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### Authors

Porter, Brady A  
Fiumera, Anthony C  
Avise, John C

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Brady A. Porter · Anthony C. Fiumera · John C. Avise

## Egg mimicry and allopaternal care: two mate-attracting tactics by which nesting striped darter (*Etheostoma virgatum*) males enhance reproductive success

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**Abstract** In a variety of fish species with paternal care of offspring, females prefer to spawn in nests that already contain eggs. This female preference has been hypothesized to explain egg thievery in male sticklebacks, allopaternal care of eggs in minnows, and the evolution of egg-mimicking body features in male cichlids and darters. Here we employ microsatellite-based parentage analyses to evaluate the reproductive success of striped darter (*Etheostoma virgatum*) males that appear to utilize two of these functionally related tactics to entice females to spawn in their nests. In an isolated population (Clear Creek, Ky.), we observed that breeding males develop conspicuous white spots on their pectoral fins. If these spots are egg mimics, as we suspect, then this represents the fourth independent evolutionary origin of egg mimicry documented to date in darters, the first based on pigmentation (as opposed to physical structures), and the first in which the egg mimics vary greatly in number among males. From direct counts of microsatellite genotypes in clutches of embryos, at least 3.8 females contributed to the progeny within a typical nest, and females tended to spawn preferentially with males that were larger and displayed more egg-mimic spots. In another population (Hurricane Creek, Tenn.) without egg mimics, the multi-locus genetic data document that allopaternal care is common, especially among the smallest males who sometimes tend nests containing their own as well as an earlier sire's offspring. Thus, these foster males had adopted egg-containing nests and then successfully spawned with subsequent females. Overall, the genetic data on paternity and maternity, in conjunction with field observations, suggest that egg mimicry and allopaternal care are two mate-attracting reproductive tactics employed by striped darter males to exploit female preferences for spawning in nests with 'eggs'.

**Keywords** Maternity · Mating systems · Molecular markers · Paternity · Reproductive strategies

### Introduction

In a common type of fish mating system, 'bourgeois' males defend territories, prepare spawning substrates, mate polygynously, and provide paternal care to the eggs and developing embryos (Blumer 1979, 1982; Taborsky 1994). In many of these species, females apparently prefer to spawn in nests that already contain eggs. For example, in aquarium trials with male river bullhead sculpins of equitable size, most females preferred to spawn with males whose nests contained eggs, even if these eggs were not sired by the guarding male (Marconato and Bisazza 1986). Similar results have been found in aquarium experiments with the threespine stickleback (Rohwer 1978; Ridley and Retchen 1981), sand goby (Forsgren et al. 1996), fathead minnow (Unger and Sargent 1988), spottail darter (Page 1974), stripetail darter (Page 1975), tessellated darter (Constantz 1985), and fantail darter (Knapp and Sargent 1989), and they have also been observed in nature with green sunfish (Hunter 1963), painted greenling (DeMartini 1987), and garibaldi (Sikkel 1989). Such proclivities for spawning in egg-containing nests may reflect female preferences for males exhibiting good parenting behavior (Sargent 1988), for males with enhanced displays (Jamieson and Colgan 1989), or for nests with decreased predation risk due to a 'dilution effect' (Whoriskey and FitzGerald 1994).

In response to this female preference for egg-containing nests, bourgeois males have evolved at least three mate-attracting tactics that potentially enhance their own reproductive success: egg mimicry, egg thievery, and nest takeovers. The first tactic relies on deception via egg-mimicking body features of the guarding male, whereas egg thievery and nest takeovers involve allopaternal care.

An example of egg mimicry is provided by the mouthbrooding haplochromine cichlids from Lake Vic-

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B. A. Porter (✉) · A. C. Fiumera · J. C. Avise  
Life Sciences Building, Department of Genetics,  
University of Georgia, Athens, GA 30602, USA  
e-mail: porter92@arches.uga.edu  
Tel.: +1-706-5421448, Fax: +1-706-5423910

toria. Lekking males attract females to their spawning pits and stimulate them to spawn by displaying a series of egg-mimicry spots ('egg dummies') on their anal fins (Wickler 1962). A female lays a portion of her eggs over the pit and then quickly snatches them into her mouth. By continuing to display his spots, a male also assures fertilization of those eggs by releasing sperm as the female attempts to gather his egg mimics (Wickler 1962; Goldschmidt 1991). A parallel system has evolved in the cichlid genera *Oreochromis* and *Nyasalapia*, where males display egg mimics in the form of tassels extending from the genital papilla (Wickler 1962, 1997).

Egg mimics have also evolved on the fin rays of several species of egg-clustering darters. In the spawning season, fleshy masses develop on the dorsal rays of males in most darter species in the subgenus *Catonotus* (Page and Swofford 1984; Mayden 1985), and on pelvic rays in members of the darter subgenus *Boleosoma* (Lindquist et al. 1981; Page and Bart 1989). Although these masses may first have served in evolution to protect the eggs from being ruptured during nest guarding (Page and Swofford 1984), in some species they have become greatly enlarged and now resemble eggs in size and color. These egg mimics have been hypothesized to stimulate females to spawn (Page and Swofford 1984; Page and Bart 1989) by taking advantage of their preference for spawning in nests that contain (or appear to contain) eggs (Knapp and Sargent 1989).

However, it has been difficult to test female preference for such egg-mimicry structures in nature because all mature darter males have had similar numbers of egg mimics. In male fantail darters, a single mimic is present on each dorsal fin spine (Knapp and Sargent 1989), and the number of spines is probably inherently constrained during development. A similar situation applies to the guardian darter, where one mimic is displayed on each soft ray of the second dorsal fin (Page and Knouft 2000). Furthermore, the size of the egg mimics in both species is strongly correlated with male body size (Knapp and Sargent 1989; Page and Knouft 2000), making it unclear whether females might prefer larger egg mimics or larger males.

