In Vitro Fertilization Reveals Offspring Recognition via Self-Referencing in a Fish with Paternal Care and Cuckoldry

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Abstract

Male bluegill sunfish (*Lepomis macrochirus*) exhibit alternative life histories: some males (parentals) delay maturation for up to 7 yr, then build nests, court females, and care for the eggs and fry, whereas other males (cuckolders) mature precociously, attempt to steal fertilizations from parentals, and provide no parental care. Parental males could avoid misdirecting their nepotism (i.e. caring for unrelated young) by abandoning entire broods if they were sired mainly by cuckolders or by discriminating between offspring and non-kin fry within broods for which they care. We tested for kin discrimination by obtaining sperm from parental and cuckolder males and eggs from several females, and using them to conceive fry in vitro. In 'blind' laboratory tests, parental males (but not cuckolder males) distinguished between sources of dripping water that had been conditioned by their own offspring vs. unrelated fry. Parental males that were in the best physical condition were especially choosy. Because the only referents available to our experimental subjects were chemical cues emanating from their own body, our results imply that parental males can use self-referent phenotype matching for kin recognition. This mechanism enables males to make the adaptive, nepotistic adjustments in paternal care that have been documented in previous studies.

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Introduction

Many animals distinguish relatives from non-relatives, close from distant kin, and one individual from another by assessing phenotypic attributes – including physical (Tibbetts 2002), vocal (Hare 1998), and especially chemical cues (Sun & Müller-Schwarze 1997, 1998; Heth et al. 1998; Hurst et al. 2001; Mateo 2002, 2003; Todrank & Heth 2003) – and then comparing those attributes against an

internal representation or 'template' of characteristics expected in various relatives. Such direct recognition (Waldman et al. 1988) occurs when phenotypes of recipients match templates closely enough (Holmes & Sherman 1982; Lacy & Sherman 1983; Reeve 1989). Although templates might be genetically determined (e.g. Grosberg & Quinn 1986; Keller & Ross 1998), typically they are learned during associations with appropriate referents (known kin) in unambiguous social circumstances, such as within a nest or burrow (Sherman et al. 1997; Holmes 2001). Templates sometimes develop through learning salient attributes of an individual's own phenotype, a mechanism known as self-referent phenotype matching (Holmes & Sherman 1982; Sherman 1991; also called the 'armpit effect' by Dawkins 1982). Theoretically, self-referencing might also occur via instantaneous comparisons not involving templates (Hauber & Sherman 2001). Such 'online' processing is possible because an individual's own phenotype is always available for comparisons with others.

Kin recognition based on social learning is well documented (Fletcher & Michener 1987; Alexander 1990; Brown et al. 1993; Sherman et al. 1997), and evidence for self-referencing is accumulating rapidly (Hauber & Sherman 2001). For example, when male peacocks (*Pavo cristatus*) that were hatched and raised in large, multiple-clutch groups were released in a park outside London and observed at sexual maturity (3–4 yr later), they lekked (displayed for females) nearer to brothers than to unrelated birds (Petrie et al. 1999). Because the initial, experimentally created associations among the young peacocks would have yielded unreliable recognition templates (i.e. because groups of hatchlings comprised kin and non-kin), the field observations imply self-referencing. Similarly, associations among brothers on lekking grounds in free-living grouse (*Tetrao tetrix*: Höglund et al. 1999; *Lagopus lagopus*: Piertney et al. 1999) and manakins (*Manacus manacus*: Shorey et al. 2000) have been interpreted as resulting from self-referencing, reinforced by natal philopatry.

Among mammals, self-referencing is the most likely mechanism enabling discrimination between full- and half-siblings within litters of Belding's ground squirrels (*Spermophilus beldingi*: Holmes & Sherman 1982) and between paternal half siblings and non-relatives among young that were reared by different mothers in those ground squirrels (Holmes 1986) and also savannah baboons (*Papio cynocephalus*: Alberts 1999; Smith et al. 2003). Among fish, juvenile rainbow trout (*Oncorhynchus mykiss*) can distinguish unfamiliar kin from unfamiliar non-kin using waterborne chemical cues (Brown et al. 1993), and rainbowfish (*Melanotaenia eachamensis*) can distinguish full siblings from half siblings using a combination of visual and chemical cues (Arnold 2000). Arctic charr (*Salvelinus alpinus*) can discriminate between full siblings that have similar and dissimilar MHC (Olsén et al. 1998), presumably by incorporating their own genetically based MHC odor into their recognition template.

To date, the strongest evidence of self-referencing comes from three studies, two in the laboratory and one in the field (Hauber & Sherman 2001). First, golden hamsters (*Mesocricetus auratus*) that were cross-fostered within 12 h of birth discriminated between flank-gland odors of siblings and non-siblings at sexual

maturity even though they were not reared with either of the stimulus animals (Mateo & Johnston 2000). Flank-gland odors are not produced until pups are about 1 mo old, implying that these hamsters did not learn kin identities before being cross-fostered. It seems most likely that hamsters incorporated their own odors into their kin-recognition template, although it is conceivable that they imprinted on some chemical with a similar structure that was produced by their mother or littermates in utero or during the first 1–12 h after birth (Mateo & Johnston 2000, 2003). Secondly, brown-headed cowbird chicks (*Molothrus ater*) were raised in visual isolation from conspecifics and their feathers were dyed black as they broke from their sheaths. As fledglings, these experimental birds approached black-dyed adult females more than undyed (brown) females, indicating that they incorporated their own feather color into their species-recognition template (Hauber et al. 2000; Hauber & Sherman 2003). Thirdly, when free-living male savannah baboons intervened in fights between juveniles, they favored offspring over non-kin (Buchan et al. 2003; Sherman & Neff 2003). This indicates phenotype matching because females mate with several different males, so indirect cues, such as time spent alone with a fertile female (e.g. Davies et al. 1992, 1996), do not reveal paternity.

