mulleri	O								
Orestias multiporis	O								
Orestias mundus	O								
Orestias olivaceus	O								
Orestias parinacotensis	O	JX155718.1					JX092182.2		
Orestias pentlandii	O								
Orestias piacotensis	O	JX155720.1					JX092187.2		
Orestias polonorum	O								
Orestias puni	O	JX155675.1					JX092139.2		
Orestias richersoni	O								
Orestias robustus	O								
Orestias silustani	O						AY155565		

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Orestias taquiri	O								
Orestias tchernavini	O								
Orestias tomcooni	O								
Orestias tschudii	O								
Orestias tutini	O								
Orestias uruni	O								
Orestias ututo	O								
Oryzias latipes	O	AP008945.1	AP008945.1	AF398344.1	EF095555.1	AB570424	AB084686	AB120889	
Oxyzygonectes dovii	O	AF449340.1	AY356581.1		EF017458		EF017510	EF017407	
Pachypanchax arnoulti	O								
Pachypanchax omalonotus	O				U73262.1	U73237	U73284		KC702067.1
Pachypanchax patriciae	O								
Pachypanchax playfairii	O			DQ533086.1	U73263.1	DQ532927	U73285	JX190914.1	
Pachypanchax sakaramyi	O							JQ073283.1	KC702069.1
Pachypanchax sparksorum	O								
Pachypanchax varatraza	O								
Pamphorichthys araguaiensis	?	AF031398.1					GU179195	GU179269	GU179284
Pamphorichthys hasemani	?	HQ857451.1					HQ857427.1	HQ857445.1	HQ857439.1
Pamphorichthys hollandi	M	HQ857452.1	GU701605.1		EF017487	EF017587.1	HQ857428.1	HQ857446.1	HQ857440.1
Pamphorichthys minor	?	AF031397.1					GU179196.1	GU179270.1	GU179285.1
Pamphorichthys pertapeh	?								
Pamphorichthys scalpridens	?	HQ857453.1					HQ857429.1	HQ857447.1	HQ857441.1
Pantanodon madagascariensis	O								
Pantanodon stuhlmanni	O								
Papiliolebias bitteri	O	AF092408.1	AF002588.1		AF002418.1	AF002520.1			
Papiliolebias hatinne	O	AF092408.1	AF002588.1		AF002418.1	AF002520.1			
Paratilapia polleni	O	AP009508.1	DQ119222.1		AP009508.1	DQ119193.1	AP009508.1	JX189869.1	
Phallichthys amates	?	EF017563.1			DQ386523.1	DQ386564.1	EF017513	EF017410	
Phallichthys fairweatheri	?								
Phallichthys quadripunctatus	?				DQ386547.1	DQ386588.1	DQ386593.1		
Phallichthys tico	L	AF412168.1			DQ386545.1	DQ386587	AF412127.1	EF017409	
Phalloceros alessandrae	?								
Phalloceros anisophallos	M								
Phalloceros aspilos	?								
Phalloceros buckupi	?								
Phalloceros caudimaculatus	M	EF017578.1			EF017477	U80053		EF017426	
Phalloceros elachistos	?								
Phalloceros enneaktinos	?								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Phalloceros harpagos	?		GU701586.1						
Phalloceros heptaktinos	?								
Phalloceros leptokeras	?								
Phalloceros leticiae	?								
Phalloceros lucenorum	?								
Phalloceros malabarbai	?								
Phalloceros megapolos	?								
Phalloceros mikrommatos	?								
Phalloceros ocellatus	?								
Phalloceros pellos	?								
Phalloceros reisi	?		GU701910.1						
Phalloceros spiloura	?								
Phalloceros titthos	?								
Phalloceros tupinamba	?								
Phalloceros uai	?		HM404946.1						
Phalloptychus eigenmanni	?								
Phalloptychus januarius	M				EF017479	EF017479	EF017530	EF017428	
Phallotorynus dispilos	?								
Phallotorynus fasciolatus	?								
Phallotorynus jucundus	?								
Phallotorynus pankalos	?								