Two tactics involving allopaternal care have also been observed in various fish species. Male sticklebacks routinely steal eggs from neighboring males and place them in their own nests (Wootton 1971; Rico et al. 1992; Mori 1995; Jones et al. 1998). In contrast, male fathead minnows often take over established nests and provide care for the foster eggs, presumably in hopes of becoming more attractive to other spawning females (Unger and Sargent 1988). In the tessellated darter, the largest males often abandon eggs in space-limited nests while searching for new nesting opportunities, and these deserted nests are quickly adopted by smaller 'floater' males who tend the foster eggs and embryos, perhaps in the expectation of future matings with other females (Constantz 1985).

The striped darter (*Etheostoma virgatum*) is distributed sporadically throughout the Cumberland River

System in small to medium sized streams with slab pool habitats. Disjunct populations inhabit the Upper Cumberland in Eastern Kentucky, the Caney Fork in Central Tennessee, and the Lower Cumberland (including the Stones, Harpeth, and Red Rivers) in Tennessee and Kentucky. These populations are interrupted by two closely related allopatric species, *E. smithi* and *E. obeyense*.

Like other members of the subgenus *Catonotus*, male striped darters construct and defend nest sites under rocks to whose ceilings females attach eggs in single-layer clusters. These eggs are tended by the male until hatching. Kornman (1980) studied the life history of striped darters at one of our current study sites (Clear Creek). By comparing counts of eggs deposited in nests to the number of ova in the ovaries, he deduced that several females laid eggs under each nest rock. Darter life-spans were 1–2 years, with reproductive maturity reached by most females by the end of the first year, and by most males at the end of the second year. Males migrated to upstream spawning grounds and developed chromatic coloration and enlarged medial fins in mid- to late March, when water temperatures approached 10°C. Breeding occurred in late March at water temperatures 12–15°C, and continued through early June with peak spawning in April and May. Under a slab rock, each male excavated an area barely deeper than his own body, leaving a downstream entrance that he vigorously defended against conspecific males and potential egg predators. Male guardians are also believed to remove fungal-infected eggs (Constantz 1985) and to tend healthy eggs by mechanical cleaning (Lindquist et al. 1981), and aerating them with their pectoral fins.

The current study was designed to reveal variation in male secondary sexual characters including size, coloration, and pectoral fin markings (believed to be egg mimics) of breeding males in geographically isolated populations of the striped darter. We then employed hypervariable molecular markers to dissect the mating system in two striped darter populations that displayed variation in these male characters. Fertilized eggs and guardian males were collected from more than 50 nests in nature, and genetic parentage and reproductive success were deduced for a subset of these nests using microsatellite assays. As we shall demonstrate, the results on genetic parentage, interpreted in the context of morphological measurements and the basic breeding biology of the species, point toward egg mimicry and allopaternal care as two functionally related tactics by which striped darter males may entice females to spawn in their nests.

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## Methods

### Field collections

Streams were initially surveyed by seine in February 1999. Based on the highest fish abundances, two study localities were selected for focused sampling during that spawning season: Hurricane

Creek at Cobbs Road bridge, Rutherford County, Tenn. (35°43'20"N, 86°17'36"W; sampled from 1 to 2 May), and Clear Creek at County Road 1787, Rockcastle County, Ky. (37°28'34"N, 84°15'44"W; sampled from 9 to 19 May). Each nest rock was surrounded by dip nets and slowly lifted, revealing guarding males and their clutches. The males were preserved in absolute ethanol and their associated clutches were scraped from the rock and preserved in a saturated NaCl solution containing 20% DMSO. Distinct clutches on a nest rock were preserved separately.

#### Microsatellite development and analysis

Microsatellite markers were cloned from a single *E. virgatum* specimen from Clear Creek following standard protocols (Choudhary et al. 1993). Positive clones from radioactive screens were sequenced, and primers flanking microsatellite repeats were designed. DNA was extracted from the adults and under-developed eggs by standard organic protocols (Maniatis et al. 1982). Embryos were classified according to developmental stages (following Cooper 1979), and then sampled for genetic analysis in proportion to their frequency in each nest sample. Developed embryos were dissected away from the eggs and yolk sacks, and their DNA was isolated using an embryo extraction protocol (DeWoody et al. 2000a). Guardian males and 47 embryos from each nest were genotyped at three microsatellite loci: Cv09 5'-TTTCTGGT-CAAGCCTCTGAG-3' + 5'-ACAGGTGGAAGGGTCACATG-3'; Cv12 5'-ACTGTTAGCCCTACTCTG-3' + 5'TTGAAGCAGG-TATTCTCACC-3'; and Cv24 5'-CTTTGACATTGGGTG-CATC-3' + 5'-TCACATAGTGGGTAATGCAC-3'. PCR was conducted in 12 µl reactions containing 1×Promega buffer, 0.1 units Promega Taq polymerase, 1.5 mM MgCl<sub>2</sub>, 0.3 mM of each primer (one with fluorescent label), and 0.1 mM of each dNTP. PCR conditions consisted of 30 s at 95°C, 30 s at an optimal annealing temperature (57°C for Cv24, 55°C for Cv09 and Cv12), and 30 s at 72°C for 32 cycles.

An aliquot of 0.7 to 1.4 µl of PCR product was combined with 2 µl deionized formamide, 0.4 µl GeneScan-500 ROX size standard (Perkin Elmer Applied Biosystems), and 0.5 µl loading buffer, and was then denatured for 2 min at 95°C. The PCR products were resolved in 4.5% denaturing polyacrylamide gels on an ABI 377 automated sequencer, and alleles were sized with respect to electrophoretic mobility. The assignment of genotypes was assisted by the software package Genotyper (ABI).

