ORIGINAL PAPER

Analysis of early embryogenesis in Rainbow and Banded Darters (Percidae: *Etheostoma*) reveals asymmetric postmating barrier

Tamra C. Mendelson · Vance E. Imhoff · M. Kathryn Iovine

Abstract This study examines early embryogenesis in two species of darters, *Etheostoma caeruleum* and *E. zonale* (Teleostei: Percidae), and their hybrids. Results document the course of ontogeny from fertilization until the onset of pigmented eyes. Comparing fertilization and developmental success of conspecific versus heterospecific crosses revealed an asymmetric postmating reproductive barrier. *E. caeruleum* eggs treated with *E. zonale* sperm exhibited fertilization and developmental success similar to that of conspecific crosses. In contrast, *E. zonale* eggs treated with *E. caeruleum* sperm exhibited reduced fertilization relative to conspecific crosses and abnormal development. Development in this latter cross was compromised at all stages, but appeared to be concentrated around epiboly, or cell migration. As epiboly represents the stage of ontogeny when zygotic genes of both species jointly contribute to embryogenesis, results provide insight into the genetic mechanisms underlying postmating barriers in *Etheostoma*. Finally, the observed asymmetry in fertilization success is consistent with predictions based on the behavioral ecology of these species. *Etheostoma zonale* spawn in the open water column, whereas *E. caeruleum* bury their eggs under the substrate. The observed fertilization barrier may have therefore resulted from selection favoring increased fertilization specificity in *E. zonale*.

Keywords Embryogenesis · *Etheostoma* · Speciation · Postmating-prezygotic isolation · Postzygotic isolation

Introduction

The study of speciation focuses largely on the evolution of reproductive isolating barriers, which are classified according to when during the reproductive cycle gene flow is prevented. Premating barriers restrict individuals of different species from mating; postmating-prezygotic barriers restrict fertilization after mating; and postzygotic barriers cause a reduction in hybrid fitness after fertilization (e.g., Coyne and Orr 2004). Postzygotic barriers are further classified as either 'intrinsic,' when genetic incompatibilities prevent normal hybrid development, or 'extrinsic,' when phenotypically intermediate hybrids are maladapted to the environment (Coyne and Orr 2004). Arguably the most well studied reproductive barriers are intrinsic postzygotic barriers, and these incompatibilities may cause a reduction in hybrid fitness at any developmental stage. Determining precisely when during ontogeny

T. C. Mendelson (✉) · V. E. Imhoff · M. K. Iovine
Department of Biological Sciences, Lehigh University, Bethlehem, PA 18015, USA
e-mail: tcm204@lehigh.edu

hybrid fitness is reduced is fundamental to identifying both the genetic changes, and ultimately the selective forces, driving the evolution of reproductive isolation.

Organisms with a transparent chorion, the outer membrane enclosing a developing embryo, provide invaluable opportunities to visualize early development. Direct observation of fertilization and early development can determine whether species are isolated by fertilization (i.e., postmating-prezygotic) barriers versus intrinsic postzygotic barriers that prevent successful hybrid development. This distinction can be exceedingly difficult to determine in internally fertilizing animals or those with opaque chorions (Lorch and Servedio 2005). Externally fertilizing, aquatic organisms with transparent chorions are especially suited for this type of study. Not only is development readily observable, but postmating and postzygotic barriers may be particularly critical to maintaining species boundaries, as gametes are often released by multiple species into a shared environment (Palumbi 1998; Howard 1999).

Darters (Teleostei: Percidae) are a group of externally fertilizing freshwater fish with transparent chorions. The group consists of four recognized genera, together comprising approximately 20% of North American ichthyofauna (Page 1981; Page 1983; Page 2000). Multiple darter species commonly reside within a single community and many exhibit overlapping habitat preferences (Page 1983; Stauffer et al. 1996; Welsh and Perry 1998); thus, behavioral and/or postmating reproductive barriers play a primary role in restricting interspecific gene flow within these communities (Winn 1958; Mendelson 2003a, 2003b).

The objective of this study was to describe early embryogenesis in rainbow darters, *Etheostoma caeruleum*, banded darters, *E. zonale*, and their hybrids, testing whether, and when during ontogeny, hybrid fitness breaks down. Previous studies suggest that hybrids of closely related darter species suffer little to no fitness consequences relative to pure species offspring, such that hybrid inviability likely only occurs between distantly related species (Hubbs 1967; Mendelson 2003a). *E. caeruleum* and *E. zonale* are distantly related members of the genus and are more likely, therefore, to exhibit some degree of hybrid inviability. *E. caeruleum* is a geographically widespread and abundant member of the subgenus *Oligocephalus* (Page 1981), with a preferred habitat of moderate to fast flowing riffles. The taxonomy of *E. zonale* is more problematic, having been assigned to subgenera *Nanostoma* (Page 1981) and *Etheostoma* (Bailey and Etnier 1988), though more recent molecular analyses support the latter hypothesis (Porter et al. 2002). Banded darters are also geographically widespread and frequently co-occur with rainbow darters. Hybridization between these syntopic species in nature has never been reported, indicating strong reproductive barriers to gene flow.