Phallotorynus psittakos	?								
Phallotorynus victoriae	?								
Pituna brevirostrata	O								
Pituna compacta	O							EF455717	
Pituna obliquoseriata	O								
Pituna poranga	O	AF092412.1	AF002586.1		AF002416.1	AF002518.1	AF002467.1		KC702070.1
Pituna schindleri	O								
Pituna xinguensis	O								
Plataplochilus cabindae	O								
Plataplochilus chalcopyrus	O								
Plataplochilus loemensis	O								
Plataplochilus miltotaenia	O								
Plataplochilus mimus	O								
Plataplochilus ngaensis	O								
Plataplochilus pulcher	O								
Plataplochilus terveri	O								
Platypanchax modestus	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Plesiolebias altamira	O								
Plesiolebias aruana	O	AF092409.1	AF002587.1		AF002417.1	AF002519	AF002468.1		
Plesiolebias canabravensis	O								
Plesiolebias filamentosus	O								
Plesiolebias fragilis	O								
Plesiolebias glaucopterus	O				AF244429.1		AF245468.1		
Plesiolebias lacerdai	O								
Plesiolebias xavantei	O								
Poecilia boesemani	?								
Poecilia butleri	?	KF276672.1	JX968651.1				JN368131.1	KF276701.1	KF276729.1
Poecilia catemaconis	?	KF276668.1					KF276610.1	KF276697.1	KF276726.1
Poecilia caucana	?	EF017589.1	JX968687.1		EF017489	EF017589.1	EF017540	EF017437	GU179286
Poecilia caudofasciata	?								
Poecilia chica	?								
Poecilia dauli	?								
Poecilia dominicensis	L								
Poecilia elegans	L								
Poecilia formosa	?						HM567260.1		
Poecilia gillii	L	AF031388.1	JX968680.1				FJ446358.1		
Poecilia hispaniolana	?		JX968690.1						
Poecilia hondurensis	?		JX968667.1						
Poecilia kempkesi	?								
Poecilia koperi	?								
Poecilia kykesis	?								
Poecilia latipinna	M	AF031389.1					FJ446153	KF276696.1	KF276725.1
Poecilia latipunctata	?	AF080489.1	JQ935927.1		EF017488	EF017488	EF017539	EF017436.1	GU179287
Poecilia marcellinoi	?								
Poecilia maylandi	?								
Poecilia mechthildae	L								
Poecilia mexicana	?	HQ677841.1	KJ661415.1			JQ612959.1	FJ178776.1	KF276717.1	KF276750.1
Poecilia nicholsi	L								
Poecilia obscura	L						GQ855738		
Poecilia orri	M	AF031400.1	JQ840648.1						
Poecilia parae	M	AF031396.1					GU179188.1	GU179264.1	GU179277.1
Poecilia petenensis	L	AF031401.1	EU751941.1						
Poecilia reticulata	?	EF017585.1	JQ667562.1	AH011837.1	EF017485	U80051	EF017536.1	EF017434	DQ912023
Poecilia rositae	L								
Poecilia salvatoris	M								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Poecilia sarrafae	L								
Poecilia sphenops	?	KF276670.1	JX968660.1				KF276612.1	KF276699.1	KF276728.1
Poecilia sulphuraria	?	AF080490.1					HQ677900.1	KF276710.1	KF276739.1
Poecilia teresae	?								
Poecilia vandepolli	L								
Poecilia velifera	L	AF031402.1	JQ667581.1						
Poecilia vivipara	?	AF031387.1	GU701904.1				HQ677905.1	HQ857448.1	HQ857442.1
Poecilia waiapi	?								
Poecilia wandae	L								
Poecilia wingei	?	GU179239.1					GQ855739.1	GU179268	GU179283
Poeciliopsis baenschi	M	AF412191.1					AF412148		
Poeciliopsis balsas	L								
Poeciliopsis catemaco	L		EU751942.1				AF412161.1		
Poeciliopsis elongata	M	AF412172.1					AF412129	KF141320.1	
Poeciliopsis fasciata	L	AF412193.1			EF017495	EF017495.1	AF412150	EF017443	
Poeciliopsis gracilis	L	AF412192.1	JN028271.1				AF412155		