#### Genetic assessment of parentage

To evaluate the power of the microsatellite markers to resolve parentage, exclusion probabilities (under the 'one parent known' and 'neither parent known' models) were calculated independently for each population using allele frequencies in the sample of presumably unrelated adults (Jamieson and Taylor 1997). Using these allele frequencies as input into the computer simulations by DeWoody et al. (2000b), we determined that sample sizes of about 47 embryos per nest should normally be sufficient to detect the gametic contributions of each dam, assuming that no more than 11 females contributed equally to each darter nest.

Genotypes were scored for embryos and corresponding nest attendant males in 8 of the 21 nests collected from Hurricane Creek, and 11 of the 28 nests from Clear Creek. Typically, the progeny within a nest proved to consist of full or half-siblings, so the maternal contribution to each embryo was evident by subtraction of the paternal alleles. Presumptive 'nest takeover' events were detected when a male guardian was genetically excluded as the sire for all embryos in the nest, or when two embryo clusters were present in the nest and many or all of the most-developed embryos had not been sired by the attendant. For each discrete set of progeny, we conservatively invoked the minimum number of sires and dams necessary to explain the data (Parker and Kornfield 1996). To statistically upward-adjust such values to accommodate

the limited empirical resolution of the genetic markers used, computer simulations (HAPLOTYPES) by DeWoody et al. (2000b) were also employed to estimate the numbers of contributing dams.

To gain further insight on female reproductive strategies, we also incorporated data from Kornman (1980) on the mean number of ripe ova per gravid striped darter female. We tested the hypothesis that a female typically lays all her eggs in one nest by dividing our observed number of eggs per nest by our genetically measured minimum and estimated numbers of dams per nest. These values then were compared by *t*-test to Kornman's (1980) tallies of ova numbers.

#### Measurement of male characters

The following features were recorded for each preserved nest-guarding male: standard body length and depth (measured with dial calipers to the nearest hundredth of a millimeter), body weight (after blotting excess ethanol), and total body coloration. Many darter species are known for the spectacular colors exhibited by males during the breeding season. Due to chromotaxis (the ability of an individual to change color rapidly by contracting and dispersing carotenoid and melanin pigments within integumental chromatophores), color variation among male striped darters is difficult to assess objectively. To circumvent this problem, we extracted and quantified total carotenoids from the integument, and standardized these values by the total surface area of the fish.

Carotenoids were extracted from males with two treatments of absolute ethanol, saponified with 10% KOH overnight, and isolated by partitioning with petroleum ether and water. The organic layers were dehydrated by filtration through NaSO<sub>4</sub> and then concentrated with a rotary evaporator at room temperature. Concentrated carotenoids were suspended in 10 ml. of hexane for spectrophotometric quantification at  $\lambda_{max}$ . Relative concentrations of carotenoids in males were calculated using 2,500 as an average pigment extinction coefficient, as discussed in Davies (1976). To standardize carotenoid measurements, the total carotenoid contents were divided by the (lateral) cylindrical surface areas of each male (calculated from standard length, body height, and body depth). Also recorded were the total numbers of white markings (presumptive egg-mimic spots) for both pectoral fins. A spot was counted when it occupied the entire area between two rays and was readily distinguished from the surrounding fin membrane.

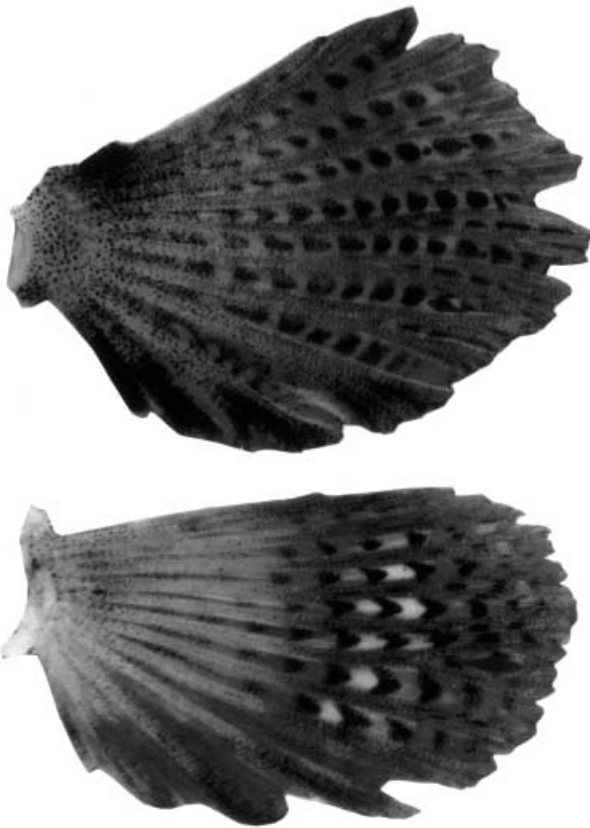
Independent regression analyses and stepwise multiple regression analyses (Minitab release 10 Xtra, 1995) were employed to examine possible correlations between various male characters and reproductive success, the latter estimated from the genetic parentage data in conjunction with total counts of eggs per nest.

## Results

### Adult characteristics

Among nest-guarding males, standard body lengths and weights were highly correlated [ $r^2=0.89$  in Clear Creek ( $df=30$ ,  $F=226.8$ ,  $P<0.001$ ), and  $r^2=0.63$  in Hurricane Creek ( $df=21$ ,  $F=34.3$ ,  $P<0.001$ )], but neither measure of body size was correlated significantly with carotenoid contents [all values of  $r^2<0.05$  ( $P>0.36$ )].