We describe embryogenesis from the first cell division until the appearance of pigmented eyes, demonstrating that development in *E. caeruleum*, *E. zonale*, and hybrids of *E. caeruleum* mothers follow similar trajectories. In contrast, hybrids of *E. zonale* mothers exhibited reduced fertilization success and abnormal or no cell division. None was observed to develop into a fully formed embryo, and hybrid breakdown was evidenced at all stages of development. Our results therefore provide evidence of both fertilization (i.e., postmating-prezygotic) and intrinsic postzygotic barriers between these species. In addition, by comparing fertilized with unfertilized eggs, this study provides a clear indicator of fertilization success, allowing us to distinguish between barriers that occur before and after fertilization in *Etheostoma*.

Materials and methods

Collection

Adult specimens of *E. caeruleum* were collected from North Deer Creek, near its confluence with French Creek in Venango County, Pennsylvania (5 mi NW Franklin). *Etheostoma zonale* were collected from French Creek, Crawford County, Pennsylvania (3 mi N Meadville), and from Middle Fork Red River, Powell County, Kentucky (7 mi E Stanton). *E. zonale* (French Creek) and *E. caeruleum* (Deer Creek) are

therefore effectively sympatric, and *E. zonale* (Red River) is allopatric with respect to these populations. Specimens were transported in aerated coolers to the laboratory at Lehigh University, Bethlehem, Pennsylvania. Collections from French and Deer Creek were kept in coolers overnight at a temperature of approximately 15°C (±3°C). *E. zonale* from the Red River, collected 2 weeks prior, were maintained in a recirculating aquarium system at a temperature of 17°C (±1°C) and fed live blackworms and frozen bloodworms twice daily.

In vitro fertilization

Four cross types, two conspecific and two reciprocal heterospecific, were performed at separate times over the course of 2 days. For each cross type, randomly selected adult specimens were anesthetized in an appropriate dilution of MS222 (Ethyl 3-aminobenzoate, methanesulfonic acid salt, 98%). Females were briefly dried with a Kim-wipe and gently squeezed to release eggs into separate, dry 35 mm Petri dishes. At the same time, a different investigator squeezed male ejaculate into a Petri dish containing approximately 5 ml of water. Ejaculate was lightly mixed into the water, and 500 µl of that mixture was transferred via pipette to each egg clutch. After one minute, approximately 1 ml water was added to each clutch and the mixture was swirled for 30 s.

For the *E. caeruleum* × *E. caeruleum* conspecific cross, 2 females were fertilized using ejaculate of 1 male. An additional clutch of *E. caeruleum* eggs was sham fertilized using 500 µl water as a negative control. For the *E. zonale* × *E. zonale* conspecific cross, 3 females from French Creek were fertilized using the ejaculate of 1 male, also from French Creek. For the *E. caeruleum* female (f) × *E. zonale* male (m) hybrid cross, 2 females were fertilized by 1 male from French Creek. For the *E. zonale* f × *E. caeruleum* m hybrid cross, 2 females from French Creek were fertilized by 1 male. Upon observing reduced fertilization success, strikingly abnormal development, and mortality in this latter cross type, 3 additional *E. zonale* females were fertilized by an *E. caeruleum* male on the following day to confirm the result. *E. zonale*

females from French Creek were not available at that time; we therefore used Red River females, which inhabit the same (Ohio) river drainage. An additional *E. caeruleum* female was also fertilized during this cross to control for viability of the male's sperm. For these crosses, 200 µl sperm mixture was used per clutch to ensure a sufficient amount of ejaculate for 4 clutches. This difference in ejaculate amount should not affect our results, as minute amounts of ejaculate are sufficient to fertilize large numbers of eggs (e.g. Hubbs and Strawn 1957).

Incubation

Eggs were incubated in a 600 l closed, recirculating system set at a temperature of 17°C (±1°C). Each cross type was kept in a separate 3 l tank on the system. The pH was approximately 7.6, conductivity was 100 ppm (~145 μS) and general hardness was 100 ppm. At the time of collection from French Creek the pH was 7.8 and the pH from Deer Creek was 8.4. The conductivity for both locations was 100 ppm and the general hardness from both locations was 68 ppm. These abiotic parameters exhibit natural daily and seasonal fluctuations; conditions in the laboratory fell within the natural range of variation.