Poeciliopsis hnilickai	L	AF412202.1			EF017496	EF017496.1	EF017547	EF017444	
Poeciliopsis infans	L	AF412176.1					AF412138		
Poeciliopsis latidens	L	AF412194.1					AF412151		
Poeciliopsis lucida	M	AF412184.1			AF042472.1		AF412139		
Poeciliopsis lutzi	?								
Poeciliopsis monacha	L	AF412173.1					AF458376		
Poeciliopsis occidentalis	M	AF412187.1	HQ556953.1				AF412142		
Poeciliopsis paucimaculata	M	AF412171.1					AF412128		
Poeciliopsis pleurospilus	L		EU751947.1						
Poeciliopsis presidionis	M	AY743254.1					AF412157		
Poeciliopsis prolifica	M	AF412190.1					AF412146		
Poeciliopsis retropinna	M						AF412130		
Poeciliopsis santaelena	?								
Poeciliopsis scarlli	L	AF412198.1					AF412159		
Poeciliopsis sonoriensis	M	DQ138947.1					DQ138944.1		
Poeciliopsis turneri	M	AF412197.1					AF412158		
Poeciliopsis turrubarensis	L	AF412204.1					AF412163		
Poeciliopsis viriosa	L	AF412175.1					AF412132		
Poropanchax hannerzi	O								
Poropanchax luxophthalmus	O								
Poropanchax normani	O								
Poropanchax rancureli	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Poropanchax stigmatopygus	O								
Priapella bonita	?								
Priapella chamulae	L						JF892548.1	KJ525830.1	KJ525810.1
Priapella compressa	?	EF017603.1			EF017503.1	EF017603.1	EF017554.1	DQ235860	KJ525811.1
Priapella intermedia	?	EF017602.1	AY356595.1		EF017502	EF017602.1	EF017553.1	EF017450.1	
Priapella lacandonae	?						JF892549.1		
Priapella olmecae	?	EF017604.1			EF017504.1	U80046.1	JF892547.1	EF017452.1	
Priapichthys annectens	L	EF017591.1			DQ386528.1	DQ386565.1	FJ518864	EF017439	
Priapichthys caliensis	?								
Priapichthys chocoensis	?								
Priapichthys darienensis	L								
Priapichthys nigroventralis	?								
Priapichthys panamensis	?				DQ386542.1	DQ386584.1	DQ386591		
Priapichthys puetzi	?				DQ386544.1	DQ386585	DQ376992		
Procatopus aberrans	O								
Procatopus nototaenia	O								
Procatopus similis	O								
Procatopus websteri	O								
Profundulus candalarius	O		HQ682636.1				JQ254931.1		
Profundulus guatemalensis	O		JN028283.1				AY155568	GQ119857	
Profundulus hildebrandi	O						JQ254932.1		
Profundulus kreiseri	O								
Profundulus labialis	O		HQ682638.1	DQ533103.1	DQ533279.1	DQ532944	AY155567		
Profundulus oaxacae	O								
Profundulus portillorum	O						JQ254929.1		
Profundulus punctatus	O		HQ691246.1				AY155566		
Pronothobranchius kiyawensis	O	EU401645.1	EF464705.1		AF002348.1	EU401665.1	AF002285.1		
Prorivulus auriferus	O								
Pseudopoecilia austrocolumbiana	?								
Pseudopoecilia festae	L	EF017592.1			EF017492	EF017592.1	EF017543	EF017440	
Pseudopoecilia fria	?								
Pseudoxiphophorus obliquus	?					JQ612950.1	JQ612895.1		
Pterolebias hoignei	O	EF455704.1			AF002421.1	AF002523.1	AF002471.1	EF455712	
Pterolebias longipinnis	O	AF092415.1	AF002595.1		AF244430.1	AF244446.1	AF245462.1	EF455709	KC702072.1
Pterolebias phasianus	O	AF092414.1	AF002596.1		AF002427.1	AF002529.1	AF002477.1	EF455710	
Ptychochromis grandidieri	O		AY263879.1	DQ533108.1	DQ533284.1	AY263811		JX189871.1	
Quintana atrizona	?	EF017605.1	FN545619.1		EF017505	EF017605.1	FJ178764	EF017453	
Rachovia brevis	O	AY850640.1	AY850640.1		AY850665.1	AY850665.1			

