

Reproductive isolation between two darter species is enhanced and asymmetric in sympatry

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(Received 28 August 2013, Accepted 30 January 2014)

Robust reproductive isolation was found between the rainbow darter *Etheostoma caeruleum* and the orangethroat darter *Etheostoma spectabile*, as more offspring were produced when conspecific males and females were crossed as compared with heterospecific crosses. Furthermore, fewer eggs resulted from heterospecific crosses involving sympatric *E. spectabile* females than those using allopatric *E. spectabile* females, while a similar pattern was not observed in heterospecific crosses using *E. caeruleum* females. These results suggest that reinforcement, *i.e.* selection for pre-zygotic reproductive barriers driven by reduced hybrid fitness, may have contributed to the evolution and maintenance of reproductive barriers between these potentially hybridizing species in sympatry.

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Key words: behavioural isolation; *Etheostoma caeruleum*; *Etheostoma spectabile*: hybridization; orangethroat darter; rainbow darter.

INTRODUCTION

Increased avoidance of heterospecific mating in sympatry, *i.e.* heightened premating isolation, has been documented across a variety of animal and plant taxa, suggesting it may be widespread (Butlin, 1987, 1989; Coyne & Orr, 1989; Howard, 1993). This pattern has generally been attributed to reinforcement, wherein pre-zygotic reproductive barriers are strengthened by natural selection against heterospecific mating, due to the cost of producing unfit hybrid offspring. Reinforcement is of much interest in evolutionary biology because it provides a mechanism by which natural selection can directly drive speciation (Dobzhansky, 1940; Blair, 1955; Servedio & Noor, 2003). Although the role of reinforcement in speciation has been historically controversial, it is supported by a growing body of theoretical and empirical research (Noor, 1995; Rundle & Schluter, 1998; Nosil *et al.*, 2003; Jaenike *et al.*, 2006; Nosil & Yukilevich, 2008; Lemmon & Lemmon, 2010; Matute, 2010). With regard to reinforcement as a process of speciation, the populations of related species in sympatry must be capable of producing fertile hybrids (Butlin 1987, 1989). If there is no possibility of gene flow between them, speciation would already be complete and reinforcement cannot occur.

Among vertebrates, teleosts potentially represent fruitful subjects for the study of reinforcement because particularly high frequencies of natural hybridization have been

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reported in many clades (Hubbs, 1958; Schwartz, 1972; Scribner *et al.*, 2001). For example, reinforcement may have contributed to the divergence of benthic and limnetic three-spined sticklebacks *Gasterosteus aculeatus* L. 1758: mating preferences for conspecifics are more pronounced in sympatric populations of benthics and limnetics than in allopatric populations (Rundle & Schluter, 1998), and benthic–limnetic F1 hybrid males are fertile, but less able to compete for females than the parental species (Vamosi & Schluter, 1999).

Darters (Percidae: Etheostomatinae) are among the most diverse groups of freshwater fishes in North America, containing > 200 species as well as numerous sub-species and geographic races (Page, 1983). In Mendelson (2003), multiple reproductive isolating barriers and genetic distance were measured between 13 pairs of darter species, and behavioural isolation was found to have evolved faster and occasionally to completion. In contrast, post-zygotic isolating barriers have evolved more slowly and remained incomplete (*i.e.* F1 hybrids have non-zero fitness). Additionally in Mendelson *et al.* (2007), behavioural isolation between the Christmas darter *Etheostoma hopkinsi* (Fowler 1945) and the redband darter *Etheostoma luteovinctum* Gilbert & Swain 1887 was stronger than pre-zygotic post-mating barriers such as gametic incompatibility (*i.e.* egg–sperm incompatibility). These data suggest that the evolution of pre-mating or behavioural isolation has played an important role in darter speciation. Reinforcement as a possible factor in darter speciation, however, has received little attention. Darter speciation is thought to have been mainly allopatric, based on the generally non-overlapping distribution pattern of sister species pairs (Wiley & Mayden, 1985; Near *et al.*, 2000, 2011; Page *et al.*, 2003). Nevertheless, many darter species occur in sympatry and natural hybridization has been widely documented (Keck & Near, 2009). Given that reproductive isolation between darter species is often incomplete, where species occur in sympatry, there is the potential for heterospecific mating and the strengthening of behavioural isolation by reinforcement.

