Report

Promiscuity Drives Self-Referent Kin Recognition

Timothy J.A. Hain[1] and Bryan D. Neff[1,*]
[1] Department of Biology
University of Western Ontario
London, Ontario N6A 5B7
Canada

Summary

Kin selection theory has been one of the most significant advances in our understanding of social behavior [1–3]. However, the discovery of widespread promiscuity has challenged the evolutionary importance of kin selection because it reduces the benefit associated with helping nestmates [4–6]. This challenge would be resolved if promiscuous species evolved a self-referent kin-recognition mechanism that enables individuals to differentiate kin and nonkin [7–9]. Here, we take advantage of an asymmetry in the level of promiscuity among males of alternative life histories in the bluegill sunfish (*Lepomis macrochirus*). We show that, as a consequence of this asymmetry, offspring of "parental" males have a high level of relatedness to nestmates, whereas offspring of "cuckolder" males have a low level of relatedness to nestmates. We find that offspring of parentals do not use a direct recognition mechanism to discriminate among nestmates, whereas offspring of cuckolders use kin recognition by self-referent phenotype matching to differentiate between kin and nonkin. Furthermore, we estimate that the cost of utilizing such self-referent kin recognition is equivalent to a relatedness (R) of at least 0.06. These results provide compelling evidence for adaptive use of kin recognition by self-referent phenotype matching and confirm the importance of kinship in social behavior.

Results and Discussion

Here, we use the bluegill sunfish (*Lepomis macrochirus*) to test the association of promiscuity with kin recognition by self-referent phenotype matching (hereafter referred to as "self-referent kin recognition"). With self-referent kin recognition (euphemistically referred to as the "armpit effect" [10]), individuals compare phenotypic cues of putative kin to their own phenotype to determine the degree of relatedness. This mechanism contrasts with familiarity, in which individuals instead learn phenotypic cues of conspecifics encountered during early development and remember these individuals as kin [7–9]. Bluegill are native to lakes and rivers of North America. The population studied here is found in Lake Opinicon, Ontario, Canada (44° 38′N, 76° 19′W), where males are characterized by a discrete life-history polymorphism termed "parental" and "cuckolder"

*Correspondence: bneff@uwo.ca

[11, 12]. Parentals construct nests, court, spawn with up to nine females (range = 1–9, mean = 5; author's unpublished data), and provide sole care for developing larvae. Cuckolders instead mature precociously and specialize in stealing fertilizations from parentals. Genetic paternity analysis has shown that a parental fertilizes an average of about 80% of the eggs in his nest largely by excluding cuckolders during spawning [13, 14]. Cuckolders fertilize the remaining 20% of the eggs and do this in part by opportunistically intruding into the nests of multiple parentals during spawning.

The difference in the levels of promiscuity (i.e., degree of multiple mating) between the two male life histories should lead to an asymmetry in the relatedness of nestmates of parentals' offspring versus cuckolders' offspring. By using microsatellite loci and pair-wise relatedness calculations, we indeed found that within nests, the average relatedness of parentals' offspring was more than three times that of cuckolders' offspring (median parental R = 0.30, median cuckolder R = 0.09; Mann-Whitney U = 152.5, n = 73, p < 0.001; Figure 1). Thus, a parental's offspring could gain more kin-selective benefits than a cuckolder's offspring simply by associating with and helping a random nestmate [1]. Such benefits may include reduced aggression and increased cooperation within shoals of fish, and such cooperation in bluegill and other fishes have been shown to lead to increased foraging efficiency and growth rate [15, 16]. Kin discrimination may be particularly important in such foraging contexts when there is an optimal group size that limits membership or when food resources are limited and must be shared among group members. Conversely, to gain a similar kin-selective benefit, a cuckolder's offspring would have to actively discriminate among nestmates. Because cuckolders' offspring are always in broods of mixed parentage with potential kin dispersed throughout the nest [17], only self-referent kin recognition would provide a reliable mechanism to distinguish kin from nonkin [7–9]. Location and familiarity (learning), two other reported kin-recognition mechanisms [18, 19], would not reliably allow discrimination between kin and nonkin.