In Clear Creek, the pectoral fins of breeding males had variable numbers of white crescent-shaped spots between the melanic markings, a feature absent in the Hurricane Creek population (Fig. 1). The contrasting white and dark fin pattern of guardian males in Clear Creek was highly conspicuous against the black recesses of the nest rock, and appeared to play a signaling function in conspecific male display. We observed males

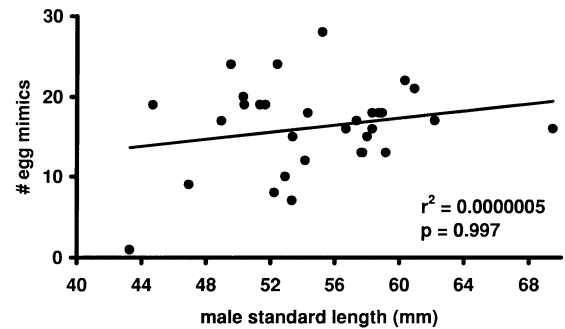


**Fig. 1** Pectoral fins of spawning striped darter (*Etheostoma virgatum*) males. *Above* A fin from Hurricane Creek. *Below* A fin from Clear Creek showing white spots believed to represent egg mimics. A spot was counted when it occupied the entire area between two fin rays, and was readily distinguished from the surrounding membrane. Using these criteria, this fin scores 7 spots

emerging from under nest rocks and orienting head-on toward approaching conspecifics, with pectoral fins splayed out to the sides and rapidly moved in a series of flipping motions. These white pectoral fin spots (averaging  $0.86 \text{ mm} \pm 0.12 \text{ SD}$ ;  $n=100$ ) are similar in size to water-hardened *E. virgatum* eggs ( $1.64 \text{ mm} \pm 0.20$ ;  $n=100$ ) and we provisionally interpret them as egg mimics. The number of egg-mimic spots ranged from 1 to 28 in Clear Creek males, and was not correlated with standard body weight ( $r^2=0.002$ ,  $df=22$ ,  $F=0.04$ ,  $P=0.85$ ), length (Fig. 2), or total carotenoid content ( $r^2=0.006$ ,  $df=21$ ,  $F=0.12$ ,  $P=0.73$ ).

#### Microsatellite markers

Twenty-eight positive clones were isolated and sequenced from the *E. virgatum* genomic library. All clones contained microsatellites, but only three proved to be highly polymorphic and consistently scorable in both populations. Each locus was amplified and scored in a total of 1,070 individuals. In a sample of 44 presumably unrelated adults from Hurricane Creek and 39 from Clear Creek, the numbers of alleles per locus ranged



**Fig. 2** Linear regression of standard length against the number of egg mimics on the pectoral fins of adult striped darter males in the Clear Creek population ( $df=22$ ,  $F=0.00001$ )

**Table 1** Summary of population attributes for three microsatellite loci in the two study populations of adult striped darters

Locus	Number of alleles	$n_e^a$	Heterozygosity		$P_E^b$
			Observed	Expected	
Hurricane Creek ( $n=44$ )					
Cv09	3	2.4	0.58	0.58	0.17
Cv12	10	4.5	0.77	0.78	0.42
Cv24	12	7.1	0.86	0.86	0.56
Mean	8.3	4.7	0.74	0.74	0.79 (0.94)
Clear Creek ( $n=39$ )					
Cv09	19	11.1	0.91	0.91	0.69
Cv12	14	10.0	0.90	0.90	0.66
Cv24	24	12.5	0.92	0.92	0.72
mean	19	11.2	0.91	0.91	0.97 (0.99)

<sup>a</sup> Effective number of alleles [ $1/(1-\text{exp. heterozygosity})$ ] following Hedrick (1985, p. 70)

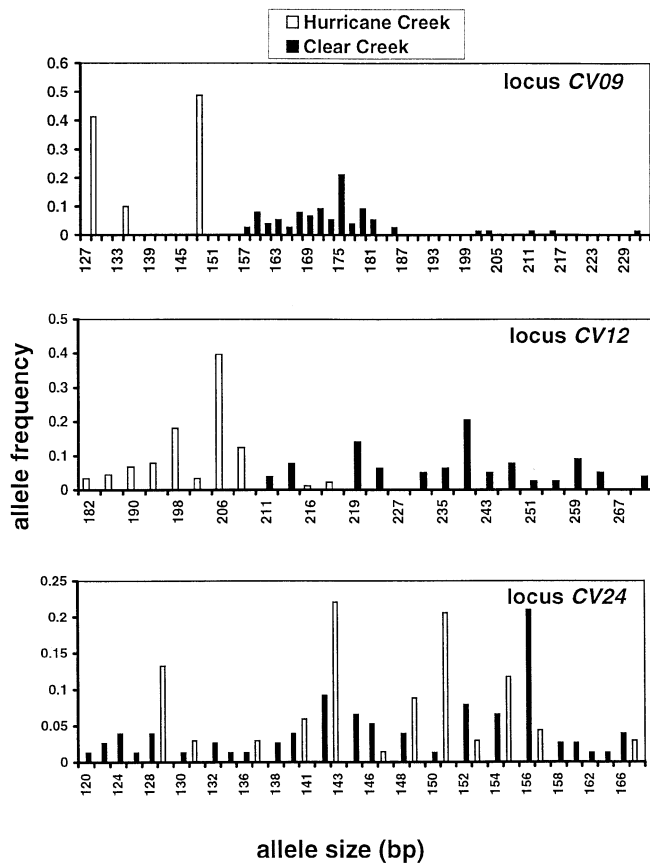
<sup>b</sup> Exclusion probability calculated for the 'neither parent known' model (or the 'one parent known' model); mean is the cumulative  $P_E$  across all three loci

from 3 to 24 (Table 1). Allele frequencies differed dramatically between the two populations (Fig. 3), and the multi-locus exclusion probabilities (one-parent-known model) were 0.94 and 0.99, respectively (Table 1).

#### Paternity

In total, 987 striped darter embryos from 19 nests were examined genetically. All of the assayed progeny in 14 nests (74%) displayed genotypes consistent with paternity by the guarding male (Table 2). The remaining nests, most of which included foster embryos, are discussed next.