The rainbow darter *Etheostoma caeruleum* Storer 1845 and the orangethroat darter *Etheostoma spectabile* (Agassiz 1854) are common species with wide, partially overlapping distributions in the eastern U. S. A. They are similar in morphology, behaviour and ecology, and often co-occur in sympatric streams (Winn, 1958; Page, 1983). The two species have the same mating system: during the breeding season, gravid females are followed by males, who attempt to guard them against conspecific rivals. The female buries herself shallowly in the substratum when she is ready to spawn; eggs and sperms are released upon the attendance of a male. Neither territoriality nor parental care is exhibited by these species (Winn, 1958). Bright bluish and reddish breeding colouration is expressed by male *E. caeruleum* and *E. spectabile*, differing primarily in the presence or absence of red on the anal fin (Page, 1983); there is little evidence that male breeding colouration is attractive to females in either species (Pyrone, 1995; Fuller, 2003). Instead, breeding colouration may be involved in male–male competition and species discrimination.

Etheostoma caeruleum and *E. spectabile* are members of separate clades within the sub-genus *Oligocephalus* (Lang & Mayden, 2007). On the basis of museum and literature records, *E. caeruleum* and *E. spectabile* are the darter species most likely to hybridize with other darters, and *E. caeruleum*–*E. spectabile* hybrids are the most common type of natural darter hybrid (Keck & Near, 2009). Furthermore, the entire mitochondrial genome of the current darter *Etheostoma uniporum* Distler 1968, a member of the *E. spectabile* clade, has been replaced by that of *E. caeruleum* (Ray *et al.*,

2008; Bossu & Near, 2009). Thus there is gene flow between the *E. caeruleum* and *E. spectabile* lineages, and consequently the potential for reinforcement to have affected the evolution of behavioural isolation between these species. In this study, a series of spawning trials were conducted with *E. caeruleum* and *E. spectabile*, so as to (1) determine the extent of reproductive isolation between these species and (2) look for evidence of reinforcement by comparing the strength of isolation between sympatric and allopatric populations.

MATERIALS AND METHODS

Fishes were caught by kick-seine (net dimensions 106 cm × 122 cm, mesh size 3 mm) in April and May 2011, during the breeding season of *E. caeruleum* and *E. spectabile*. The sympatric site for *E. caeruleum* and *E. spectabile* was a small tributary of the Salt Fork River (Champaign Co., IL; 40·06° N; 88·089° W). The allopatric sites for *E. caeruleum* and *E. spectabile* were Prairieville Creek (Barry Co., MI; 42·426° N; 85·428° W) and Kaskaskia Creek (Champaign Co., IL; 40·141° N; 88·344° W). Although the sympatric and allopatric *E. spectabile* sites are geographically close to one another, they occur in distinct river drainages: the sympatric site is in the Vermillion–Wabash–Ohio River drainage, and the allopatric site is in the Kaskaskia–Mississippi River drainage. All three sites are narrow (width: <3 m), shallow (depth: <1 m) streams with riffles over mixed sand and gravel substrata (typical grain diameter ranging from <1 to 30 mm).

The spawning trials were conducted in a no-choice format, in which a single male was paired with a single female. This protocol is often employed in studies of pre-zygotic isolation (Noor, 1995; Hatfield & Schluter, 1996; Rundle & Schluter, 1998). A spawning trial consisted of a male and a female being placed in a 38 l aquarium with gravel substratum for 7 days, during which they were allowed to interact freely. No fish was used for more than one trial. Two sets of trials were performed, one using fish from the sympatric populations and the other using fish from the allopatric populations. For each set, there were two conspecific and two heterospecific crosses (♀ *E. caeruleum* × ♂ *E. caeruleum*, ♀ *E. spectabile* × ♂ *E. spectabile*, ♀ *E. caeruleum* × ♂ *E. spectabile*, ♀ *E. spectabile* × ♂ *E. caeruleum*), with each cross replicated four to five times. The experiment thus involved 36 spawning trials divided between eight treatments. The aquaria were kept at 19 °C, under a 14 L:10 D cycle. The fishes were fed daily with frozen chironomid larvae; feeding took place after egg collection and followed the same procedure across all crosses.