We used in vitro fertilization and two-choice behavioral trials to determine the kin-recognition mechanisms employed by offspring of parentals and offspring of cuckolders. The two-choice trials presented pairs of larvae with the choice of associating with odor cues of broods differing in their degree of relatedness or familiarity, but not both, relative to the focal larvae. Specifically, four types of trials were conducted: (1) familiar full sibling versus unfamiliar full sibling, (2) unfamiliar full sibling versus unfamiliar nonkin, (3) unfamiliar full sibling versus unfamiliar half sibling, and (4) unfamiliar half sibling versus unfamiliar nonkin. These trials enabled us to test for the independent roles of familiarity and relatedness (and the degree of relatedness) in kin recognition.

Consistent with our kin-recognition hypothesis, offspring of parentals did not discriminate between the
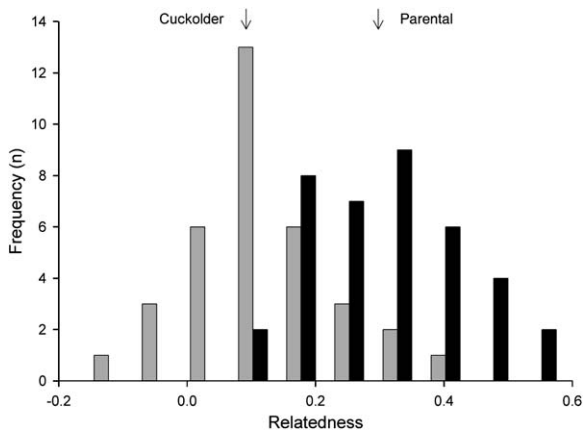
Figure 1. Average Pairwise Relatedness of Nestmates Sired by Cuckolders or Parentals

Arrows represent the median relatedness value for each life history. Cuckolder offspring (gray bars) were more than three times less related to nestmates than were parental offspring (black bars).

odors of unfamiliar full siblings and unfamiliar nonkin (mean of differences between kin and nonkin = −0.06 ± 0.16 SE; Wilcoxon Z = 0.55, n = 15, p = 0.58), unfamiliar full siblings and unfamiliar half siblings (mean of differences = 0.07 ± 0.14; Z = 0.53, n = 11, p = 0.59), unfamiliar half siblings and unfamiliar nonkin (mean of differences = 0.24 ± 0.18; Z = 1.31, n = 11, p = 0.19), or unfamiliar full siblings and either unfamiliar half siblings or nonkin (mean of differences = −0.01 ± 0.11; Z = 0.04, n = 26, p = 0.97; Figure 2A). Thus, we could rule out that offspring of parentals use self-referent kin recognition. Furthermore, we found that offspring of parentals do not use familiarity because they did not discriminate between the odors of familiar full siblings versus unfamiliar full siblings (mean of differences = 0.09 ± 0.28; Z = 0.20, n = 10, p = 0.84).

In contrast, although offspring of cuckolders did not discriminate between odors of familiar full siblings versus unfamiliar full siblings (mean of differences = 0.24 ± 0.19; Wilcoxon Z = 1.19, n = 10, p = 0.24) or unfamiliar half siblings and unfamiliar nonkin (mean of differences = −0.07 ± 0.16; Z = 0.56, n = 20, p = 0.57), they did prefer to associate with odors from unfamiliar full siblings versus unfamiliar half siblings or nonkin (mean of differences = 0.63 ± 0.18; Z = 2.64, n = 16, p = 0.008; Figure 2B). Thus, offspring of cuckolders do not use familiarity as a recognition mechanism and do not discriminate between half siblings and unrelated individuals. They do discriminate between full siblings and all other less-related individuals. It is unlikely that cuckolder offspring simply prefer the odor of any cuckolders' offspring because in all full-sibling-versus-paternal-half-sibling trials where cuckolders sired both stimulus broods (n = 5), focal larvae still preferred to associate with full siblings (binomial test: p = 0.03; see Figure 2B).