Photography

Initial photographs of a haphazard selection of four eggs per clutch were taken within 10 min of fertilization (or sham). After the eggs adhered to the Petri dish (eggs generally adhered within 10–30 min), we monitored the development of four marked eggs per clutch, including the sham control. Eggs were marked by drawing on the bottom exterior of the Petri dish. Eggs were visualized using a Nikon SMZ 1500 stereoscopic zoom microscope at 3× magnification. Digital photographs were taken with a Nikon DXM 1200 digital camera using Automatic Camera Tamer (ACT-1) Software, Version 2.63.

Marked eggs were photographed once every hour (±10 min) for the first 62 h, then at 72 h, and daily thereafter until day 9. Embryos that died during this time were photographed until failure and then removed to deter fungal growth. If an
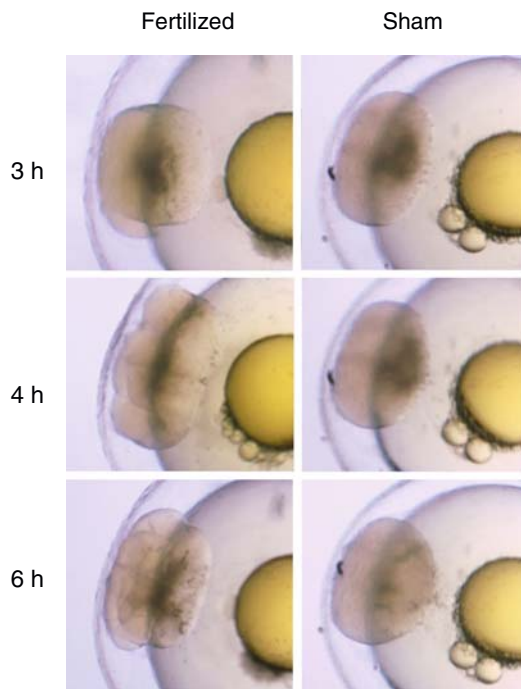
Fertilized   Sham

3 h

4 h

6 h

Fig. 1 Fertilized and unfertilized eggs of *E. caeruleum* at 3 h, 4 h, and 6 h post-treatment. Both fertilized and unfertilized eggs exhibit expansion of the chorion and development of a single cell. This cell did not undergo cleavage in unfertilized eggs. Fertilized egg is pictured in 2-cell, 4-cell, and 16-cell cleavage stage. All eggs contained the characteristic large oil droplet in the middle of the yolk

egg failed or became unadhered (and therefore no longer identifiable), another developing, adhered egg was selected and marked (#5, #6, etc.) to ensure photographs of at least 4 unique eggs per clutch per hour. To photograph, the Petri dish was removed from the system, photos were taken, and the dish was replaced. Due to the necessary removal of Petri dishes from the closed system for photographing, fluctuations larger than 1 degree in temperature were possible.

Results

Fertilization

Eggs in both the fertilized and unfertilized (sham) clutches of *E. caeruleum* exhibited an expansion of the chorion from the egg within 10 min, and all developed a single cell within 1 h (Fig. 1). This cell was larger in the fertilized ($x = 1.0 \pm 0.10$ mm) versus the sham ($x = 0.86 \pm 0.03$ mm) eggs (assuming equal variances, $t = 2.51$, df = 14, $P = 0.025$; Table 1). No cell division was observed in the sham clutch. Therefore unfertilized eggs were distinguishable from fertilized eggs within 3 h, after fertilized eggs underwent the first cell division.

Early embryogenesis in *E. caeruleum* and *E. zonale*

Early stages of development in *E. caeruleum* and *E. zonale* were documented to establish a frame of reference for normal embryogenesis in conspecific crosses. The first cell division in fertilized eggs at 17°C was observed within 2–3 h. Four-cell stage occurred within the next hour. The rate of cleavage increased to greater than once per hour, with a blastula (128-cell stage) typically observed by hour 8 (Fig. 2). The transition from 'high' stage to 'oblong' stage, when the animal–vegetal axis begins to shorten, was noted when the length of the blastula in direct contact with the yolk began to increase. Oblong stage began at 10–11 h. The transition from oblong to 'sphere' stage was less discrete; therefore the timing of this stage was not estimated.