The tanks were checked for eggs daily during the spawning trials, and eggs were collected with a siphon. Dead eggs were distinguished from live eggs by their opacity and buoyancy. Live eggs were kept in water-filled plastic tubs, which were treated with methylene blue and placed in an incubator set to 11 °C and a 14 L:10 D cycle. The methylene blue and lower temperature served to inhibit fungal growth. Emerged fry were fed live brine shrimp *Artemia* sp. larvae three times per week. For each spawning trial, the total number of eggs, the number of live eggs and the number of fry that survived until feeding were recorded.

DATA ANALYSIS

As *E. caeruleum* and *E. spectabile* females differed in fecundity, statistical analyses were performed separately for the females of each species ($n = 17$ trials using female *E. caeruleum* and $n = 19$ trials using female *E. spectabile*). Indices of reproductive isolation (R_1) were calculated as $R_1 = 1 - (N_{\text{HC}}/N_{\text{TOT}})^{-1}$, where N_{HC} = fry number from heterospecific crosses and N_{TOT} = total fry number (adapted from Ramsey *et al.*, 2003). An index of 0 would indicate no difference in progeny number between conspecific and heterospecific crosses (no reproductive isolation), while an index of 1 would indicate that progeny resulted from conspecific crosses only (complete reproductive isolation).

Statistical analyses were performed in SAS (v. 9.3, SAS Institute; www.sas.com). The total numbers of eggs collected per spawning trial were compared among crosses using a generalized linear model (GLIMMIX procedure in SAS); as the egg count data were left-skewed due to trials that yielded few or no eggs, the data were fitted to a negative binomial distribution with

a log link function instead of a Poisson distribution. The predictor variables were male identity (conspecific or heterospecific), range (sympatric or allopatric) and their interaction; when a male identity \times range interaction effect was found, *post hoc* comparisons were made between the four cross types using differences of least square means.

To further elucidate the nature of potential reproductive isolation, the proportions of live eggs:total eggs and fry:live eggs were also analysed using ANOVA (GLM procedure in SAS), again for the effects of male identity, range and their interaction. The proportion of eggs released by the female that were successfully fertilized by the male is reflected by live eggs:total eggs, while the proportion of fertilized eggs that successfully developed to hatching is reflected by fry:live eggs. The number of observations in the analyses was largest for total eggs ($n = 17$ for female *E. caeruleum* and $n = 19$ for female *E. spectabile*). Because some females did not spawn any eggs, the number of observations was smaller for the proportion of live eggs:total eggs ($n = 9$ for female *E. caeruleum* and $n = 17$ for female *E. spectabile*). Similarly, because some replicates yielded no live eggs, the sample size for the proportion of fry:live eggs was reduced ($n = 7$ for female *E. caeruleum* and $n = 16$ for female *E. spectabile*).

RESULTS

Reproductive isolation was observed and was particularly strong between *E. caeruleum* and *E. spectabile*. For sympatric populations, R_1 was 0.98 for crosses involving *E. caeruleum* females and 1 for crosses involving *E. spectabile* females, indicating that heterospecific crosses yielded almost no viable offspring. R_1 was lower for allopatric populations: 0.09 for crosses involving *E. caeruleum* females and 0.42 for crosses involving *E. spectabile* females. The low observed R_1 for allopatric *E. caeruleum* females was due to low hatching success in the corresponding conspecific crosses, and thus may be an artefact of the condition of the females sampled for this study.

Both *E. caeruleum* and *E. spectabile* preferred to spawn with conspecific fish, with consistently more eggs being produced by females paired with a conspecific male (Fig. 1 and Table I). On average, 9.7 times more eggs were spawned by *E. caeruleum* females paired with conspecific than heterospecific males. Similarly, on average 1.6 times more eggs were spawned by *E. spectabile* females paired with conspecific than heterospecific males.

