Temporal variation in cuckoldry and paternity in two sunfish species (*Lepomis* spp.) with alternative reproductive tactics

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Abstract: Male alternative reproductive tactics have been described in many mating systems. In fishes, these tactics typically involve a territorial male that defends a spawning site or nest and a parasitic male that uses sneaking or female mimicry to steal fertilizations from the territorial male. In this paper, we use molecular genetic markers to examine the success of males that adopt alternative reproductive tactics in two sunfishes, comprising the bluegill (*Lepomis macrochirus* Rafinesque, 1819) and the pumpkinseed (*Lepomis gibbosus* (L., 1758)). In sunfishes, the tactics are referred to as parental (territorial male) and cuckolder (parasitic male). We show that cuckoldry rates peak in the second trimester of the breeding season in bluegill, whereas cuckoldry rates are lowest during this period in pumpkinseed. We also show that paternity of parental male bluegill is positively correlated with body condition, but not body length or mass. No relationship between these phenotypic variables and paternity in pumpkinseed was found. We discuss the patterns of cuckoldry in relation to differences between the species in mating opportunities, parental male defence ability, and cuckolder density. Finally, we discuss how the paternity data can be used to differentiate between two mechanisms underlying the expression of alternative reproductive tactics, comprising the condition strategy and alternative strategies.

Résumé : La description de plusieurs systèmes d'accouplement comporte des tactiques reproductives de rechange chez les mâles. Chez les poissons, ces tactiques mettent typiquement en scène un mâle territorial qui défend un site de fraie ou un nid et un mâle parasite qui se faufile ou qui mime une femelle pour soutirer des fertilisations au mâle territorial. Dans notre étude, nous utilisons des marqueurs génétiques moléculaires pour évaluer le succès de mâles qui utilisent chacune de ces tactiques reproductives de rechange chez deux crapets, soit le crapet arlequin (*Lepomis macrochirus* Rafinesque, 1819) et le crapet soleil (*Lepomis gibbosus* (L., 1758)). Chez les crapets, ces tactiques sont désignées comme parentale (mâle territorial) ou trompeuse (mâle parasite). Nous montrons que les taux de tromperie atteignent leur apogée dans le second trimestre de la saison de reproduction chez le crapet arlequin, alors qu'ils sont minimaux durant cette période chez le crapet soleil. Il existe aussi chez les crapets arlequins une corrélation positive entre la paternité d'un mâle parental et sa condition corporelle, mais non avec la longueur ou la masse de son corps. On n'observe aucune relation entre les variables phénotypiques et la paternité chez le crapet soleil. Nous discutons des patrons de tromperie en fonction des différences entre les espèces en ce a trait aux occasions d'accouplement, à la capacité de défense des mâles parentaux et à la densité des mâles trompeurs. Nous examinons enfin comment les données de paternité peuvent servir à différencier entre deux mécanismes qui sous-tendent l'expression des tactiques reproductives de rechange, soit la stratégie reliée à la condition et les stratégies de rechange.

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Introduction

Many mating systems are characterized by males that adopt alternative reproductive tactics (ARTs) and such systems have been especially well documented within the fishes (Taborsky 1994). Within this taxon, ARTs typically involve a territorial male that defends a spawning site or nest and a parasitic male that uses sneaking or female mimicry to steal fertilizations from the territorial male. Territorial and parasitic males are often specialized morphologically and behav-

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iourally for their respective reproductive roles (e.g., Dominey 1980; Goodson and Bass 2000). Multiple hypotheses exist to explain the evolution of ARTs, and central to differentiating between these hypotheses is genetic paternity data that can be used to calculate the relative fitnesses of the tactics (Gross 1996). To this end, it is important to understand temporal patterns in paternity and cuckoldry rate. For example, if cuckoldry rates vary across the breeding season, then sampling during only one time period could provide biased estimates of the relative reproductive success of the tactics.