The onset of gastrulation was identified by the appearance of the germ ring, a distinct and

Table 1 Mean and standard deviation (in mm) of chorion diameter, yolk diameter, and the length of the first cell (long axis). *Etheostoma zonale* eggs from French Creek are shown separately from those of the Red River. Measurements were taken using ImageJ computer software (Abramoff et al. 2004)

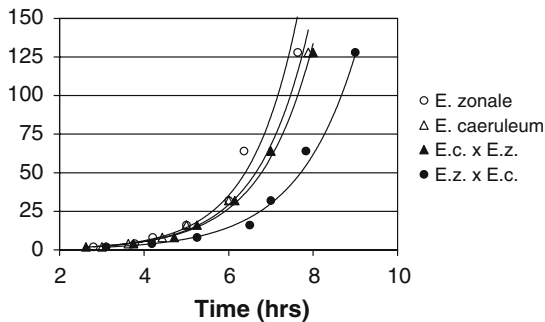|	Chorion	Yolk	First cell
E. caeruleum conspecific	2.5 ± 0.10	2.0 ± 0.07	1.0 ± 0.10
E. caeruleum heterospecific	2.5 ± 0.10	1.9 ± 0.07	1.2 ± 0.12
E. caeruleum unfertilized	2.5 ± 0.12	1.9 ± 0.09	0.86 ± 0.03
E. zonale (French Cr) heterospecific	2.0 ± 0.10	1.8 ± 0.08	0.97 ± 0.11
E. zonale (Red R) heterospecific	2.0 ± 0.10	1.6 ± 0.04	0.93 ± 0.17
E. zonale conspecific	2.1 ± 0.06	1.7 ± 0.06	0.97 ± 0.10

Fig. 2 Time course from first cell division to blastula formation (128-cell stage) in *E. caeruleum*, *E. zonale*, and the reciprocal hybrid crosses. *E. zonale* f × *E. caeruleum* m hybrids included the analysis are restricted to those that completed epiboly and developed somatic tissue. Development in *E. zonale* f × *E. caeruleum* m is delayed relative to that of the other three cross types

thickened band of cells at the migrating end of the cell mass (Kimmel et al. 1995). This band of cells was apparent between 21 and 23 h, either immediately preceding or concurrent with the appearance of the shield. In *E. caeruleum* (and in hybrids of *E. caeruleum* females) an additional 'band' was observed at 12–14 h, concurrent with early sphere stage. This band appeared to result from a line of absent cells at the migrating end of the cell mass, but because there was no associated thickening of the margin, and due to its early appearance, this band does not likely coincide with involution.

The shield was identified as an asymmetric thickening at one end of the germ ring, the thickened side ultimately developing into the dorsal side of the embryo (Fig. 3). The shield typically appeared by hour 23 (Table 2). Epiboly was 50% complete within 27–28 h and 100% complete within 32–34 h (Table 2). The head bud, a triangular mass of cells stemming from the animal pole of the shield, appeared concurrently with 100% epiboly.

Embryos developed with the greater part of the dorsal side down, underneath the yolk, such that it was difficult to identify the precise time at which somites and eyes first formed. Based on a best approximation, somites were apparent between 46 and 51 h and developed in the anterior–posterior direction, and eyes were apparent between 44 and 53 h. Pigmented melanocytes were visible on the yolk between 51–59 h for *E. caeruleum*, 50–54 h for *E. zonale*, and 53–55 h

for *E. caeruleum* f × *E. zonale* m hybrids, and eyes were pigmented black between 5 and 6 days.

Overall hatching success was not calculated, as our original intent was restricted to documenting embryogenesis. Hatching success is approximated, however, by calculating the percentage of photographed eggs that successfully hatched. Based on this criterion, *E. caeruleum* × *E. caeruleum* exhibited 83% hatching success (10 of 12); *E. zonale* × *E. zonale* had 80% success (12 of 15); *E. caeruleum* f × *E. zonale* m had 80% success (8 of 10); *E. zonale* f × *E. caeruleum* m had 0% hatching success (0 of 33). As this study establishes a clear indicator of fertilization success, we will be able in future studies to identify viable eggs (those producing at least a single cell), and to document the number of successfully hatched eggs as a percentage of viable eggs in a given clutch.

Early embryogenesis in *E. zonale* f × *E. caeruleum* m hybrids

The development of hybrid offspring of *Etheostoma caeruleum* females was indistinguishable from that of conspecific crosses. In contrast, hybrid offspring of *E, zonale* females, from both French Creek and Middle Fork of Red River, exhibited reduced fitness at all stages, including a prezygotic fertilization barrier (Fig. 4). Of 33 eggs observed until failure (i.e., until taken over by fungus), 13 failed to undergo the first cell division and were therefore presumed unfertilized; 4 underwent abnormal cell division; 13 underwent abnormal cell migration; and 3 underwent normal cell division and migration but produced deformed embryos that failed prior to hatching. None successfully hatched.