Reinforcement, heightened reproductive isolation in sympatry, was also supported, but only in *E. spectabile*. When paired with a heterospecific male, more eggs were laid by *E. spectabile* females from an allopatric population than from a sympatric population, but the same pattern was not present in *E. caeruleum* (Fig. 1). This is borne out by the interaction effect between male (conspecific *v.* heterospecific) and range (sympatric *v.* allopatric), which was significant for *E. spectabile* females, but not for *E. caeruleum* females (Table I).

From the analyses of fertilization success (live eggs:total eggs), no difference was indicated between cross (conspecific *v.* heterospecific) or range (allopatric *v.* sympatric) or their interaction for female *E. caeruleum* (Table I). The mean \pm s.e. proportion of live eggs out of total eggs was 0.97 ± 0.01 (sympatric) and 1.00 ± 0.00 (allopatric) for conspecific crosses, and 0.22 ± 0.22 (sympatric) and 1.00 (allopatric) for heterospecific crosses. In female *E. spectabile*, there was no range or interaction effect but fertilization success was higher in conspecific than in heterospecific crosses (Table I). The mean \pm s.e. proportion of live eggs was 0.98 ± 0.01 (sympatric) and 1.00 ± 0.00 (allopatric) for conspecific crosses, and 0.90 ± 0.10 (sympatric) and 0.51 ± 0.17 (allopatric) for heterospecific crosses. Although the eggs spawned by

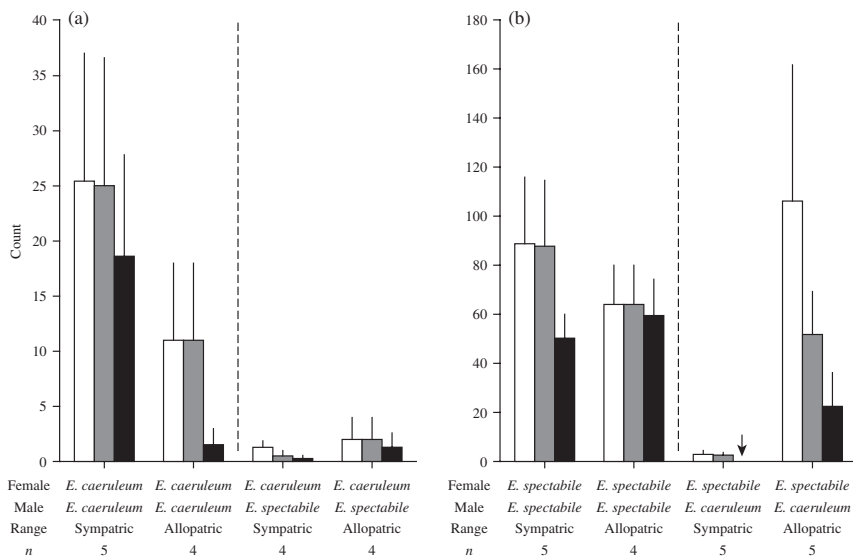


FIG. 1. Mean + s.e. number of total eggs (□), live eggs (▒) and fry (■) from crosses involving (a) *Etheostoma caeruleum* females and (b) *Etheostoma spectabile* females.

E. spectabile females in heterospecific crosses showed decreased fertilization success, it is difficult to attribute this result to any particular reproductive isolating mechanism. This pattern may be explained by males failing to fertilize eggs released by heterospecific females, reduced egg viability due to egg retention by the females in the absence of a conspecific male and intrinsic isolating mechanisms (e.g. gametic incompatibility).

A similar pattern to fertilization success was observed in survival from the fertilized egg to the fry feeding stage. Hatching success in eggs from female *E. caeruleum* were not different across treatments (Table I); the mean ± s.e. proportion of fry that hatched and survived to feeding was 0.72 ± 0.15 (sympatric) and 0.20 ± 0.20 (allopatric) for conspecific crosses, and 0.50 (sympatric) and 0.63 (allopatric) for heterospecific crosses. In *E. spectabile*, hatching success was higher in conspecific v. heterospecific crosses, and in sympatric v. allopatric crosses (Table I). The mean ± s.e. proportion of surviving fry was 0.69 ± 0.09 (sympatric) and 0.92 ± 0.03 (allopatric) for conspecific crosses, and 0.00 ± 0.00 (sympatric) and 0.43 ± 0.24 (allopatric) for heterospecific crosses. Fry that survived to feeding were obtained from all cross types except sympatric female *E. caeruleum* × male *E. spectabile*, indicating that complete hybrid inviability was not present between these species.