In Clear Creek (the population with putative egg mimics), all assayed embryos from 10 of the 11 nests (91%) were sired exclusively by the nest attendant (Table 2). The exception, nest Cvi185, contained two distinct egg clusters, one (with more advanced embryos) consistent with paternity by one uncollected male, and the other (with less developed embryos) having been



**Fig. 3** Allele frequencies at microsatellite loci in samples of striped darter adults from the two study populations

sired by the attendant male. It is not known if the male captured with nest Cvi185 actually tended both clusters. Perhaps a portion of nest rock or substrate formed a partition between the nests in situ, isolating the two clusters and possibly concealing another guardian (who was not captured). Alternatively, the captured nest attendant may have taken over the nest from a previous male or adopted and spawned on an abandoned portion of the nest rock.

However, in Hurricane Creek (the population lacking egg mimics), embryos from only four of the eight nests (50%) were sired exclusively by the nest attendant. Additionally, three of the eight nests (Cvi101, Cvi115, and Cvi136) involved at least some allopaternal care by the guarding male (for one other nest, Cvi102, no guarding male was captured, but offspring genotypes were consistent with a single sire). In nest Cvi101, no offspring genotypes were consistent with paternity by the guardian, but they were consistent with a single hypothetical but unsampled sire, indicating a recent nest takeover or adoption of an abandoned nest. In nest Cvi115, which contained embryos of mixed developmental stages, at face value 30 of the 47 specimens (64%) were consistent with paternity by the guardian male, and the remainder were consistent with one other sire who was not collected. However, considering the developmental stages of the embryos and their spatial intermixture, it seems likely that the guardian male was not the true sire of any embryos in nest Cvi115, because cuckoldry must then have taken place between the same two sires over a period of many days to explain both the genetic and developmental data. Thus, we favor an alternative possibility, also

**Table 2** Summary of striped darter spawning behavior as assessed by molecular markers

Nest ID	Male standard length (mm)	No. egg mimics	Paternity by guardian male	No. eggs in nest	Embryonic stages <sup>d</sup>	Minimum no. of dams	Estimated no. dams (95% conf. limits)
<b>Hurricane Creek:</b>							
Cvi101	47.20	0	0 of 47	338	B C D	5	5 (3, 11)
Cvi102	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	207	D	3	3 (2, 7)
Cvi103	54.36	0	47 of 47	177	D	4	4 (3, 8)
Cvi115	48.00	0	0 of 47	243	A B C D	3–4 <sup>b</sup>	3 (2, 3)
Cvi123	51.76	0	47 of 47	339	D E	4	5 (3, 12)
Cvi136a	50.00	0	47 of 47	520	B C D	4	4 (2, 7)
Cvi136b			22 of 47	189	D	2 <sup>c</sup>	1 (1, 3) <sup>c</sup>
Cvi144	49.73	0	47 of 47	376	D	4	8 (4, 21)
Cvi148	51.50	0	47 of 47	292	D E	3	6 (4, 16)
<b>Clear Creek:</b>							
Cvi150	53.33	7	47 of 47	346	B C D	5	8 (6, 16)
Cvi164	58.00	14	47 of 47	264	A B C D	6	9 (6, 16)
Cvi168	60.92	20	47 of 47	565	A B C D E	7	19 (11, 30)
Cvi172	57.63	10	47 of 47	251	E	5	6 (5, 10)
Cvi183	54.15	15	47 of 47	269	A B C D	5	4 (4, 7)
Cvi185a	48.92	15	47 of 47	150	D E	4	5 (4, 8)
Cvi185b			0 of 47	178	A B	4–5 <sup>b</sup>	3–6 (3, 10) <sup>b</sup>
Cvi187	60.33	18	47 of 47	290	D	7	7 (5, 13)
Cvi190	52.22	7	47 of 47	147	D	3	4 (3, 7)
Cvi194	58.73	17	47 of 47	258	D E	7	8 (5, 14)
Cvi198	52.40	23	47 of 47	302	B C D	6	5 (4, 9)
Cvi200	69.47	16	47 of 47	221	A B C	5	8 (6, 16)

<sup>a</sup> Guarding male not captured; nest consistent with a single hypothetical sire

<sup>b</sup> Nests consistent with a single hypothetical sire; range of dams is due to alternative genotypes that can be deduced for the sire

<sup>c</sup> Estimates of dams exclude embryos that were not sired by the tending male

<sup>d</sup> Following Cooper (1979): *A* morula with no distinct blastomeres, developing area covered 20% of yolk surface; *B* embryonic axis developed, developing area covered 33% of yolk surface; *C* tail-bud embryo, somites developed, lens placodes present but not pigmented; *D* free-tail embryo, pectoral fin buds present, eyes pigmented; *E* late embryo, mouth formed, often hatch when collected

consistent with the genetic data, in which all of the assayed embryos from this nest were sired by a single male who was not the captured guardian. If so, this is another instance of a recent nest takeover or adoption. Finally, in nest Cvi136, two distinct egg clusters were present. For the first (more developed) cluster of embryos, the guardian apparently sired 22 of the 47 specimens assayed (47%), the remainder having been sired by another male who was not collected (Table 2). The second clutch (with the less advanced embryos) was sired exclusively by the guardian male. Thus, Cvi136 is another apparent example of a nest takeover or adoption of a nest abandoned by a previous attendant.