Here we detail the temporal patterns in cuckoldry and paternity for two sunfish species: bluegill (*Lepomis macrochirus* Rafinesque, 1819) and pumpkinseed (*Lepomis gibbosus* (L., 1758)). These sunfishes are found in eastern and central North America (Scott and Crossman 1973). In our study population in Lake Opinicon, Ontario, Canada (44°34′N, 76°19′W), bluegill and pumpkinseed are characterized by male ARTs (Gross 1979). Some males called parentals delay

maturation and become territorial during the breeding season. These males build nests, court and spawn with females, and then provide sole parental care for the young in their nest. Parental care can last up to 10 days and involves actively fanning eggs and defending eggs and fry (newly hatched larvae) from predators. The care can be energetically costly, as males do not actively forage during the care period and lose about 10% of their body mass (Colgan and Gross 1977; Coleman and Fischer 1991). Conversely, other males called cuckolders mature precociously and use a parasitic tactic to steal fertilizations from parental males. Cuckoldry can involve either a sneaking tactic, whereby the male streak spawns in nests, or a satellite tactic, whereby the male uses female mimicry to enter the nests of parental males (Gross 1979; Dominey 1980). To date, satellite males have not been reported in pumpkinseed. Previous research has shown that cuckolders in both species can be highly successful at obtaining paternity within the nests of parental males (e.g., Fu et al. 2001; Rios-Cardenas and Webster 2005).

In Lake Opinicon, breeding begins in late May or early June and continues into July (Cargnelli and Neff 2006). Breeding, especially for bluegill, occurs in distinct periods called bouts that consist of a group of males spawning within several days of each other. Spawning is initiated by males who enter the littoral zone, construct a nest in the substrate, and actively defend their nest from other males. In bluegill, nests are built side-by-side in colonies that vary in size from a few nests to 300 nests. By contrast, pumpkinseed males do not congregate in colonies, and breed in lower densities (Gross and MacMillan 1981). One to several days after the nests are constructed, females approach the parental males and enter nests to spawn. Spawning can last for several hours to a full day before the females leave for deeper waters. Males that do not successfully spawn generally abandon their nest following the spawning bout. At the end of the care period, fry swim-up, leave the nest, and become independent. Successful parental males then abandon their nests and return to deeper waters presumably to replenish their energy reserves before potentially nesting a second time (Cargnelli and Neff 2006).

In this paper, we sample offspring and parental males throughout the breeding season. We use microsatellite genetic markers to determine the paternity of each nest-tending parental male and detail variation in cuckoldry rates across the season. We examine phenotypic correlates of reproductive success and propose three hypotheses to explain the temporal variation in paternity and cuckoldry. These data contribute to our understanding of the genetic mating systems of sunfishes.

Materials and methods

Bluegill (1993) or pumpkinseed (2003) reproductive activity was surveyed daily by swimmers within our study area (~2 km stretch of shoreline) on Lake Opinicon, Ontario, Canada (44°34′N, 76°19′W). For selected nests, a small tissue sample was collected from the tending parental male and a sample of several hundred fry was collected from the nest. Tissue and fry samples were preserved in 95% ethanol for later microsatellite DNA analysis. For bluegill, samples were collected from parental males (n = 60 nests) that spawned during 10 June and 6 July; for pumpkinseed, samples were collected from parental males (n = 15 nests) that spawned during 10 June and 7 July. For all of the pumpkinseed parental males and a subset of the bluegill parental males (n = 16), we also obtained mass (nearest 0.1 g) and total length measurements (nearest millimetre). These measurements were used to calculate Fulton's condition factor (mass / length$^3 \times 10^5$; Ricker 1975). This factor correlates with the nonpolar lipid density of parental males (Neff and Cargnelli 2004).