However, this experiment could not definitively rule out the possibility that these larvae had instead formed their kin template by using cues from their nestmates and not by themselves [20]. Thus, we conducted another experiment in which we scrambled the cues of kinship for offspring of cuckolders to confirm that these larvae use self-referent kin recognition. We accomplished this
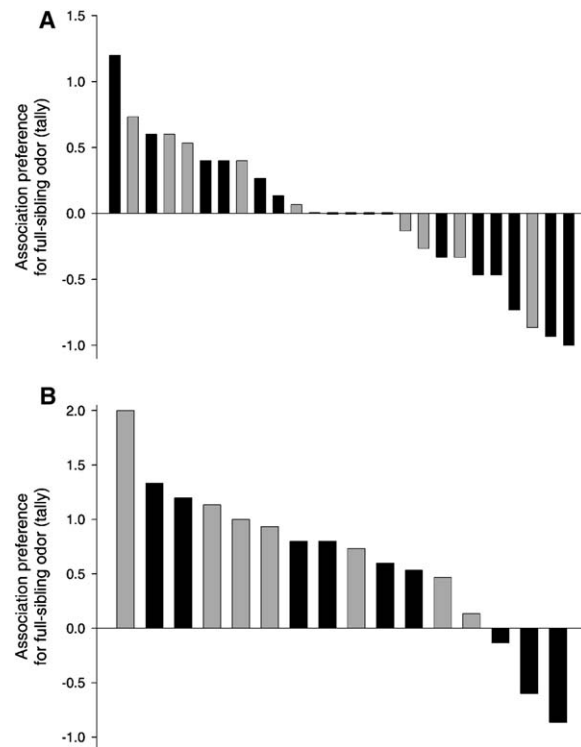

Figure 2. Association Preference for Full-Sibling Odors

Preference for full-sibling odors by parentals' offspring (A) and cuckolders' offspring (B). Preference was calculated as the average count of larvae in the full-sibling association zone minus average count of larvae in the nonkin (black bars) or half-sibling (gray bars) association zone. Trials are arranged in order of decreasing preference for full-sibling odor.

by generating focal fish from broods mixed at fertilization and consisting of two full-sibling families, one sired by a cuckolder and the other sired by a parental. We then reared from these larvae together throughout their lives. The focal larvae were then presented in pairs with odors from "pure" unfamiliar full siblings and "pure" unfamiliar nonkin (see Figure S1 in the Supplemental Data available online).

In these mixed-brood trials, when there was at least one larva sired by a cuckolder in the focal pair (in which case an asymmetry in association preference is expected), there was a significant preference for associating with the odor from the pure cuckolder-sired brood versus the pure parental-sired brood (mean of differences = 0.50 ± 0.12; Wilcoxon Z = 2.54, n = 11, p = 0.011; Figure 3). This latter result remained significant when corrected for multiple comparisons (corrected α = 0.0125). A kin template formed from nestmates would not allow a cuckolder's offspring to differentiate between the two referent odors because both odors would have been present in its nestmates since fertilization. Thus, our results cannot be explained by learned or environmental cues for kin recognition [19, 21] but instead conclusively demonstrate that offspring of cuckolders use self-referent kin recognition.

