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Author for correspondence:

Bryan D. Neff

e-mail: bneff@uwo.ca

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Alternative male reproductive tactics drive asymmetrical hybridization between sunfishes (*Lepomis* spp.)

Shawn R. Garner and Bryan D. Neff

Department of Biology, Western University, 1151 Richmond Street, London, Ontario, Canada N6A 5B7

The potential role of alternative reproductive tactics in circumventing pre-mating isolating mechanisms and driving hybridization between species has long been recognized, but to date there is little empirical support from natural systems. Hybridization occurs between bluegill (*Lepomis macrochirus*) and pumpkinseed sunfish (*Lepomis gibbosus*) and it is known to be asymmetrical (male bluegill × female pumpkinseed). Here, we test whether this pattern is driven by a recognition failure by pumpkinseed females or by an alternative cuckold reproductive tactic in bluegill males. Using genetic parentage data, we found that bluegill cuckolders fathered 24.9% of the larvae in bluegill nests, but no evidence that pumpkinseed females spawned in bluegill nests. Pumpkinseed cuckolders fathered 8.7% of the larvae in pumpkinseed nests, whereas bluegill cuckolders fathered 13.6% of the larvae in those nests. Bluegill cuckolders thus frequently spawn in pumpkinseed nests and are responsible for the asymmetrical hybridization between the species. We discuss the evolutionary consequences of interactions between bluegill and pumpkinseed and the role of alternative reproductive tactics in adaptation and introgression.

1. Introduction

Alternative reproductive tactics are an important component of mating systems in all major taxonomic groups [1]. For example, in sunfish (*Lepomis* spp.) most males mature late in life and use a parental tactic: they construct nests where they spawn with females, then provide sole parental care to the developing eggs. Other males mature early in life and use a cuckold tactic: they fertilize eggs by releasing sperm in the nest of a parental male while a female is spawning. Specifically, small cuckolders act as ‘sneakers’ that dart into the nest at the moment of spawning, whereas large cuckolders act as ‘satellites’ that deceive parental males by mimicking female characteristics [2]. This variation has led sunfish to become a model system for the study of alternative reproductive tactics [2,3].

The presence of alternative reproductive tactics has consequences for the evolution of mating systems. In sunfish, for example, cuckolders diminish the opportunity for mate choice. Females can choose a parental male as a mate, but that choice is often negated by a cuckold intrusion during spawning [4]. Sperm competition and agonistic interactions with parental males mean that cuckolders must rapidly enter a nest [5], which may limit the opportunity for mate choice by cuckolders. In the most extreme cases, it is conceivable that cuckolders do not effectively discriminate among sunfish species. Consequently, it has been suggested that cuckold males may facilitate interspecies hybridization in sunfish and in other taxonomic groups [6,7], but to date there is little empirical evidence that cuckolders drive hybridization patterns in natural systems.

Bluegill (*Lepomis macrochirus*) and pumpkinseed (*Lepomis gibbosus*) sunfish frequently hybridize in Lake Opinicon [7]. Based on allozyme loci and mitochondrial