Of the 13 unfertilized eggs, nine were from the French Creek population and four were from the Red River population. Unfertilized eggs remained in single cell stage until failure. The observed fertilization barrier likely is not due to low sperm viability, as the sperm used to fertilize Red River females was used on an additional *E. caeruleum* clutch, yielding high fertilization and hatching success.

Four eggs underwent abnormal cell division. Eggs in this group failed to undergo the first cell division, yet the single cells underwent movement

Fig. 3 Stages of development in (**a**) *E. caeruleum*, (**b**) *E. caeruleum* f × *E. zonale* m hybrids, and (**c**) *E. zonale*. Arrows point to developing shield
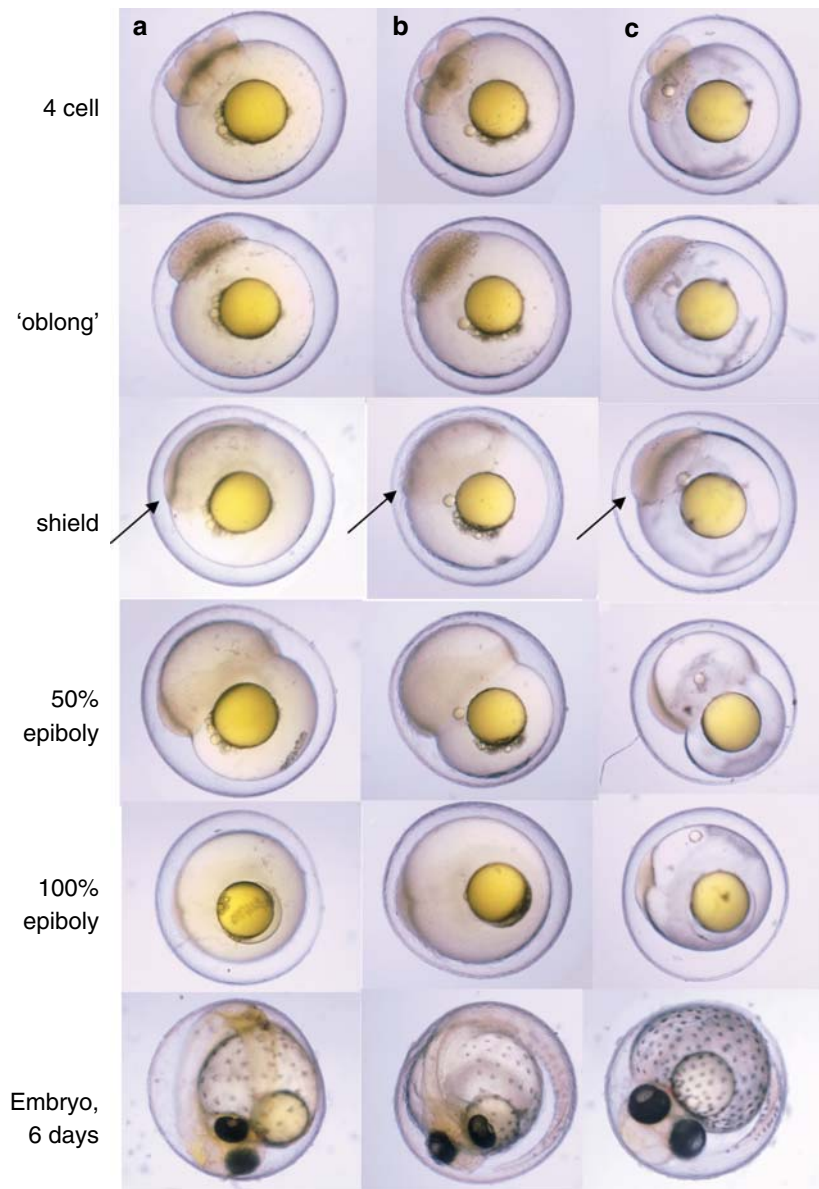

Table 2 Mean and standard deviation of time (hours) until 3 successive stages of ontogeny

	Shield	50% epiboly	100% epiboly
E. caeruleum	21.5 ± 0.53	27 ± 0.53	31.6 ± 0.53
E. zonale	22.0 ± 0.77	27.2 ± 1.17	33.3 ± 0.90
E. caeruleum f × *E. zonale* m *hybrid*	21.8 ± 1.28	27.1 ± 0.83	32.1 ± 0.83

reminiscent of epiboly, appearing to migrate around the yolk. These eggs died within 36 h. One explanation is that these eggs were unfertil-ized and this 'migration' was gynogenetic; how-ever, this behavior was never observed in unfertilized eggs and thus may represent a prob-lem with cell cleavage.