DISCUSSION

Generally, fewer viable offspring were produced from heterospecific pairings between *E. caeruleum* and *E. spectabile* than from conspecific crosses, indicating robust reproductive isolation between these species. R_I was nearly complete for sympatric populations, with indices of 0.98 and 1 for *E. caeruleum* and *E. spectabile* females, respectively. R_I was much weaker for allopatric populations, at 0.09 for

TABLE I. Summary of type 3 tests for the effects of male (conspecific or heterospecific), range (sympatric or allopatric) and their interaction on reproductive metrics in *Etheostoma caeruleum* and *Etheostoma spectabile* crosses

	Effect	d.f.	F	P
<i>E. caeruleum</i> females				
Total number of eggs	Male	1,13	6.28	<0.05
	Range	1,13	0.04	>0.05
	Male × range	1,13	0.48	>0.05
Live eggs:total eggs	Male	1,5	4.54	>0.05
	Range	1,5	4.88	>0.05
	Male × range	1,5	4.54	>0.05
Fry:live eggs	Male	1,3	0.21	>0.05
	Range	1,3	0.73	>0.05
	Male × range	1,3	1.97	>0.05
<i>E. spectabile</i> females				
Total number of eggs	Male	1,15	11.94	<0.01
	Range	1,15	15.08	<0.01
	Male × range	1,15	21.80	<0.001
Live eggs:total eggs	Male	1,13	7.14	<0.05
	Range	1,13	3.18	>0.05
	Male × range	1,13	3.75	>0.05
Fry:live eggs	Male	1,12	18.05	<0.001
	Range	1,12	6.03	<0.05
	Male × range	1,12	0.42	>0.05

E. caeruleum females and 0.42 for *E. spectabile* females. Reproductive isolation was enhanced when *E. caeruleum* was crossed with *E. spectabile* from a sympatric population compared to equivalent crosses using allopatric fish.

Reproductive isolation among darters varies substantially depending on species relatedness and the isolating mechanism examined. In the study by Mendelson (2003), behavioural isolation was quantified between 13 pairs of allopatric darter species as ranging from almost none (0) to complete (1). In this study, reproductive isolation between *E. caeruleum* and *E. spectabile* was virtually complete in sympatry, but not in allopatry; this pattern is consistent with natural selection against heterospecific mating, *i.e.* reinforcement (Dobzhansky, 1951; Howard, 1993). Enhanced reproductive isolation in areas of sympatry has also been reported from multiple taxa of mammals, birds, fishes, invertebrates and plants (Butlin, 1987, 1989; Coyne & Orr, 1989; Howard, 1993). Interpreting these data as evidence for reinforcement has been criticized on the grounds that alternative explanations such as environmental effects, may produce similar patterns (Noor, 1999). As only one sympatric and one allopatric population per species was examined in this study, the possibility that the results stem from factors other than reinforcement cannot be excluded. Nevertheless, there is compelling reason to believe that reinforcement may have contributed to the evolution of reproductive isolation between *E. caeruleum* and *E. spectabile*. The physical environments inhabited by the study populations are not substantially different from each other. Furthermore, natural hybrids between the two species have been well documented across their sympatric range (Keck & Near, 2009), and viable F1 hybrids have been artificially produced

(Hubbs & Strawn, 1957; Hubbs, 1958; Linder, 1958); these data demonstrate gene flow between the two species, which is necessary for reinforcement.

Although strong reproductive isolation is present between *E. caeruleum* and *E. spectabile*, the mechanism for this isolation remains unclear. Fertilization and hatching success were reduced when female *E. spectabile* were paired with heterospecific males, but this pattern is insufficient to explain the strength of isolation given that the same was not observed for female *E. caeruleum*. Reproductive isolation thus appears to be largely driven by differences in the total number of eggs collected from conspecific v. heterospecific crosses. A possible explanation for this difference is that most unfertilized eggs were resorbed rather than released when gravid females were faced with the prolonged absence of a suitable (conspecific) male. This is unlikely, however, as large quantities of unfertilized eggs being expelled by gravid females of both species after extended periods of isolation have been observed (pers. obs.), suggesting that female *E. caeruleum* and *E. spectabile* are incapable of resorbing eggs once ovulated. If gravid females must eventually release their eggs, then the reduction in total egg number (as opposed to live egg number) in heterospecific crosses cannot be readily explained by generally recognized pre- or post-zygotic isolating barriers. Instead, higher rates of egg cannibalism in heterospecific crosses may be responsible for these differences in total egg number.