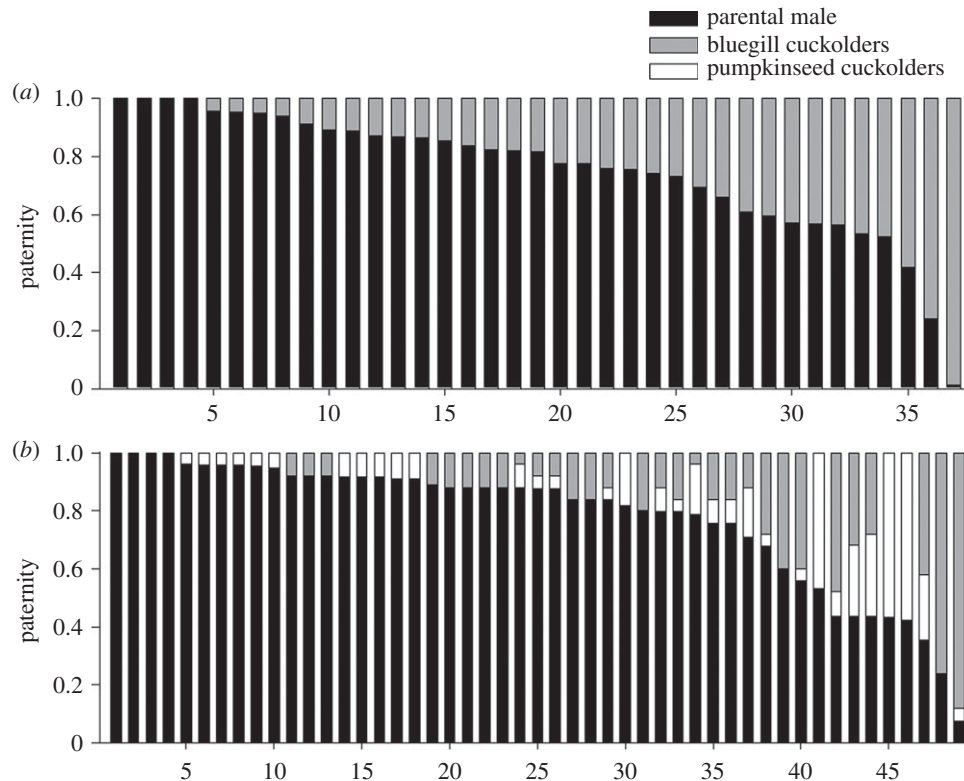


Figure 1. Allocation of paternity in (a) bluegill (*L. macrochirus*) and (b) pumpkinseed (*L. gibbosus*) nests. Each bar shows a different nest, arranged in decreasing order of paternity by the parental male.

DNA, all hybrids appear to be first generation (F_1) and have a pumpkinseed mother [7]. This unidirectional hybridization could be a result of mate recognition errors by either pumpkinseed females or bluegill cuckolders. Here, we examine the relative contributions of pumpkinseed females and bluegill cuckolders to hybridization patterns by measuring the abundance of hybrid offspring in the nests of bluegill and pumpkinseed males. If hybridization is driven primarily by pumpkinseed females, then hybrids should be abundant in bluegill nests but rare in pumpkinseed nests. If hybridization is driven primarily by bluegill cuckolders, then hybrids should be rare in bluegill nests but abundant in pumpkinseed nests.

2. Material and methods

(a) Sample collection

All sunfish were collected in Lake Opinicon, Ontario, Canada ($44^{\circ}34'N$, $76^{\circ}19'W$). Parental males were captured using dip nets, and a small tissue sample from the male and approximately 25 larvae from his nest were preserved in 95% ethanol. Bluegill nests were sampled in 2005 ($n = 37$, described in [8]), and additional parental males were sampled in 2010 ($n = 22$) for the interspecies comparisons of allele frequencies. Pumpkinseed nests were sampled in 2003 ($n = 12$, described in [4]) and 2010 ($n = 37$).

(b) Genetic analysis

Each individual was genotyped at three microsatellite loci: RB7, LMAR10 and either RB20 or LMAR14 (for details of these loci see [9,10]). RB20 was used for the parentage analysis of the 2005 bluegill samples, whereas LMAR14 was used for the interspecies allele frequency comparisons and the parentage analysis of the 2003 and 2010 pumpkinseed samples.

We identified microsatellite loci that were informative for species identification by comparing the distribution of allele sizes in parental males from each species. We found that LMAR10 and LMAR14 were each partially informative for species identification, and in combination would correctly identify 100% of pure bluegill, 100% of pure pumpkinseed and 97.6% of hybrids (detailed calculations are available in the electronic supplementary material). These loci were then used to assign each larva as a bluegill, pumpkinseed or hybrid. Additionally, for a small number of larvae with ambiguous ancestry ($n = 9$), we used a mitochondrial restriction assay (described in the electronic supplementary material) to determine whether the female parent was a bluegill or pumpkinseed.

For each nest, we calculated the proportion of paternity that could be attributed to the parental male and to cuckolders of each species. Hybrid larvae have low probability of being genetically compatible with a parental male by chance, so paternity for these larvae was assigned based on simple exclusion. For the remaining larvae, the share of paternity for the parental male and for cuckolders of his species was determined using the two-sex paternity model [11]. In pumpkinseed nests, the paternity of pumpkinseed parental males, pumpkinseed cuckolders and bluegill cuckolders did not differ significantly between 2003 and 2010 (t -tests, all $p > 0.05$), and data from these years were thus combined in our analyses.

3. Results

We genotyped a total of 865 larvae to measure paternity in the nests of 37 bluegill males (mean = 23.4 larvae per nest, range = 10–48). We found that the average parental male bluegill sired 75.1% of the larvae in his nest (figure 1a). Paternity for the remaining 24.9% of the larvae was always assigned to bluegill cuckolders. Pumpkinseed ancestry was not inferred for any of the larvae in bluegill nests.