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Rachovia hummelincki	O	AY850642.1							
Rachovia maculipinnis	O	AF092417.1	AF002590.1		AF002420.1	AF002522.1	AF002470.1	EF455714	
Rachovia pyropunctata	O	AY850641.1	AY850641.1		AY850666.1	AY850666.1			
Renova oscari	O	AF092413.1	AF002594.1		AF002425.1	AF002527.1	AF002475.1	EF455721.1	KC702074.1
Rheocles wrightae	O	KC133769.1	AY290803.1	AY655658.1	AY268896.1	AY266069	KC133646.1	JX189788.1	
Rhexipanchax kabae	O								
Rhexipanchax lamberti	O								
Rhexipanchax nimbaensis	O								
Rhexipanchax schioetzi	O								
Rivulus cylindraceus	O	AF092371.1	FN544247.1		U41800.1	AF002533	U41781		KC702077.1
Rivulus formosensis	O								
Rivulus insulaepinorum	O		FN545681.1						
Rivulus roloffi	O	AF092372.1	AF002602.1		U41798.1	AF002534	U41780.1		KC702084.1
Rivulus staecki	O								
Rivulus tomasi	O								
Scolichthys greenwayi	L	EF017590.1			EF017490	EF017590.1	EF017541	EF017438	
Scolichthys iota	L								
Scriptaphyosemion banforense	O				AF000679.1		AF000701		KC702093.1
Scriptaphyosemion bertholdi	O	JX124261.1		JX124247.1	AF000680.1	JX124233.1	AF000702		
Scriptaphyosemion brueningi	O	JX124263.1		JX124249.1	AF000681.1	JX124235.1	AF000703		
Scriptaphyosemion cauveti	O	JX124264.1		JX124250.1		JX124236.1	JX044137.1		
Scriptaphyosemion chaytori	O	JX124265.1		JX124251.1	AF000682.1	JX124237.1	AF000704		
Scriptaphyosemion etzeli	O				AF000685.1		AF000707		
Scriptaphyosemion fredrodi	O	JX124266.1		JX124252.1		JX124238.1			
Scriptaphyosemion geryi	O	JX124267.1	EF464684.1	FJ872047.1	AF092292	FJ872033	JX044136.1		
Scriptaphyosemion guignardi	O	JX124268.1		JX124253.1	EF455700	JX124239.1	JX044135.1	EF455706	
Scriptaphyosemion liberiense	O	JX124269.1		JX124254.1	AF000684.1	JX124240.1	AF000706		
Scriptaphyosemion roloffi	O	JX124270.1		JX124255.1	AF000686.1	JX124241.1	AF000709		
Scriptaphyosemion schmitti	O	JX124271.1		JX124256.1	AF000683.1	JX124242.1	AF000705		
Scriptaphyosemion wieseae	O	JX124275.1		JX124260.1		JX124246.1			
Simpsonichthys alternatus	O								
Simpsonichthys auratus	O								
Simpsonichthys boitonei	O								KC702051.1
Simpsonichthys bokermanni	O				AF092300.1	AF092300.1			KC702052.1
Simpsonichthys brunoi	O								
Simpsonichthys carlettoi	O								
Simpsonichthys chacoensis	O								
Simpsonichthys cholopteryx	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Simpsonichthys constanciae	O								KC702053.1
Simpsonichthys delucai	O								
Simpsonichthys fasciatus	O								
Simpsonichthys filamentosus	O								
Simpsonichthys fulminantis	O								
Simpsonichthys gibberatus	O								
Simpsonichthys inaequipinnatus	O								
Simpsonichthys izecksohni	O								
Simpsonichthys margaritatus	O								
Simpsonichthys marginatus	O								
Simpsonichthys multiradiatus	O								
Simpsonichthys nielseni	O								
Simpsonichthys nigromaculatus	O								
Simpsonichthys notatus	O		AF002578.1		AF002410.1	AF002512.1	AF002461.1		
Simpsonichthys ocellatus	O								
Simpsonichthys parallelus	O								
Simpsonichthys perpendicularis	O								
Simpsonichthys picturatus	O								
Simpsonichthys punctulatus	O								
Simpsonichthys radiosus	O								
Simpsonichthys reticulatus	O								
Simpsonichthys rosaceus	O								
Simpsonichthys rufus	O								
Simpsonichthys santanae	O								
Simpsonichthys semiocellatus	O								
Simpsonichthys similis	O								
Simpsonichthys stellatus	O								
Simpsonichthys suzarti	O								
Simpsonichthys trilineatus	O				AF244428.1				
Simpsonichthys virgulatus	O								
Simpsonichthys zonatus	O								
Skiffia bilineata	M						AF510749		
Skiffia francesae	M		AY356582.1				AF510845		
Skiffia lermae	M		AY356584.1				AF510782		
Skiffia multipunctata	M		AY356585.1				AF510844		
Spectrolebias brousseaui	O								
Spectrolebias costai	O		AF002578.1		AF002410.1	AF002512.1	AF002461.1		
Stenolebias bellus	O								