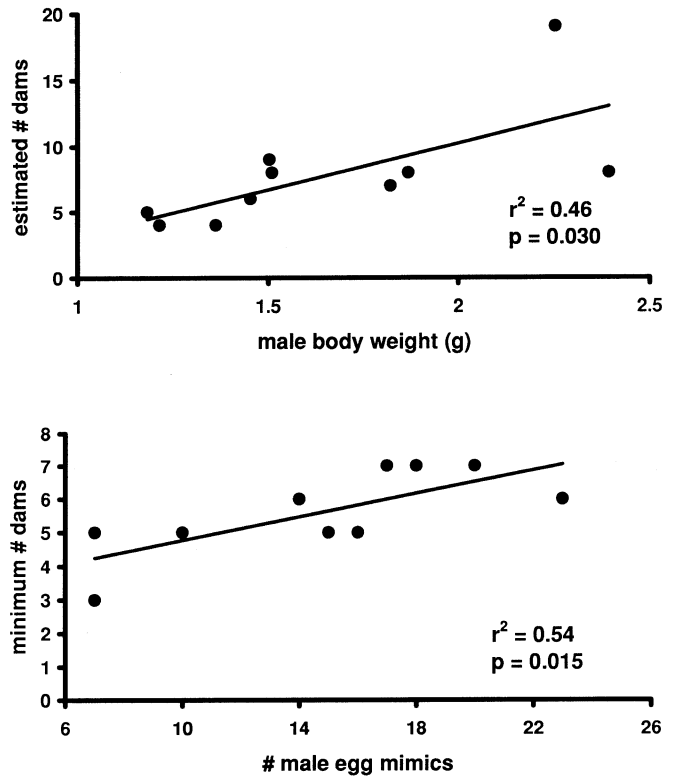
### Maternity

From direct genotypic counts of single cluster nests, an average of 3.9 ( $n=7$ ) dams and 5.6 ( $n=10$ ) dams successfully spawned per nest in the Hurricane Creek and Clear Creek samples; from the computer simulations, the upward-adjusted estimates were 5.0 and 7.8 dams per nest, respectively, and did not differ significantly between populations (two-tailed  $t$ -test assuming unequal variances;  $t=-1.9$ ,  $df=12$ ,  $P=0.09$ ). Also, the mean number of eggs per nest in Hurricane Creek (316.2, based on 16 nests) was not significantly different from the mean number (235.0, based on 27 nests) in Clear Creek (two-tailed  $t$ -test assuming unequal variances;  $t=1.6$ ,  $df=24$ ,  $P=0.12$ ).

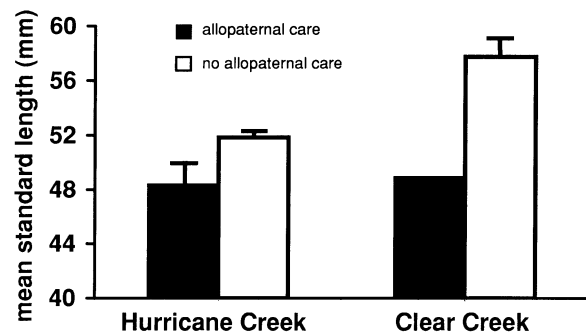
Because several to many dams contributed to each nest, explicit maternal genotypes could not be reconstructed from the available data. Thus, we were unable to determine genetically if a female lays her entire clutch in one nest or, alternatively, partitions her eggs among several nests. However, based on our observed numbers of eggs per nest in Clear Creek, and our genetically estimated numbers of contributing dams (based on HAPLOTYPES), the mean number of ova per nest contributed by each dam was 41.2, a value not significantly different from Kornman's (1980) tally of 39.3 eggs carried per gravid female (two-tailed  $t$ -test;  $df=32$ ,  $t=0.4$ ,  $P=0.72$ ). This indicates that most females in our study probably do indeed lay most or all of their ripe eggs in single nests.

### Correlation of male characters with reproductive success

Striped darter males from Clear Creek displayed the presumptive egg-mimic spots. Does variation in this feature (or the other morphological variables monitored) correlate with the genetically deduced numbers of dams per nest? Ten of the 11 nests from Clear Creek contained eggs that were sired exclusively by the guarding males, and these provide the basis for the statistical tests. Independent regression analyses revealed only two significant correlations between male features and the number of dams per nest (Fig. 4): male body weight versus the



**Fig. 4** Linear regressions of male morphological features [body weight ( $df=9$ ,  $F=6.9$ ) and numbers of egg-mimic spots on the pectoral fins ( $df=9$ ,  $F=9.6$ )] against the genetically deduced numbers of dams that spawned in their respective nests



**Fig. 5** Body lengths for guardian striped darter males that provided at least some allopaternal care versus those that sired all of the eggs in their respective nests. Males providing allopaternal care were significantly smaller than those not showing this behavior [a two-way ANOVA including locality and reproductive strategy shows a small but significant effect for reproductive strategy ( $F_{1,14}=4.75$ ,  $P=0.047$ ) but no significant interaction by locality]. A standard error bar is not present for Clear Creek population since only one male exhibited this behavior

estimated number of dams, and egg-mimic spots versus the minimum dam numbers. However, in a stepwise multiple regression analysis using male body weight, carotenoid content, and number of egg mimics, only the latter was a significant predictor of the minimum number of dams ( $r^2=0.54$ ,  $df=9$ ,  $F=9.6$ ,  $P=0.015$ ), and none of

these measured parameters was a significant predictor of the estimated number of dams.

In the Hurricane Creek population, the guardian male proved to be the true sire of all offspring he tended in only four of the eight nests assayed, thus generally precluding meaningful analyses of possible correlations between male characters with reproductive success. However, one point merits mention. Although the sample size is small, allopaternal care was documented in 3 of 8 surveyed nests in Hurricane Creek and in 1 of 11 nests in Clear Creek (Table 2). In both populations, males exhibiting this behavior averaged smaller in size than those that sired all offspring in their respective nests (Table 2; Fig. 5). In the two nests with partial allopaternal care (Cvi0136 and Cvi0185), this male behavior was associated with subsequent mating success by the foster father. In other words, in both cases the less developed of the two clusters of embryos proved to have been sired exclusively by the current guardian (Table 2).