Our analysis also enabled us to determine the potential fitness cost of utilizing self-referent kin recognition. First, by examining trials involving offspring of cuckolders, we found that in four out of 14 trials that involved
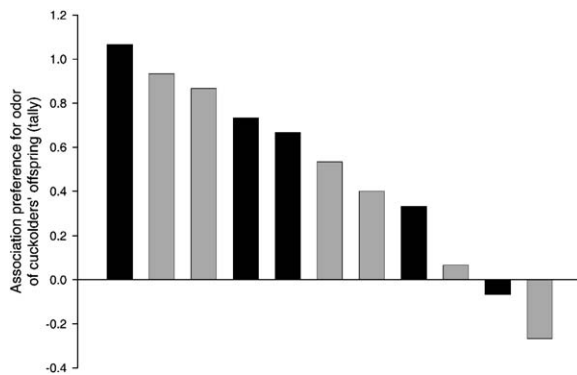
Figure 3. Association Preference for Odors from the Cuckolder-Sired Broods

Preference was calculated as the average count of larvae in the cuckolder-sired association zone minus average count of larvae in the parental-sired association zone. Trials involved focal pairs of larvae that were either full-sibling offspring of a cuckolder (black bars) or one offspring of a cuckolder and one offspring of a parental (gray bars). Trials are arranged in order of decreasing preference for cuckolder-sired odor.

full-sibling versus unrelated referents, the focal pair incorrectly associated with the unrelated referent (see Figures 2 and 3). This represents a maximum error rate of 28%; the actual error rate may be lower because we do not know whether the focal pair chose to associate with the unrelated referent. With this error rate, a cuckolder's offspring would be expected to associate with, on average, an individual of relatedness 0.36 (= 0.28 × 0 relatedness + 0.72 × 0.5 relatedness). Thus, a cuckolder's offspring could increase its kin-selective benefits by as much as 4-fold (= 0.36/0.09) by actively discriminating kin from nonkin. If a parental's offspring discriminated kin from nonkin with the same accuracy as a cuckolder's offspring, it too could expect to associate with, on average, an individual of relatedness 0.36. The level of relatedness would instead be 0.30 (see Figure 1) if an individual randomly associated with a nestmate. Thus, a parental's offspring could increase its kin-selective benefits only 1.2 times (= 0.36/0.30) by using self-referent kin recognition. Because offspring of parentals do not use self-referent kin recognition (see Figure 2A), our data suggest that there is a fitness cost in excess of a relatedness value of 0.06 (= 0.36 − 0.30).

It is likely that both types of offspring possess the genetic architecture for self-referent kin recognition, and that the differential expression represents phenotypic plasticity. Some of a parental's offspring become parentals themselves and use self-referent kin recognition in the context of parental care as adults [22, 23]. The differential gene expression in the larva could be mediated by RNA or transcription factors released by the spermatozoa into the ovum, as has been recently discovered in several mammals [24, 25]. In bluegill, spermatozoa of cuckolders have more ATP than do those of parentals, and they have more ATP than the amount that is required to travel to and fertilize eggs [26]. Given that ATP is a precursor to cyclic AMP, an important signal transmitter implicated in many cellular activities [27], it is possible that ATP is one of the transcription factors involved in the differential expression.

The evolution of social behavior, which is the association and interaction with conspecifics, has interested biologists for decades, and in many species, such behavior can be explained by kin selection [1–3]. Although kin selection has been one of the most significant advances in our understanding of social behavior, its importance has recently been challenged with the discovery of widespread promiscuity in mating systems because promiscuity reduces the benefit associated with helping nestmates [4–6]. Several other studies have attempted to quell this challenge by demonstrating an association between promiscuity and self-referent kin recognition [28–32]. However, our study is among the first to provide evidence of self-referent kin recognition that cannot be explained by in utero or indirect recognition [20, 33]. Furthermore, although self-referent kin recognition should evolve in promiscuous species because, in these species, other mechanisms cannot reliably identify kin ([7–9], but see also [34]), there has been little empirical evidence directly linking promiscuity with the mechanism. Here, we used an intraspecific approach that eliminates phylogeny as a potential confounding variable [35] and showed that an asymmetry in promiscuity between alternative male reproductive life histories is associated with differential expression of self-referent kin recognition in their offspring. These data suggest that promiscuity, and specifically its consequence on the relatedness of nestmates, is a driving force behind the expression of self-referent kin recognition. This recognition mechanism allows individuals to discriminate between kin and nonkin even when nestmates are not reliably kin. Future studies will investigate context-dependent kin recognition, the ontogeny of self-referent kin recognition in larvae sired by parentals, and the benefits of kin discrimination by larvae.