The paternity of each nest-tending parental male was determined using three microsatellite loci and the two-sex paternity model (Neff et al. 2000a; Neff 2001). This model determines the proportion of offspring sired by the nest-tending parental male. The remaining offspring are assumed to be sired by the specialized cuckolder males (e.g., Neff and Gross 2001). The microsatellite loci comprised *RB7*, *LMAR10*, and *RB20* (bluegill) or *LMAR14* (pumpkinseed); the primer sequences are published in DeWoody et al. (1998) and Schable et al. (2002). First, DNA was isolated from the samples using a proteinase K (EC 3.4.21.64) digestion (Neff et al. 2000b). DNA concentration was quantified using a spectrophotometer. Second, we used a Whatman Biometra® T1 thermocycler to amplify the microsatellites with the following program: 60s at 92 °C; 7 cycles of 30s at 92 °C, 30s at 54 °C, and 30s at 72 °C; and 28 cycles of 15s at 92 °C, 30s at 54 °C, and 20s at 72 °C. Each 10 μL polymerase chain reaction (PCR) contained ~75 ng of total DNA, 2 mmol/L MgCl$_2$, 1× PCR buffer (Fisher Scientific, Ottawa, Ontario), 0.4 mmol/L of each deoxynucleotide (Fisher Scientific), 0.25 units *Taq* DNA polymerase (Fisher Scientific), and 0.2 μmol/L of each forward and reverse primer (Invitrogen™ Life Technologies, Eugene, Oregon). PCR product was run following standard protocol for the Ceq. 8000 Genetic Analysis System (Beckman Coulter, Fullerton, California). Genotypes were obtained from a total of 75 parental males and 3724 offspring (mean 50 offspring per nest, range 10–95). The genotypes at these loci did not deviate from Hardy–Weinberg equilibrium. The mean exclusion probabilities for the parentage analysis were 0.90 (range 0.80–1.00) for bluegill and 0.93 (range 0.85–0.98) for pumpkinseed.

Statistical analyses were performed using JMP® version 4.0.4 (SAS Institute Inc. 2000). Temporal variation in cuckoldry rates and paternity was assessed by first dividing the breeding season into trimesters (early, mid, and late) of approximately equal duration. Analysis of variance (ANOVA) with a Tukey's post hoc test was then used to examine the temporal patterns in paternity for each species. For pumpkinseed, we also examined temporal variation in parental male body length, mass, and condition. We could not perform the equivalent analysis for bluegill because we had phenotypic data for only a subset of males (n = 16) that were collected during the third trimester. Simple linear regression was used to examine the relationships between paternity and parental male length, mass, and condition. For the regression analysis, we did not correct the critical alpha value for multiple comparisons because common corrections assume that the statistical tests are independent; such tests are otherwise conservative as is the case for our data because body length and mass are highly correlated and condition is a composite of length and mass (Sokal and Rohlf