Two non-exclusive factors can potentially lead to increased cannibalism in heterospecific crosses. First, most of the eggs produced in heterospecific crosses may have been non-viable. In several species of nest-building fishes, unfertilized, diseased or dead eggs are selectively consumed as part of brood maintenance (Mrowka, 1987; Smith, 1991). While parental care is not exhibited by either *E. caeruleum* or *E. spectabile* (Winn, 1958), non-viable eggs may nevertheless be recognized and preferentially consumed by these species. Unfertilized eggs have been observed being expelled and immediately consumed by isolated female *E. caeruleum* (pers. obs.). A higher proportion of non-viable eggs in heterospecific crosses may result from male and female behaviour. Heterospecific female darters may not have been recognized as potential mates by males, and thus their eggs were not fertilized. In the swordtail *Xiphophorus birchmanni* Lechner & Radda 1987, males are able to discriminate between conspecific and heterospecific female odour (Wong *et al.*, 2005); as female darters are cryptically coloured, any species recognition by males may also be based on olfactory cues. Conversely, heterospecific male darters may have been discriminated against by females. In banded darters *Etheostoma zonale* (Cope 1868) and splendid darters *Etheostoma barrenense* Burr & Page, 1982, there is a preferential conspecific association between females and males based on visual signals (Williams & Mendelson, 2010); identification of conspecific males in female *E. caeruleum* and *E. spectabile* may similarly be based on species-specific breeding colouration. Egg viability in *E. caeruleum* declines over time: egg fertilization success decreases by *c.* 20% or more if a ready female is prevented from spawning with a conspecific male for 24 h (R. C. Fuller, unpublished data). If heterospecific males were regarded by females as unsuitable mates, the females' clutches may have been withheld long enough for significant declines in egg viability.

The second possible cause of increased egg cannibalism in heterospecific crosses is discrimination against hybrid eggs. Kin recognition based on olfaction has been documented across multiple teleost clades (Olsén, 1992). Moreover, filial cannibalism is modulated by relatedness in the common guppy *Poecilia reticulata* Peters 1859, the

black molly *Poecilia sphenops* Valenciennes 1846 and the fathead minnow *Pimephales notatus* (Rafinesque 1820), with cannibalism occurring at higher rates on unrelated eggs and younger than on own offspring (Loekle *et al.*, 1982; Green *et al.*, 2008). It is possible that hybrid eggs produced between *E. caeruleum* and *E. spectabile*, however viable, lacked essential chemical cues (or expressed inappropriate heterospecific cues), which caused them to be regarded as non-kin by one or both parents and preferentially consumed. As intraspecific and interspecific predation on eggs and fry is widespread among fish taxa (Smith & Reay, 1991; Manica, 2002), selective cannibalism may be an overlooked mechanism for maintaining reproductive isolation in the face of heterospecific mating.

Curiously, reproductive isolation in sympatry *v.* allopatry was greater for *E. spectabile* but not *E. caeruleum*. Compared to conspecific crosses, fewer eggs were spawned by sympatric *E. spectabile* females paired with heterospecific males, whereas no such reduction was observed in allopatric *E. spectabile* females. In contrast, fewer eggs were spawned by both allopatric and sympatric *E. caeruleum* when paired with heterospecific males. This pattern suggests that enhanced reproductive isolation may be asymmetrical: if females are responsible for behavioural species discrimination, then reinforcement would seem to have acted on *E. spectabile*, but not *E. caeruleum*. Asymmetrical reproductive character displacement has been reported in a number of different taxa, and may result from differing selection on reproductive isolation in the two species (Bordenstein *et al.*, 2000; Pfennig & Simovich, 2002; Smadja & Ganem, 2005; Cooley *et al.*, 2006). For example, if one species were more abundant than the other within the sympatric zone, the selective pressure for evolving species recognition would be stronger for the less abundant species due to the increased probability of heterospecific mating (Cooley, 2007). Unfortunately, the selective forces that drive the evolution of isolating mechanisms in darters are not well understood. While this study suggests that there may be selection against heterospecific mating between *E. caeruleum* and *E. spectabile*, the form of this selection and whether it differs between species are uncertain.