## Discussion

### Genetic paternity and maternity

No instances of cuckoldry were detected among the 19 striped darter nests genetically assayed. This finding contrasts with microsatellite-based observations on paternity in several other nest-tending fishes. In three species of *Lepomis* sunfish, sneaked fertilizations from cuckolding males occurred in 43–90% of the assayed nests (DeWoody et al. 1998; Neff 2001), and lower levels of cuckoldry have also been detected in the fifteen-spine stickleback (Jones et al. 1998), sand goby (Jones et al. 2001), tessellated darter (DeWoody et al. 2000b), and largemouth bass (DeWoody et al. 2000c). Striped darter nests are constructed under a rock and have only a single small opening. This physical arrangement (contrasting with the open nests of sunfish, for example) probably improves nest defense against potential cuckolders.

As field observations suggest (Kornman 1980) and the genetic data confirm, striped darter males mate with multiple females. From direct counts of deduced maternal gametes in offspring arrays, an average of at least 3.9 dams contributed to a nest, and computer simulations (based on population allele frequencies) suggest that the true number may be nearly twice that high in many cases. Developmental stages of the embryos proved to be poor indicators of the number of contributing dams. For example, nest Cvi0187 contained embryos only of late development 'stage D', but our genetic data demonstrate that at least seven mothers contributed to that cohort. Similar results, in which genetic data revealed more dams for a nest than distinct developmental stages of embryos, were obtained in the redbreast sunfish (DeWoody et al. 1998) and tessellated darter (DeWoody et al. 2000b).

In general, when many dams spawn in a nest, their reproductive success is inherently difficult to quantify in genetic parentage studies. As the total number of contrib-

uting dams increases, the probability of allelic sharing by two or more parents increases dramatically (see Fiumera et al. 2001), and this obscures the reconstruction of exact female genotypes (DeWoody et al. 2000d). For this reason also, it is extremely difficult to determine if each female distributes her clutch among nests. Furthermore, even if dams' genotypes were deduced accurately, the probability of finding multiple nests that shared a dam could be low unless most of the nests in the population were sampled. Despite these difficulties, we did gain some information on female reproductive modes. The number of eggs we observed per nest, divided by our genetic estimates of the number of dams per nest, suggests that individual females in Clear Creek laid on average 41.2 eggs per nest, a number nearly identical to Kornman's (1980) data on the mean number of ripe eggs per female in that population. This suggests that female striped darters in Clear Creek normally lay most or all of their eggs at one site. Measurements on ripe eggs were not available for the Hurricane Creek population.

Several factors probably constrain polyandry in striped darters. Suitable nest rocks are thought to be limiting in most *Catnotus* darter species (Page and Schemske 1978; Constantz 1985), and males in our study area were in competition with *Pimephales notatus*, *Etheostoma crossopterygum* (in Hurricane Creek) and *E. flabellare* (in Clear Creek) for nesting sites. Striped darter males mature more slowly than females (Kornman 1980), and all else being equal this creates a biased operational sex ratio with fewer male breeders and nests. Under circumstances of low nest density, perhaps the energetic costs or dangers associated with nest searching are unusually high for females. Alternatively, a guardian male may be efficient at encouraging a female to lay all of her eggs in his nest. Another likely contributing factor is the mode of ova development. Unlike the situation in many other darter species, striped darter ova develop synchronously in the ovary (Kornman 1980), probably further favoring an 'all-at-once' egg laying strategy.

### Sexual selection

The estimated number of dams contributing to a striped darter nest ranged from 4 to 19 in Clear Creek and was strongly correlated with the total number of eggs per nest. Given the large range of dams per nest, the polygynous mating system of this species, and the low cuckoldry levels observed, the variance in reproductive success among striped darter males must be heavily influenced by the number of spawning females they can attract. Thus, the intensity of sexual selection on bourgeois males is presumably high.

### Carotenoid content

Variation in body color (as measured by standardized carotenoid content) among males ranged widely (from



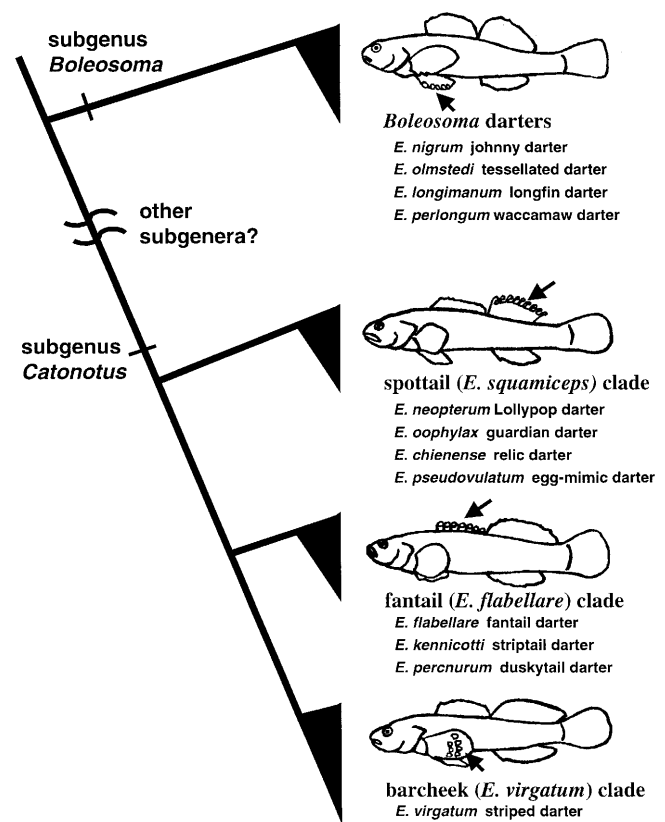
$1.607 \times 10^{-9}$  to  $6.62 \times 10^{-8}$  g/mm<sup>2</sup>), but did not significantly explain the variation in male reproductive success. Similar studies of orangethroat darters also failed to demonstrate associations between male coloration and mating success (Moerchen 1973; Pyron 1995). One possible explanation is that body coloration in darters is involved primarily in male-male competition and species recognition, rather than in mate attraction.