Fig. 1. Summary of the paternity of parental male (*a*) bluegill (*Lepomis macrochirus*) or (*b*) pumpkinseed (*Lepomis gibbosus*) in Lake Opinicon, Ontario, Canada. Paternity is the proportion of fry sired by a male in his nest. The data are ordered based on rank and each bar represents an individual parental male.
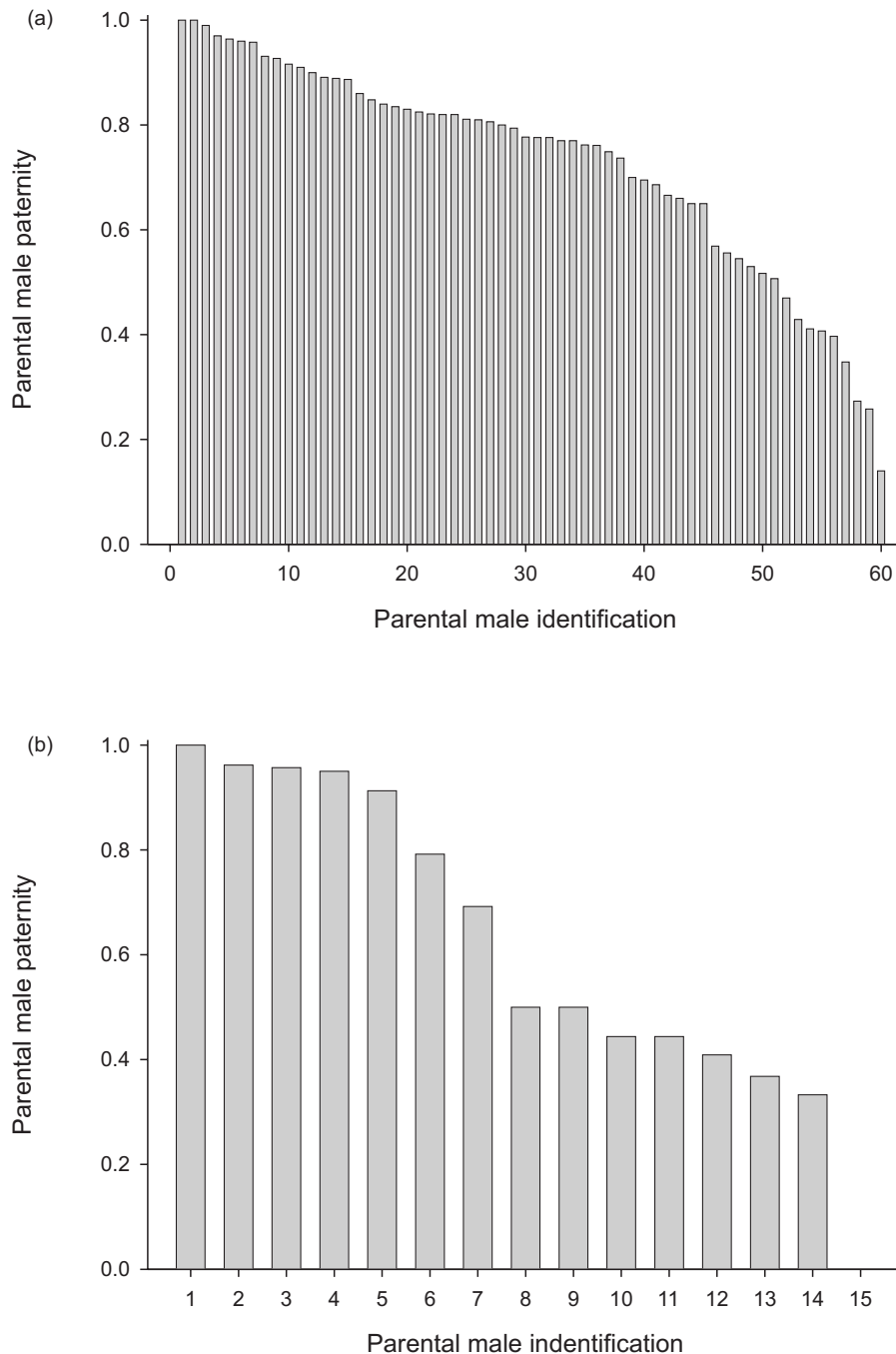
Paternity data were arcsine square root transformed for statistical analyses, but are presented in figures in their raw form for clarity.

Results

For bluegill, parental male paternity (expressed as a proportion of the offspring analysed from each nest) had a mean (1 SD) of 0.73 ± 0.20 and ranged from 0.14 to 1.0 (Fig. 1A). For pumpkinseed, parental male paternity had a mean (1 SD) of 0.62 ± 0.30 and ranged from 0.0 to 1.0 (Fig. 1B). These

means were not significantly different (Wilcoxon: $Z = 1.08$, $n = 75$, $p = 0.28$). Examination of the microsatellite genetic data for the pumpkinseed male with zero paternity revealed that the male was incompatible with 18 of the 20 offspring analysed at two or more loci. He was incompatible with the remaining two offspring at one locus. Thus, it is unlikely that mutation explains the low paternity value.