Thirteen eggs exhibited abnormal cell migra-tion. Four of these underwent premature epiboly, with essentially normal cell division up to 16–64 cell stage, but then beginning cell migration be-fore reaching the blastula stage. All died before 100% epiboly. Six other eggs in this abnormal epiboly group exhibited mostly normal cell divi-sion leading to the normal formation of a blastula
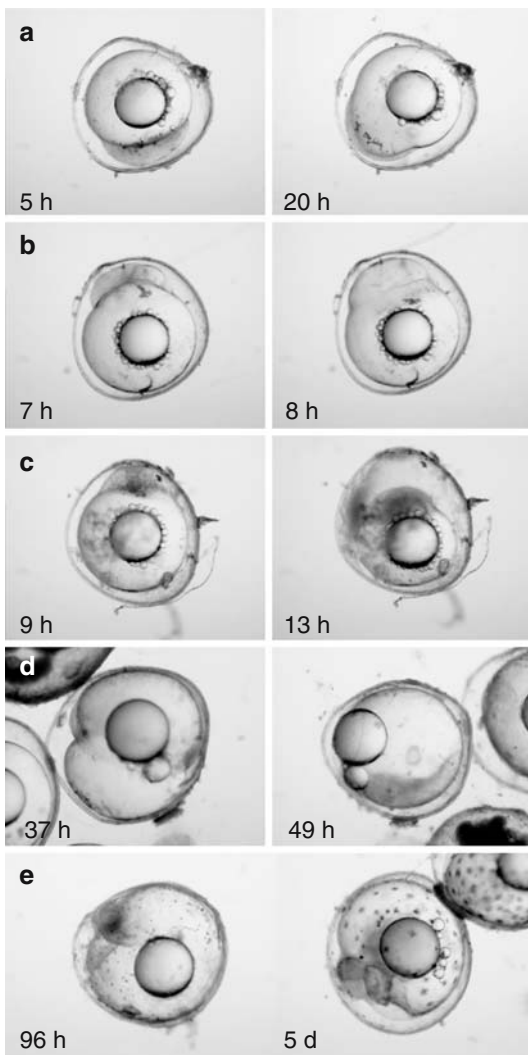
Fig. 4 Developmental abnormalities observed in *E. zonale* f × *E. caeruleum* m hybrids. (**a**) Migration without normal cell division, (**b**) Premature migration, (**c**) Abnormal or absent migration, (**d**) Delayed migration, deformed embryo, (**e**) Normal migration, deformed embryo

but failed to either begin or complete cell migration. Three other eggs exhibited delayed but otherwise apparently normal cell migration, showing 50% epiboly at 37 h (as compared to 27–28 h in conspecific crosses). These resulted in the formation of a deformed embryo that did not hatch.

Finally, a last group of eggs ($n = 3$) underwent apparently normal cell division and migration but nonetheless produced deformed, asymmetric embryos that failed before hatching. Thus, defects

were observed in all stages of development in *E. zonale* f × *E. caeruleum* m hybrids.

Discussion

Embryogenesis

A primary objective of this study was to describe early embryogenesis in two species of darters. Previous studies of development in *Etheostoma* have described later stages of ontogeny (Paine 1984; Simon et al. 1995). Here, we provide a detailed description of embryogenesis in *E. caeruleum* and *E. zonale* from the first cell division through the appearance of pigmented eyes. Although the rate of development will vary with temperature, this study serves to document the chronological order in which early developmental stages occur in *Etheostoma*. In general, development proceeds as in the well-studied zebrafish, *Danio rerio*, with some exceptions (Kimmel et al. 1995). First, likely due to the difference in temperature (17°C for darter species versus 28°C typically reported for zebrafish), there is a significant difference in the overall rate of development. Zebrafish embryos achieve the first cell division by 40 min (2–3 h in darters), and the following cell divisions occur once every 15 min (about once per hour in darters) (Kimmel et al. 1995). A second difference appears to be in the relative onset of involution, or gastrulation, which occurs earlier in darters. Zebrafish embryos proceed past 50% epiboly before development of the germ ring and subsequently the embryonic shield. In contrast, darter embryos began involution by approximately 25% epiboly, and the shield is evident well before 50% epiboly is reached. The consequences of this difference in timing are not clear; however, it is of interest to note that some of the heterospecific embryos that failed to develop were the result of premature ($n = 4$) or delayed ($n = 3$) epiboly.