### Egg mimicry

Previous studies have found that female darters spawn preferentially in nests that already contain eggs (Page 1974; Constantz 1985; Knapp and Sargent 1989). In principle, males could evolve at least three mate-attracting tactics to exploit this partiality: egg thievery, egg mimicry, and allopaternal care via nest takeovers. Egg thievery is probably not an option for egg-clustering darters, since darter eggs lose their adhesive properties immediately after deposition and could not be reattached in a natural position following their removal from a neighbor's nest. However, we have provisionally identified the other two tactics as two related ways in which striped darter males have exploited a female sensory bias for egg-containing nests.

Males from the Clear Creek population acquire 1–28 egg-mimicking spots during the breeding season and use them in nesting display. If these spots truly are egg mimics, they provide the fourth case of parallel evolution of egg mimicry in the egg-clustering darters (Page and Bart 1989; Porterfield et al. 1999), the first documentation of this phenomenon in the barcheek clade of *Catnotus* (Fig. 6), and the first case of egg mimicry involving fin pigmentation as opposed to structural body features. The egg-mimicry hypothesis posits that a male's pectoral spots exploit a preexisting female preference for spawning in nests that already contain eggs, but these showy spots might also play a role in territorial defense against conspecific males, or in the recognition of conspecific mating partners (Paterson 1985). In the latter case, they might act primarily as an isolating mechanism that inhibits interspecific hybridization with the sympatric fantail darter, *Etheostoma flabellare*. These alternative hypotheses for the functional significance of pectoral fin spots are not mutually exclusive.

For the Clear Creek males, our genetic data provide some evidence that higher numbers of spots are associated with increased numbers of dams per nest. Also, our field observations reveal that these males orient head-on toward conspecifics and readily display their pectoral fins while defending nesting sites, whereas in other darter species with egg mimics on the dorsal rays, males display laterally to conspecifics (Page 1974, 1975; Page and Knouft 2000), presumably to maximize the visual effect on females. Taken together, these findings suggest that the pectoral markings of male striped darters may serve a dual function: to attract females via egg mimicry, and to deter rival males from pirating prime nesting loca-



**Fig. 6** Phylogeny of egg clustering darters based on morphological characters (modified from Page and Bart 1989), showing here the parallel evolution of egg mimics on breeding males. The phylogenetic relationships of the subgenus *Boleosoma* to other subgenera, including *Catonotus*, is not fully resolved, but the latter is comprised of three clades: the spottail (*E. squamiceps*) clade with ten species, four of which (listed in the figure) have egg mimics on the soft rays of the second dorsal fin; the fantail (*E. flabellare*) clade with three species, all of which have egg mimics on the spines of the first dorsal fin; and the barcheek (*E. virgatum*) clade with five species, only one of which (*E. virgatum*) has egg mimics, and only in some populations. Males in the subgenus *Catonotus* display their egg spots in a lateral and upright posture, near or under the nest rock. Four of the five species in the subgenus *Boleosoma* often exhibit egg mimics at the end of the pelvic and (sometimes) pectoral fin rays; these egg mimics are used in inverted displays on the nest rock

tions. In the future, these behavioral possibilities might be experimentally evaluated under controlled conditions.

### Allopaternal care via nest takeovers

Suitable nest rocks are probably a limiting factor in male reproductive success for most egg-clustering darter species, and large body size is probably important in the successful defense of nests from conspecifics (Page and Schemske 1978; Constantz 1985). In the current study, males who were foster parents for some or all of their tended embryos had smaller mean body sizes than males who sired 100% of the embryos that they guarded. Given the known tendency for large tessellated darter males to

abandon egg-containing nests (Constantz 1985), it seems likely that striped darter foster males tend to be small or sub-optimal individuals who adopt a larger male's nest after it is abandoned (although it remains possible that the foster males in our study had usurped even smaller males from their nests).

In support of the 'nest-adoption' scenario, we have documented intermediate biological situations that could represent all stages of the process— from nest abandonment (nest Cvi102), to recent nest adoption without reproduction as yet by the foster male (Cvi101 and Cvi115), to the successful fertilization of recently laid eggs by a male who had adopted a nest and was also tending older foster embryos (Cvi136 and Cvi185). Thus, allopaternal care was associated with spawning benefits for the foster fathers in two of the four adopted nests assayed. Because male mating success appears to be correlated with body size, allopaternal care via nest adoption may be a common strategy by which smaller striped darter males increase their mean fitness by exploiting a female's preference to spawn in egg-containing nests.

Allopaternal care was more common in the Hurricane Creek population (where males lack egg-mimic spots) than in Clear Creek (where they possess them). Given that egg-mimic spots on the pectoral fin appear to be a novel evolutionary trait that arose in only one isolated population of *E. virgatum*, the Hurricane Creek males probably never had this evolutionary option available to them as a mate-attracting tactic.

## Conclusions

In summary, our genetic analysis of the mating system of striped darters has revealed several aspects of spawning behavior that were not apparent in a previous life history study of this species (Kornman 1980). These include a polygynous mating system without cuckoldry, variable numbers of conspicuous pectoral fin markings (potential egg mimics) associated with male reproductive success, and allopaternal care via nest adoption as a tactic by which small males can gain reproductive success. Because darter females prefer to mate with males that have (or appear to have) eggs in the nest, sexual selection via sensory exploitation probably has helped to drive both egg mimicry and allopaternal care by males, two distinct but functionally related mate-attracting tactics.

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