Analysis by trimester indicated different temporal patterns for bluegill and pumpkinseed (Fig. 2). Paternity among bluegill parental males was high early and late in the breeding season, and low during the middle part of the season

Fig. 2. Box plots summarizing the paternity of parental male (*a*) bluegill (*Lepomis macrochirus*) or (*b*) pumpkinseed (*Lepomis gibbosus*) in Lake Opinicon, Ontario, Canada, across the three trimesters. Paternity is the proportion of fry sired by a male in his nest. Box plots show the 10th, 25th, 50th, 75th, and 90th percentiles and the dots are data lying outside this range. The numbers below the box plots denote sample sizes. In both panels, the second trimester was significantly different from the other two trimesters (Tukey's post hoc test, $p < 0.05$). Paternity data were arcsine square root transformed for statistical analysis, but are presented in their raw form for clarity.
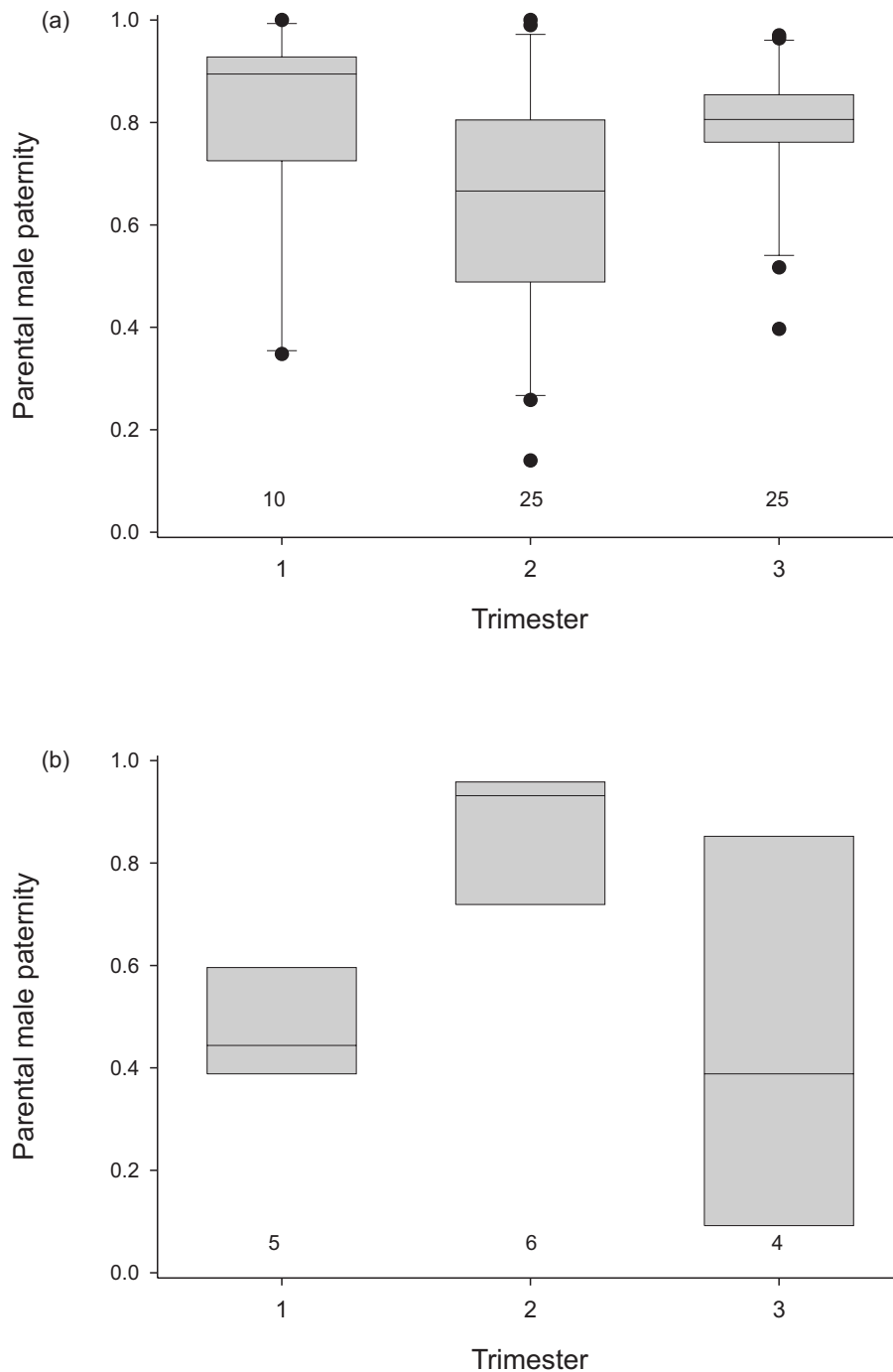
(ANOVA: $F_{[2,57]} = 3.52$, $p = 0.036$). Conversely, paternity among pumpkinseed parental males was low early and late in the breeding season, and high during the middle part of the season (ANOVA: $F_{[2,12]} = 3.92$, $p = 0.049$). In pumpkinseed, there was no significant variation in male phenotype (i.e., body length, mass, or condition) across the three trimesters (ANOVAs: all $p > 0.49$).