Fertilization

Another main objective of the study was to document the activity of unfertilized eggs. A clear indicator of fertilization is necessary for

estimating fertilization success in experimental crosses, and thus for determining whether hybridization (in relevant cases) is prevented by barriers to fertilization, or, rather, by postzygotic barriers that reduce hybrid fitness after fertilization. Comparing the activity of fertilized eggs with that of sham controls, we conclude that the 4-cell stage is a reliable indicator of fertilization in these species. We identify the 4-cell stage as a more reliable indicator than the 2-cell stage because, although no sham egg in our study was observed to reach the 2-cell stage (Fig. 1), cells of unfertilized zebrafish eggs have been observed occasionally to undergo a single, asymmetric cleavage (M.K. Iovine, personal observation). Because some of the *E. zonale* f × *E. caeruleum* m eggs in our study underwent an asymmetric first cleavage qualitatively indistinguishable from that of some unfertilized zebrafish eggs, the 4-cell stage provides a better indication of fertilization. Of course, the most reliable indicator of fertilization is a genetic analysis of diploidy. Until such a test is conducted, we argue the 4-cell stage provides the best proxy for assessing fertilization in *Etheostoma* embryos.

This work thus extends that of Hubbs and Strawn (1957) and Hubbs (1967), who examined development in multiple darter species and used the appearance of pigmented eyes to indicate fertilization. Our results demonstrate that fertilization should be assessed at an earlier stage. The majority of hybrid failure in our study occurred before the onset of pigmented eyes, but after the formation of a blastula. Using pigmented eyes as an indicator of fertilization may, therefore, fail to identify successful fertilization, thereby incorrectly implicating fertilization barriers, rather than postzygotic barriers, as the primary barriers to gene flow. Indeed, Hubbs (1967) reported evidence of a fertilization (i.e., postmating-prezygotic) barrier between some species but little evidence of hybrid inviability.

Hybrid development

Our final objectives were to characterize the development of hybrid embryos and to determine when during ontogeny hybrid fitness breaks down. Documenting the fate of hybrid crosses revealed both postmating-prezygotic (i.e., fertilization) and postzygotic (i.e., developmental) reproductive barriers between *E. caeruleum* and *E. zonale*. *Etheostoma caeruleum* eggs crossed with *E. zonale* sperm were fertilized at rates typical of conspecific crosses and yielded normally developing embryos. However, the reciprocal cross exhibited dramatically reduced fertilization success, and fertilized eggs subsequently failed to produce a viable embryo. Unfertilized eggs were observed in all five clutches of *E. zonale* f × *E. caeruleum* m, and one clutch had no fertilized eggs. A reasonably strong postmating-prezygotic fertilization barrier thus appears to exist between these species, although in one direction only.

A strong postzygotic barrier was also observed, as no fertilized egg of the *E. zonale* f × *E. caeruleum* m clutch developed into a normal embryo. Fitness was reduced at several stages, including blastula formation, cell migration, and body formation. The ontogenetic stage presenting the biggest obstacle to development appeared to be cell migration, as the majority of fertilized eggs (13 of 20) exhibited essentially normal cell cleavage but failed to initiate or complete normal epiboly. These results are consistent with the observation that 'maternal [non-zygotic] influence typically slows at gastrulation and is not likely to be preponderant beyond hatching' (Hubbs 1967). Further, in zebrafish and *Xenopus*, zygotic (including paternal) transcription begins approximately at mid-blastula transition, before gastrulation but around the timing of the first cell movements (Kane and Kimmel 1993). Interspecific incompatibilities therefore appear to manifest in hybrid offspring when zygotic genes of both species jointly contribute to the process of cell migration. Results therefore support the hypothesis that epistatic interactions contribute to proper cell migration and development.

We further observed that eggs from the allopatric population had lost most of the yellow color to their yolks, and their yolks were significantly smaller than the sympatric population ($t = 6.95$, df $= 18$, $P < 0.0001$; Table 1), possibly because these fish had been held in the laboratory for two weeks prior to fertilization (see Hubbs and Strawn 1957). These differences did not

appear to negatively affect hybrid success, as a greater number of allopatric eggs were fertilized and reached a more advanced stage of development as compared to the sympatric clutches.

Asymmetry and behavioral ecology

Asymmetric postmating barriers are a common feature of plants (Tiffin et al. 2001) and animals (references in Zeh and Zeh 2000), and both general and specific explanations have been proposed. In the case of *E. caeruleum* and *E. zonale*, the observed asymmetry, at least in fertilization success, may be explained in the context of their spawning behavior. Darters exhibit at least five distinct spawning modes (Winn 1958) that are largely phylogenetically conserved (Page 1985). *Etheostoma caeruleum* belongs to a clade of 'buriers,' in which females dive into the benthos to spawn, leaving developing eggs buried under the substrate. *Etheostoma zonale* belongs to a clade of 'attachers,' in which females attach eggs to rocks or vegetation. The eggs of attachers are thus exposed to the water column while the eggs of buriers are not. Selection therefore may favor increased fertilization specificity in the eggs of *E. zonale*, for which direct competition between conspecific and heterospecific sperm is more likely. This hypothesis invokes a general prediction that, on average, attachers will be more likely to exhibit fertilization specificity than buriers; however, sufficient data are not yet available to test this prediction.