Experimental Procedures

Relatedness Calculation

We calculated average relatedness within a nest by using microsatellite analysis of larvae and putative parents collected in June 1996 [13, 14]. Genotypes were determined for an average of 46 larvae (range = 43–48) from 38 nests at 11 microsatellite loci. For each nest, each larva was first assigned to either the nest-tending parental or a cuckolder by exclusion methods [14]. Next, the larva's mean relatedness to all other larvae within the nest was calculated with the formula (equation 6) developed by Queller and Goodnight [36]. These data were then used to determine the mean level of relatedness within nests for parental-sired larvae and for cuckolder-sired larvae.

Experimental Fish

During the summer of 2005, swimmers equipped with snorkelling gear conducted daily surveys of breeding activity along the littoral zone of the northern edge of Lake Opinicon. When spawning was discovered, mature parentals, cuckolders, and females were netted opportunistically and transported by boat to aquarium facilities at the Queen's University Biological Station, which resides on the lake's shore. These fish were used to generate offspring via in vitro fertilization [37]. Full and half siblings were generated by the fertilization of 100 eggs from either one or each of two females in 500 ml glass jars with milt from either a parental or a cuckolder. Full-sibling fertilizations were performed in duplicate; one replicate was used for focal fish, and the other replicate was used to provide an "unfamiliar" odor source. This design ensured that we could control for the effects of familiarity as a recognition mechanism because we could select referent odors with which the focal fish had never come into contact. We generated mixed broods by dividing a jar in half with

a removable barrier. Eggs from one of two females were placed on either side of the barrier; one batch was fertilized with milt from a parental, and the other batch was fertilized with milt from a cuckolder. Five minutes after fertilization, when sperm had ceased activity [26], the barrier was removed, and the eggs were gently mixed. As above, replicate families of "pure" full siblings were also generated for both families in the mixed broods to serve as unfamiliar full-sibling referent odors (Figure S1).

Recognition Trials

Behavioral trials were conducted between 10:00 and 17:30 EST within 4 days of postlarval swim-up (i.e., when the larvae switch to exogenous feeding and are free swimming). The two-choice trial aquarium measured 34.4 cm × 18.9 cm × 20.4 cm (l × w × h) and was filled with fresh lake water to a depth of 8.1 cm. Two 10 cm association zones were defined at either end of the tank (Figure S1); the remaining 14.4 cm defined the middle, neutral zone. A trial began by the placement of two larvae from the same brood into the center of the tank. Simultaneously, water conditioned by one of two broods differing from the focal fish in either relatedness or familiarity (but not both) was introduced at a distance of 5 cm from either end of the aquarium at a rate of 6.6 ± 1.5 ml/min (sd). The conditioned water was taken directly from the jars that had contained the referent brood for 16–23 hr, and the side on which the referents were placed was determined by the flipping of a coin. Fish, including bluegill, have a well-developed olfactory system that has been shown to be involved in mate choice and kin recognition [30, 38, 39]. Two focal larvae were used because preliminary trials with only a single focal larva revealed erratic and agitated swim behavior consistent with a flight response. This behavior was not displayed by pairs of larvae. Each trial lasted 5.0 min, during which an observer who was naïve to the sources of conditioned water recorded the number of focal fish that were in the neutral zone or either association zone at 10 s intervals. All analyses examined the average of the counts of the scan samples for the second half of trials to ensure that there was sufficient time for odor cues to accumulate in the test aquarium and for focal larvae to assess these cues [23]. For the trials involving mixed broods, we used microsatellite loci and exclusion paternity techniques to determine the paternal origin of the two focal larvae (methods in [13]). Focal fish were never used in more than one trial. Between trials, the lake water was changed and the aquarium was cleaned with ethanol.

Supplemental Data

Supplemental data include one figure and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/18/1807/DC1/.

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