There was no relationship between parental male body length or mass and paternity in bluegill ($R^2 < 0.05$, $p > 0.43$ for both), but there was a positive relationship between Fulton's condition factor and paternity ($R^2 = 0.41$, $n = 16$, $p = 0.008$; Fig. 3). In pumpkinseed, there was no relationship between any of these three variables and paternity ($R^2 < 0.08$, $p > 0.31$ for all; Fig. 3).

Fig. 3. Phenotypic correlates of parental male paternity in bluegill (*Lepomis macrochirus*) or pumpkinseed (*Lepomis gibbosus*) in Lake Opinicon, Ontario, Canada. The phenotypic variables include body length, mass, and Fulton's condition factor. The regression line denotes the only significant relationship. Paternity data were arcsine square root transformed for statistical analysis, but are presented in their raw form for clarity.
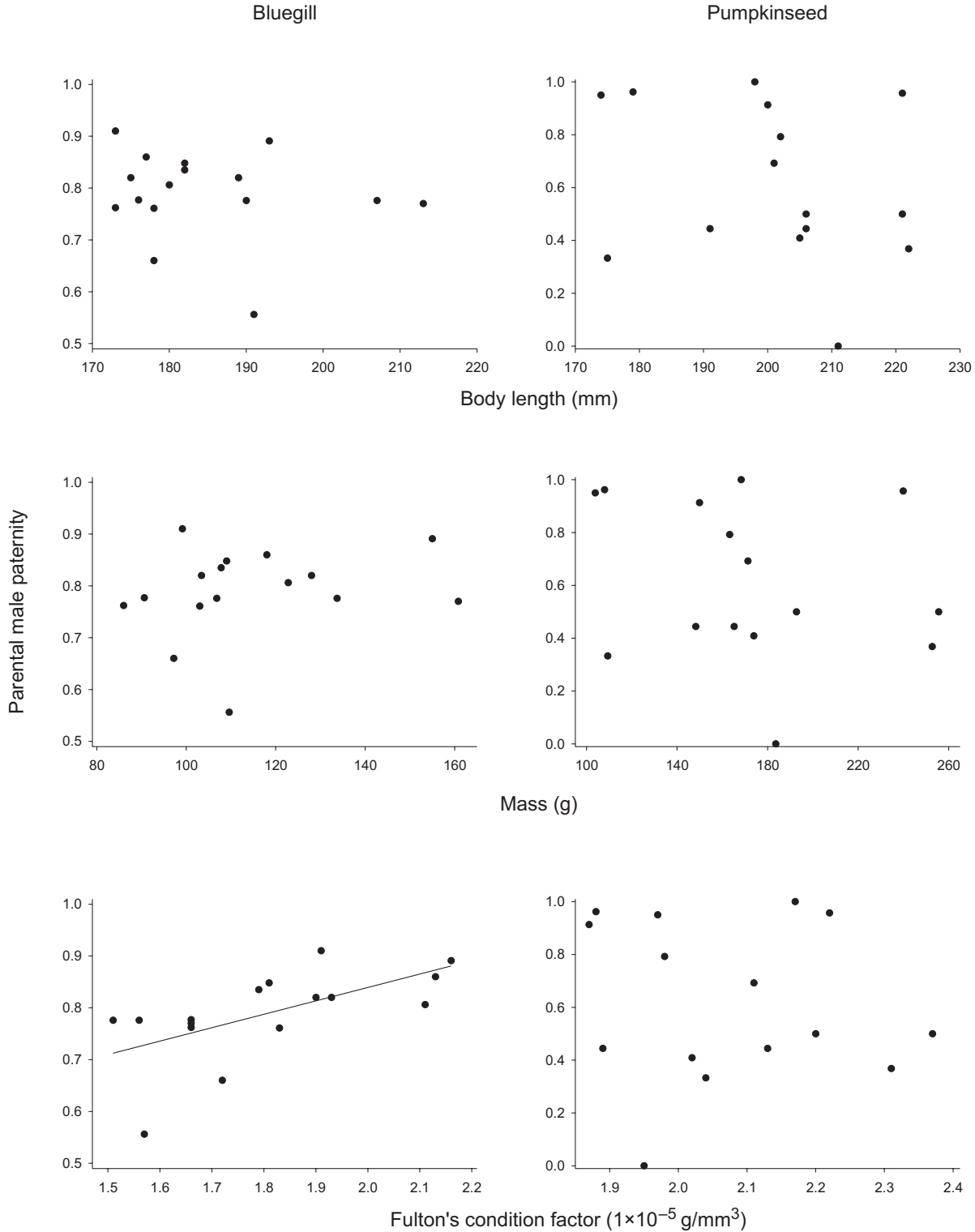
Discussion