Hybrid fry were obtained from heterospecific crosses in both directions, indicating that post-zygotic isolation is incomplete between *E. caeruleum* and *E. spectabile*. The results indicated that eggs produced by a female *E. spectabile* paired with a heterospecific male have reduced fertilization and hatching success, and that the same is not true of eggs produced by *E. caeruleum*. Owing to the extremely low number of live eggs obtained from heterospecific crosses overall (except in the allopatric female *E. spectabile* × male *E. caeruleum* cross), the ability to assess hybrid inviability from this study is very limited. The hatching success of hybrid eggs may also have been disproportionately affected if the raising conditions (*e.g.* water quality) were sub-optimal, as hybrid eggs may exhibit decreased resilience to environmental challenge relative to conspecific eggs (Kozak *et al.*, 2012). As a result, further experiments are needed to characterize post-zygotic isolation between *E. caeruleum* and *E. spectabile*. Previous studies have found little evidence for hybrid inviability between these species in at least the F1 generation (Hubbs & Strawn, 1957; Hubbs, 1958; Linder, 1958).

The diversification of darters is thought to have been driven primarily by allopatric speciation (Wiley & Mayden, 1985; Near *et al.*, 2000, 2011; Page *et al.*, 2003). At the same time, many darter species are found in sympatry and hybridization has also played a significant role in darter evolutionary history. Natural hybrids have been reported involving over 25% of darter species (Keck & Near, 2009), and mitochondrial

introgression has been found in over 12% of darter species (Near *et al.*, 2011). The *E. spectabile* clade has been the subject of multiple introgression events, both ancient and recent. Introgression has resulted in the fixation of heterospecific mitochondrial haplotypes in both the strawberry darter *Etheostoma fragi* Distler 1968 and in *E. uniporum*. In the case of *E. uniporum*, the heterospecific alleles originated from *E. caeruleum*, though conversely the *E. caeruleum* clade shows no evidence of introgression from the *E. spectabile* clade (Ray *et al.*, 2008; Bossu & Near, 2009). The recent formation of a hybrid swarm between *E. spectabile* and the redbelly darter *Etheostoma radiosum* (Hubbs & Black 1941) sub-species *cyanorum* in the Blue River may illustrate the initial process of sympatric interaction and introgression in darters, as the species had previously been largely separated by a dam (Branson & Campbell, 1969; Echelle *et al.*, 1974). On the other hand, introgression does not follow inevitably from darter hybridization; hybrids between the logperch *Percina caprodes* (Rafinesque 1818) and the blackside darter *Percina maculata* (Girard 1859) are well-reported (Keck & Near, 2009), yet there is no evidence of introgression between the two lineages (Near, 2002; Near & Bernard, 2004).

Given the prevalence of past and present hybridization between darter species, there is ample opportunity for reinforcement to act in strengthening reproductive barriers. Furthermore, high levels of hybridization are common among other teleosts as well (Hubbs, 1955; Slavenko, 1957; Schwartz, 1972; Scribner *et al.*, 2001). Although it is premature to conclude that reinforcement has occurred between *E. caeruleum* and *E. spectabile*, reproductive isolation between these species is stronger in sympatry than in allopatry and appears to be at least partially based on behaviour. Furthermore, this pattern was based on crosses between female *E. spectabile* and male *E. caeruleum*, which also exhibited reduced fertilization and hatching success, factors that could have driven selection on enhanced behavioural isolation. The results of this study suggest a promising avenue of study for better understanding speciation and diversity in darters, and in fishes as a whole.

The authors would like to thank E. Berdan, A. Johnson, G. Kozak, M. Schrader and D. Welsh for their helpful comments on the manuscript.

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