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Stenolebias damascenoi	O								
Terranatos dolichopterus	O	AF092421.1	AF002593.1		AF002424.1	AF002526.1	AF002474.1	EF455716.1	KC702094.1
Tomeurus gracilis	O	EF017607.1			EF017507	EF017607.1		EF017455	
Trigonectes aplocheiloides	O	AF092400.1			AF092333.1	AF092333.1			
Trigonectes balzanii	O	AF092401.1	AF002641.1		AF002459.1	AF002572	AF002509		KC702095.1
Trigonectes macrophthalmus	O								
Trigonectes rogoaguae	O								
Trigonectes rubromarginatus	O	AF092402.1	AF002642.1		U73279.1	U73257	U73301	EF455723.1	KC702096.1
Trigonectes strigabundus	O								
Valencia hispanica	O	AF449339.1	KF767525.1		AF449401	AF449339.1			
Valencia letourneuxi	O	AF449337.1	KF767526.1		AF449399	AF449337.1			
Xenodexia ctenolepis	M	EF017606.1			EF017506	EF017606.1	EF017557	EF017454	
Xenoophorus captiva	M		AY356586.1				AF510758		
Xenophallus umbratilis	L	AF412169.1			EF017475	EF017577.1	FJ518845	EF017424	
Xenotaenia resolanae	M		AY356590.1				AF510825		
Xenotoca eiseni	M	NC_011381.1	AY356587.1		NC_011381.1	NC_011381.1	AF510766		
Xenotoca melanosoma	M		AY356588.1				AF510763		
Xenotoca variatus	M		AY356589.1				AF510808		
Xenurolebias myersi	O	AF092366.1			AF092299.1	AF092299.1			
Xiphophorus alvarezi	L		EU752036.1				U06494	KJ525812.1	KJ525792.1
Xiphophorus andersi	L						U06495	DQ235870.1	
Xiphophorus birchmanni	L		JQ935946.1				U06496	KJ525813.1	KJ525793.1
Xiphophorus clemenciae	L	EF017601.1			EF017501	EF017601.1	EF017552	DQ235883.1	KJ525794.1
Xiphophorus continens	L						U06502	KJ525815.1	KJ525795.1
Xiphophorus cortezi	L		JQ935948.1				U06503	KJ525816.1	KJ525796.1
Xiphophorus couchianus	L						U06504	KJ525817.1	KJ525797.1
Xiphophorus evelynae	L	EF017599.1			EF017499	EF017599.1	EF017550	EF017447	
Xiphophorus gordoni	L		EF017523				JX856171.1	KJ525818.1	KJ525798.1
Xiphophorus hellerii	L	EF017597.1	JQ667588.1		EF017497	FJ234985	JQ612909.1	KJ525819.1	GU454735
Xiphophorus kallmani	L						JX988790.1	JX988798.1	
Xiphophorus kosszanderi	L								
Xiphophorus maculatus	L	AP005982.1	AP005982.1		AP005982.1	AP005982.1	AP005982.1	DQ235880	
Xiphophorus malinche	L						U06516	DQ235867.1	
Xiphophorus mayae	L						AF404296	JX988799.1	
Xiphophorus meyeri	L						U06517	KJ525820.1	KJ525800.1
Xiphophorus milleri	L						U06518	DQ235864	
Xiphophorus mixei	L						JX988792.1	JX988800.1	
Xiphophorus montezumae	L		JQ935955.1				U06519	KJ525821.1	KJ525801.1