We found significant variation across the breeding season in cuckoldry and paternity patterns in bluegill and pumpkin-seed sunfish. Interestingly, there was an opposite pattern of cuckoldry rates in the two species. Bluegill cuckoldry was highest (i.e., low parental male paternity) during the middle of the breeding season, whereas pumpkinseed cuckoldry was

Table 1. Summary of paternity analysis of nest-tending parental male bluegill (*Lepomis macrochirus*) in Lake Opinicon (Ontario, Canada).

n	Mean (%)	Range (%)	Source
40	75.1	41–100	Philipp and Gross 1994
38	76.9	26–100	Neff 2001
28	81.3	45–100	Fu et al. 2001
60	72.6	14–100	This study
166	**76.5**	**14–100**	

Note: Data comprise the number of nests from which progeny were collected, as well as the mean and range of paternity of parental males. Values in boldface type are the total or average of the data from the four studies.

lowest during this part of the season (Fig. 2). Although relatively little is known about the breeding ecology and dynamics of pumpkinseed in Lake Opinicon, considerable data are available for bluegill. We thus initially focus our discussion on possible explanations for the variation in cuckoldry rates in bluegill.

First, cuckoldry rates in bluegill may depend on mating opportunities for cuckolder males. In Lake Opinicon, spawning activity peaks during the middle part of the breeding season. For example, there are nearly twice as many parental males nesting during the middle part of the season compared with the first or third trimester (Cargnelli and Neff 2006). Males nesting during the middle part of the breeding season also obtain a larger brood, which suggests that proportionately more females are spawning during this part of the season (Cargnelli and Neff 2006). Given that spawning activity trades off with nest defence (Gross 1982), cuckolders may be more successful at gaining access to nests during the middle trimester. Furthermore, the increased opportunity of spawning may facilitate cuckoldry because less time is spent by cuckolders seeking out spawning opportunities (Gross 1991).

Second, parental males spawning during the middle part of the breeding season may be less able to defend their nests from cuckolders. Based on a subset of our bluegill parental males, we found a positive relationship between male condition and paternity (Fig. 3; also see Neff and Cargnelli 2004). Other research has shown that the mean condition (as measured by Fulton's condition factor) of nesting parental males changes during the breeding season, and is lowest during the middle part of the season and highest early and late in the season (L.M. Cargnelli, unpublished data). We could rule out roles of body length and mass because body size of nesting parental males decreases as the breeding season progresses (Cargnelli and Neff 2006). Thus, data from another study suggest that parental males nesting during the second trimester are in lower condition and may have less energy to allocate to nest defence during spawning.