We genotyped 1169 larvae to measure paternity in the nests of 49 pumpkinseed males (mean = 23.9 larvae per nest, range = 12–26). We found that the average parental male pumpkinseed sired 77.7% of the larvae in his nest (figure 1b). Paternity for 8.7% of the larvae in pumpkinseed nests was attributed to cuckoldry by other pumpkinseed males, while paternity for the remaining 13.6% of the larvae was attributed to cuckoldry by bluegill males. Overall, there were 160 larvae in pumpkinseed nests for which we inferred bluegill ancestry: 151 larvae (94.4%) did not match the nest-tending parental male pumpkinseed and had microsatellite genotypes that were consistent with an F₁ hybrid produced by a bluegill cuckold and pumpkinseed female. One larvae (0.6%) had two different bluegill alleles at both informative microsatellite loci and a bluegill mitochondrial haplotype, consistent with a mating between a bluegill cuckold and bluegill female. Three larvae (1.9%) had two different bluegill alleles at one microsatellite locus and a pumpkinseed mitochondrial haplotype, consistent with a backcross between a bluegill cuckold and a hybrid female. The remaining five larvae (3.1%) were a genetic match for the nest-tending parental male pumpkinseed, but had both bluegill and pumpkinseed microsatellite alleles and a pumpkinseed mitochondrial haplotype, consistent with either a backcross between the nest-tending parental male pumpkinseed and a hybrid female, or with a chance genetic match between the pumpkinseed parental male and a hybrid produced by a bluegill cuckold and pumpkinseed female.

4. Discussion

Our results show that bluegill cuckolders are responsible for the asymmetry in the direction of hybridization between bluegill and pumpkinseed. We found no evidence that female pumpkinseed spawn in bluegill nests, whereas our data indicate that bluegill cuckolders frequently spawn in pumpkinseed nests. Indeed, a parental pumpkinseed is more likely to be cuckolded by a bluegill than by another pumpkinseed. Interestingly, our data suggest that hybrid females occasionally spawn in pumpkinseed nests, and may backcross with either bluegill or pumpkinseed males. Second generation hybrids (F₂) may thus be present at low frequency in Lake Opinicon, even though they were not detected in a previous survey [7]. Overall, our data show that bluegill cuckolders drive unidirectional hybridization between bluegill males and pumpkinseed

females, and suggest that hybrid females may subsequently mate with either bluegill or pumpkinseed males.

Alternative reproductive tactics may be an important determinant of evolutionary patterns. Hybridization typically affects evolutionary patterns through genetic introgression, with effects that can range from species fusion to introducing novel genes that can enable adaptive radiation [12,13]. We observed second generation hybrids in pumpkinseed nests, which suggest that hybridization could be leading to introgression between bluegill and pumpkinseed. Conversely, the amount of introgression is probably small, as hybrids between bluegill and pumpkinseed are known to have inferior reproductive characteristics [14]. Indeed, our study identified 151 F₁ hybrids and at most eight F₂ hybrids, suggesting that F₁ hybrids produce considerably fewer progeny than might be expected based on their abundance. Nevertheless, we have shown that cuckolders lead to frequent hybridization and are thus a potential vector for introgression between these fishes.

Interestingly, despite a lack of widespread introgression, hybridization by cuckolders may independently influence evolutionary patterns. Interspecies cuckoldry reduces the fitness of parental male pumpkinseed by approximately 14% (the rate of interspecies cuckoldry), but in contrast to intraspecific cuckoldry, hybridization similarly reduces the fitness of pumpkinseed females because hybrid fitness is so low. Consequently, pumpkinseed of both sexes would benefit from phenotypic and behavioural traits that minimize cuckoldry by bluegill. Additionally, because cuckold success is frequency-dependent in sunfish [15], bluegill cuckoldry may reduce the success and ultimately the frequency of pumpkinseed cuckolders. Indeed, pumpkinseed cuckolders fertilized 15% of the eggs at a site without bluegill [16], but less than 9% of the eggs in our study. Finally, given that bluegill cuckolders drive hybridization, it will be interesting to determine why bluegill cuckolders spawn in pumpkinseed nests. It is possible that the behaviour results from a low fitness cost of spawning in a heterospecific nest, some barrier to effective mate recognition or some undetected fitness benefit of hybrid production.