Species	Character State	ND2	COI	28S	12S	16S	Cyt b	Rag1	rhodopsin
Xiphophorus monticolus	L						JX988793.1	JX988801.1	KJ525802.1
Xiphophorus multilineatus	L						U06522	KJ525823.1	KJ525803.1
Xiphophorus nezahualcoyotl	L		JQ935960.1				U06524	KJ525824.1	KJ525804.1
Xiphophorus nigrensis	L	AF031386.1					U06526	KJ525825.1	KJ525805.1
Xiphophorus pygmaeus	L		JQ935962.1				U06528	KJ525826.1	KJ525806.1
Xiphophorus roseni	L								
Xiphophorus signum	L						U06531	KJ525827.1	KJ525807.1
Xiphophorus variatus	L		JQ935964.1				U06532	KJ525828.1	KJ525808.1
Xiphophorus xiphidium	L	EF017598.1			EF017498	EF017598.1	EF017549	KJ525829.1	KJ525809.1
Zoogoneticus purhepechus	?								
Zoogoneticus quitzeoensis	M		AY356592.1				EU679476		
Zoogoneticus tequila	?		AY356591.1				AF510757		

Table S3.2. Models of molecular evolution chosen by PartitionFinder (Lanfear et al. 2012) on the basis of the Akaike Information Criterion.

Partition	Model
12S, 16S	GTR + I + G
28S	GTR + G
COX1 codon 1 & 2	GTR + I + G
COX1 codon 3	GTR + I + G
Cyt B codon 1	GTR + G
Cyt B codon 2	GTR + I + G
Cyt B codon 3, RHO codon 3	GTR + I + G
ND2 codon 1	GTR + G
ND2 codon 2	GTR + I + G
ND2 codon 3	GTR + I + G
Rag1 codon 1	GTR + I + G
Rag1 codon 2, RHO codon 2	GTR + I + G
Rag1 codon 3	GTR + I + G
RHO codon 1	GTR + I + G

Table S3.3. Fossils and secondary calibrations used in molecular dating (time tree) analysis

Group	Mean (Ma)	Log (st dev)	Offset	Lower (Ma)	Upper (Ma)	Prior Distribution	Reference
Cichlidae	70.35	-	-	40.2	100.5	Truncated Normal	
Poeciliidae + Anablepidae	55.55	-	-	39.9	71.2	Truncated Normal	
Perciformes	97.3	7.48	-	37.3	157.3	Truncated Normal	Betancur-R et al. (2013)
Atheriniformes	77.4	11.6	-	37.4	117.4	Truncated Normal	Betancur-R et al. (2013)
Beloniformes	67.5	12.1	-	27.5	107.5	Truncated Normal	Betancur-R et al. (2013)
Orestiini	19.5	1	15.97	-	-	Lognormal	Costa (2011)
Aphanius	21.73	1	20.43	-	-	Lognormal	Reichenbacher and Kowalke (2009)
Fundulus	17.015	1	13.6	-	-	Lognormal	Lugaski (1977)
Cyprinodon	3.955	1	2.58	-	-	Lognormal	Smith (1981), Miller (1945)
Emeptricthys	3.955	1	2.58	-	-	Lognormal	Smith (1981), Miller (1945)