Third, the cuckoldry patterns in bluegill may reflect a greater proportion of cuckolders spawning during the second trimester than the first or third trimesters. Gross (1991) has shown that, although there is negative frequency-dependent selection on cuckolders, the reproductive success of all cuckolders increases with their numbers relative to parental males. Thus, the increased cuckoldry observed during the

second trimester may simply reflect a greater relative number of cuckolders spawning during this time. Currently, however, there are no data available on the relative numbers of cuckolders spawning in each trimester.

We could not adequately address these three hypotheses in pumpkinseed because of a paucity in data. It is possible that the cuckoldry patterns relate to mating opportunities if, unlike the bluegill, fewer females spawn during the second trimester and parental males are better able to defend their nest during this period compared with the other two trimesters. A different pattern from the bluegill for the frequency of mating opportunities has been reported in the longear sunfish (*Lepomis megalotis* (Rafinesque, 1820)) in which mating opportunities decreased across the breeding season (Dupuis and Keenleyside 1988). It is also conceivable that the relative numbers of cuckolders spawning in each trimester mirrors the paternity patterns, although currently no data are available. It seems unlikely that differences in parental male phenotype, and their ability to defend their nest from cuckolders during spawning, can explain the paternity pattern. First, we found no variation in phenotype across the three trimesters, albeit our sample size and hence statistical power was low. Nevertheless, we also found no relationship between parental male body length, mass, or condition and paternity.

Interestingly, for the pumpkinseed, we found one parental male that had a paternity of zero. It is possible that the male represents a nest takeover, as has been reported in another sunfish (DeWoody et al. 1998). However, reconstruction of potential multilocus genotypes could not account for more than ~10% of the offspring. Alternatively, the nest-tending parental male that we collected may have constructed and spawned in the nest, but he may have been infertile.

Finally, genetic paternity data can be used to differentiate between two competing hypotheses to explain the evolution of ARTs. These hypotheses comprise the conditional strategy and the alternative strategies; the former predicts unequal fitnesses of the ARTs, whereas the latter predicts equal fitnesses (Gross 1996). Gross and Charnov (1980) developed a life-history model that can be used to calculate the relative fitnesses of ARTs. Specifically, Gross and Charnov (1980) show that the relative fitness of cuckolders to parentals can be calculated according to the following equation: $[q \times (1 - h)] / [(1 - q) \times h]$, where q is the proportion of males that enter the parental life history and h is the proportion of offspring sired by parentals in the population. To this end, the data presented here show that there is temporal variation in paternity and thus sampling should include appropriate representation of nests from each trimester in the breeding season. Indeed, the mean paternity estimate for bluegill noted here is lower than those previously published from the same population (Table 1). As a simple example of the potential consequence of sampling variation, assume that the difference in mean paternity reported across studies actually reflects temporal variation in sampling (it may also reflect interannual variation or methodological differences as one study used allozymes and the others used microsatellite loci). Next, for the Lake Opinicon bluegill population, assume that the proportion of males that mature as parentals is $q = 0.82$ (Gross and Charnov 1980; Neff and Lister 2007). Using the model of Gross and Charnov (1980), the estimates

of the relative fitness of cuckolders to parentals range from 1.05 (for $h = 0.813$) to 1.72 (for $h = 0.726$; see Table 1). The former value suggests close to equal fitnesses and would be consistent with the alternative strategies hypothesis, whereas the latter value suggests that cuckolders have higher fitness and would be consistent with the conditional strategy hypothesis (for further discussion see Neff and Lister 2007).

In conclusion, we have detailed the temporal patterns of paternity in two sunfish species. These patterns differed between species, and this difference may reflect temporal variation in mating opportunities, parental male defence ability, or cuckolder densities.

Acknowledgements

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