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Supplementary Methods

Genetic analysis

Using the adult samples, Micro-checker was used to determine if microsatellite allele frequencies deviated significantly from the expectations of Hardy-Weinberg equilibrium [1]. In bluegill, LMAR10 showed a significant departure from Hardy-Weinberg equilibrium in 2005 ($n = 37$, $P < 0.01$), although there was not a significant departure in 2010 ($n = 22$, $P > 0.05$). In pumpkinseed, LMAR14 showed a significant departure from Hardy-Weinberg equilibrium in both 2003 ($n = 12$, $P < 0.05$) and 2010 ($n = 37$, $P < 0.001$). In both cases these departures were caused by a large excess of homozygotes, consistent with the presence of a null allele at LMAR10 in bluegill (but not pumpkinseed) and at LMAR14 in pumpkinseed (but not bluegill). We subsequently used the second Brookfield equation to calculate the frequency of the null allele and adjust the population allele frequencies at these loci [1, 2].

We identified two microsatellite loci that were informative for identifying bluegill and pumpkinseed ancestry. LMAR10 had a size distribution that did not overlap between species; an allele smaller than 282 bp had a bluegill origin and an allele larger than 282 bp had a pumpkinseed origin (Supp. Fig. 1a). This locus could identify all individuals that had at least one pumpkinseed parent, but would not detect bluegill ancestry in a hybrid that inherited a null allele from its bluegill parent. Locus LMAR14 had a size distribution that partially overlapped between species; an allele smaller than 308 bp had a bluegill origin and an allele larger than 308 bp had an unresolved origin (Supp. Fig. 1b). In combination, these two loci would correctly identify 100% of pure bluegill, 100% of pure pumpkinseed, and 97.6% of hybrids, while misclassifying

only those hybrids that inherited from their bluegill parent both a null allele at LMAR10 ($p = 0.099$) and a non-informative allele at LMAR14 ($p = 0.238$; combined: $0.099 \times 0.238 = 0.024$).

Additionally, we developed a mitochondrial restriction assay that could be used to determine if an individual had a bluegill or a pumpkinseed mother. The cytochrome c oxidase subunit I (COI) gene was PCR amplified using novel primers (LepCOI-F: TCG ACC AAT CAC AAA GAC ATC GGC AC; LepCOI-R: ACT TCG GGG TGA CCG AAA AAC CAG AA), which bound the same location as previous COI primers [3], but with sequences that matched the bluegill mitochondrial sequence (Genbank accession: NC_015984). These primers reliably amplified the COI gene in both bluegill and pumpkinseed. Based on 22 bluegill and 32 pumpkinseed COI sequences from Genbank, we then identified a fixed difference in a HindIII restriction site (AAGCTT), which was present in all bluegill sequences but absent in all pumpkinseed sequences. We validated this restriction polymorphism by using HindIII to digest the amplified COI gene of 25 bluegill parental males and 33 pumpkinseed parental males from Lake Opinicon, which resulted in the predicted restriction banding patterns for all individuals (2 small bands for each bluegill, 1 large band for each pumpkinseed). This assay was then used to determine the maternal ancestry of 9 larvae whose ancestry was not fully resolved by microsatellite genotypes.

To correct our paternity estimates using the two-sex paternity model [4], we first calculated the proportion of the larvae in each nest that were genetically compatible with the parental male (i.e. they shared at least one allele at each locus; ng_{dad} in [4]). At loci with null alleles (LMAR10 in bluegill, LMAR14 in pumpkinseed), parental males with homozygous genotypes were considered genetically compatible with larvae that were homozygous or could not be amplified at that locus. Second, we estimated the proportion of the larvae in the

population that would be expected to be genetically compatible with each parental male by chance (NG_{dad}). We estimated NG_{dad} for each parental male by randomly generating 10,000 larvae based on the population allele frequencies, and then determining the proportion that were genetically compatible with that male. Values of NG_{dad} ranged from 0.01 to 0.24 in bluegill (mean = 0.09) and from 0.01 to 0.22 in pumpkinseed (mean = 0.05). Paternity for each parental male was calculated as $(ng_{dad} - NG_{dad}) / (1 - NG_{dad})$ [4].

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Supplementary Figure 1. Microsatellite allele frequencies in bluegill (*Lepomis macrochirus*) and pumpkinseed (*L. gibbosus*). The dashed lines indicate size boundaries that were used to determine if an allele had a bluegill or pumpkinseed origin.