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Concluding Remarks

Parent-offspring conflicts have been a key player in the evolution of vertebrate reproductive mode. Variation in the intensity of conflicts is hypothesized to have specific impacts on transitions from one mode of reproduction to another and the likelihood that post-zygotic reproductive isolation will evolve within species. In this dissertation I examined how differences in reproductive mode among species of Cyprinodontiformes affect the likelihood of evolutionary transitions and the evolution of reproductive barriers.

Chapter one demonstrated that oviparous species in Cyprinodontiformes have properties that predispose them to the evolution of matrotrophic adaptations following the transition to livebearing. Eggs in these species are capable of acquiring molecules from their surrounding environment and concentrating them within the yolk, most likely via pinocytosis. The ability to actively acquire nutrients from the surrounding environment would serve as a preadaptation to the evolution of matrotrophy following the transition to viviparity, because eggs would already have a mechanism for obtaining any form of provisioning emitted by the mother. Moreover, the ability to move molecules into and potentially out of the egg sets the stage for offspring to manipulate their mothers into providing increased investment. This result provides a potential explanation for why matrotrophy has evolved so readily in viviparous fish, but so infrequently in viviparous squamate reptiles.

Chapter two extended the predictions of the viviparity-driven conflict hypothesis (VDCH), specifically that post-zygotic reproductive isolation evolves more quickly in placental species than in non-placental species. The placental species *P. prolifica* showed

detectable levels of reproductive incompatibilities as a function of interpopulation genetic distance, but no such pattern existed in either non-placental species. The reproductive incompatibilities manifested as strong decreases in offspring body size which would have negative fitness impacts on offspring in a natural setting. The findings are consistent with patterns observed in *H. Formosa*, another placental fish, but the negative epistatic interaction appears to have occurred over traits that determine levels of maternal provisioning instead of offspring development. The findings of this chapter provide strong support for the hypothesis that conflict-driven speciation is more likely in placental species than non-placental species as a function of increased parent-offspring conflicts in the former.

Chapter three examined the effect of reproductive mode on the evolution of reproductive isolation and diversification at macroevolutionary scales. Consistent with the predictions of the viviparity-driven conflict hypothesis and its extensions, I find post-zygotic reproductive isolation to evolve faster among viviparous species than among oviparous species. The transition from lecithotrophy to matrotrophy was not associated with an accelerated rate of post-zygotic barrier formation, but estimated levels of post-zygotic isolation were higher for matrotrophs than for lecithotrophs at all observed interspecific genetic distances. These patterns corroborate the hypothesis that parent-offspring conflicts accelerate how quickly post-zygotic reproductive isolation evolves among taxa. Against my predictions, I did not find stepwise pattern in which speciation rates were slowest for oviparous taxa, intermediate for viviparous lecithotrophs, and fastest in viviparous matrotrophs. However, speciation rates were significantly higher for

viviparous taxa relative to oviparous taxa. The results from this chapter support the predictions of the VDCH as viviparity led to accelerated rates of post-zygotic isolation evolution and subsequently speciation. The impacts lecithotrophy and matrotrophy have on the process speciation are less obvious, and further work is required to determine if the absence of patterns observed in this study are due biological differences or a lack of statistical power.

Accumulating evidence continues to point to the strong relationship between parent-offspring conflicts and the evolution of vertebrate reproductive mode. Conflicts have helped to drive the evolution of more and more complex reproductive adaptations, and have contributed to the vast diversity in vertebrate reproductive systems. In addition, the relationship between conflict and reproductive mode creates differences in the probability that reproductive barriers will evolve among taxa. Conflict is sure to have played a role in the diversification of extant species, but how big of a role remains unknown. Future work will uncover how universal the signature of conflict is in other systems, and how important conflict has been to the origin of species.