Conclusions

This study describes the early stages of embryogenesis in darters, from the first cell division to the onset of pigmented eyes. Early development in *Etheostoma* is largely similar to that of the model organism *Danio rerio*, with minor exceptions. This study also identifies a reliable indicator of fertilization success, critical for distinguishing between pre- and post-zygotic reproductive barriers. Finally, both postmating and postzygotic barriers were observed. *E. zonale* eggs crossed with *E. caeruleum* sperm exhibited reduced fertilization and zero hatching success. The majority of developing hybrids in this cross failed in the epiboly phase, consistent with the hypothesis that proper cell migration requires the contribution and successful interaction of genes from both parents. This study therefore provides a critical foundation for extended research into the genetic mechanisms and selective forces governing the evolution of postmating reproductive barriers in *Etheostoma*.

References

Abramoff, MD, Magelhaes PJ, Ram SJ (2004) Image processing with imageJ. Biophotonics Int, 11(7):36–42

Bailey RM, Etnier DA (1988) Comments on the subgenera of darters (Percidae) with descriptions of two new species from southcentral United States. Misc Publ Univ Michigan Museum Zool 175:1–48

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Inc., Sunderland, Massachusetts

Howard DJ (1999) Conspecific sperm and pollen precedence and speciation. Ann Rev Ecol Syst 30:109–132

Hubbs C (1967) Geographic variations in survival of hybrids between etheostomatine fishes. Bull Texas Memorial Museum 13:1–72

Hubbs C, Strawn K (1957) Survival of F1 hybrids between fishes of the subfamily Etheostominae. Journal of Experimental Zool 134: 33–62

Kane DA, Kimmel CB 1993. The zebrafish midblastula transition. Development 119: 447–456

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203: 253–310

Lorch PD, Servedio MR (2005) Postmating-prezygotic isolation is not an important source of selection for reinforcement within and between species in *Drosophila pseudoobscura* and *D. persimilis*. Evolution 59:1039–1045

Mendelson TC (2003a) Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae: *Etheostoma*). Evolution 57:317–327

Mendelson TC (2003b) Evidence of intermediate and asymmetrical behavioral isolation between Orangethroat and Orangebelly darters (Teleostei: Percidae). Am Midland Nat 150:343–347

Page LM (1981) The genera and subgenera of darters (Percidae, Etheostomatini). Occasional Papers of the Museum of Natural History, University of Kansas 90:1–69

Page LM (1983) Handbook of darters. TFH Publications, Saddle Brook, New Jersey

Page LM (1985) Evolution of reproductive behaviors in Percid fishes. Illinois Nat Hist Surv Bull 33:275–295

Page LM (2000) Etheostomatinae. In: Craig JF (eds) Percid fishes: Systematics, ecology and exploitation. Blackwell Science Ltd., Oxford, pp 225–253

Paine MD (1984) Ecological and evolutionary consequences of early ontogenies of darters (Etheostomatini). Environ Biol Fish 11:97–106

Palumbi SR (1998) Species formation and the evolution of gamete recognition loci. In: Howard DJ, Berlocher SH (eds) Endless forms: Species and speciation, Oxford University Press, New York, pp 271–278

Porter BA, Cavender TM, Fuerst PA (2002) Molecular phylogeny of the snubnose darters, subgenus *Ulocentra* (Genus *Etheostoma*, Family Percidae). Molecular Phylogenetics and Evolution 22:364–374

Simon TP, Brandt TM, Graves KG, Whiteside BG (1995) Ontogeny and description of eggs, larvae, and early juveniles of the fountain darter, *Etheostoma fonticola*. Southwestern Nat 40:208–215

Stauffer JR, Boltz JM, Kellogg KA, van Snik ES (1996) Microhabitat partitioning in a diverse assemblage of darters in the Allegheny River system. Environ Biol Fish 46:37–44

Tiffin P, Olson MS, Moyle LC (2001) Asymmetrical crossing barriers in angiosperms. Proc Roy Soc London Ser B 268:861–867

Welsh SA, Perry SA (1998) Habitat partitioning in a community of darters in the Elk River, West Virginia. Environ Biol Fish 51:411–419

Winn HE (1958) Comparative reproductive behavior and ecology of fourteen species of darters (Pisces: Percidae). Ecol Monogr 28:155–191

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