In general, social learning and indirect cues are unreliable indicators of paternity if cuckoldry or intraspecific brood parasitism commonly occur (Neff & Sherman 2002). When social learning yields unreliable templates, only direct recognition mechanisms would enable a parent to discriminate its own young from foreign young. For example, parental male bluegill sunfish (*Lepomis macrochirus*) care for eggs and newly hatched fry in their nest, but parental males' genetic relatedness to broods is variable because cuckoldry is frequent and its genetic outcome can be unpredictable (Gross 1982; Neff 2001). Previously, we discovered that males adjust their paternal efforts in response to cuckoldry based on: (1) an indirect cue, the frequency with which cuckolders streak-spawned in their nest; and (2) a direct cue, the odors emanating from fry after eggs hatch (Neff & Gross 2001; Neff 2003). In a recent follow-up study, Neff & Sherman (2003) reported that parental males can differentiate between fry collected from their own nest in the field (most of which were offspring) and fry collected from another male's nest (none of which were offspring) using direct (water-borne) cues. However, parental males were unable to distinguish between eggs that were collected from their own nest and eggs collected from another male's nest, implying that chemical recognition cues are produced only after hatching (Neff & Sherman 2003; also see Brown & Colgan 1986).

The present study was undertaken to test if either parental or cuckolder males can distinguish between water conditioned by fry sired with their sperm via in vitro fertilization, but whom they have never contacted, from water conditioned by fry they have not sired and also have never contacted. Such discrimination would provide strong evidence of use of chemical cues in recognition and of recognition via self-referencing. It would also provide a mechanism by which parental males could adjust nepotism toward broods depending on their paternity.

Methods

Study Animals

Bluegill sunfish are native to lakes and rivers of North America (Lee et al. 1980). Males exhibit a polymorphism in life histories and behaviors termed 'parental' and 'cuckolder' (Gross 1982, 1991). At our study site, Lake Opinicon, Ontario (44°16′N, 76°30′W), parental males delay maturation for 7 yr and then establish breeding sites near the shore in colonies consisting of nests (approx. 45 cm in diameter) that are tightly packed together. Females arrive at these colonies in schools, visit males' nests, choose a mate, and lay eggs in his nest. Multiple females often spawn with the same parental male. Parental males remain at their nest for 7–10 d because eggs hatch 2–3 d after fertilization and fry linger in the nest for up to 7 more days before dispersing.

Cuckolder males, by contrast, mature precociously when they are 2 yr old, and they do not compete for territories, build nests, or associate with young. Instead, cuckolders gain access to nests by either mimicking female behaviors or stealthily sneaking in, and then stealing inseminations by ejaculating simultaneous with the pair's spawning. Molecular genetic paternity analyses have revealed that, on average, cuckolders fertilize 21% of eggs in a nest and parentals fertilize 79% (Neff 2001).

Experimental Fish

Experiments and observations were conducted at the Queen's University Biological Station on Lake Opinicon, during May–July 2002 and 2003. Breeding colonies of bluegill were located along the lake's shore and surveyed by snorkeling (see Gross 1982). Just prior to spawning, parental and cuckolder males and gravid females were collected using dip nets. Parental males always were non-neighbors whose nests were at least 1 m apart (e.g. see Fig. 1 in Neff 2001). Cuckolders and females were collected opportunistically from schools that formed above breeding colonies. Captive males were housed individually in aquaria measuring approx. $60 \times 60 \times 60$ cm, supplied with fresh lake water via a flow-through system that pumped water from near the shore, and exposed to natural light cycles through several windows. Captives were fed blood worms ad libitum at 08:00 EST each day until discrimination experiments began.

On the day each female was captured, approx. 500 eggs were collected by applying gentle pressure to the abdomen. These eggs were placed into 500 ml jars containing 50 ml of fresh lake water. Milt was immediately collected from a parental or a cuckolder male by holding him over a 2 ml graduated syringe and gently applying pressure to his gonadal region. The milt was squirted over the eggs and the mixture was left alone for 2 min. Then the jar was filled with fresh lake water and a bubbler was inserted to maintain oxygen levels. For the next 5–6 d, 30% of the water was exchanged every 12 h (08:00 and 20:00).

Eggs typically hatched 3 d after fertilization, and fry were allowed to develop for another 2–3 d before use in discrimination experiments. By then fry had eyes

but were still feeding endogenously on their yolk-sac, and they were a few days away from 'swim-up' (i.e. when they would have left the nest in nature).

Kin Discriminations using Olfactory Cues

Experiments were conducted between 09:00 and 19:00. The parental or cuckolder male that was to be tested was weighed (g) and measured (mm), then placed into a large aquarium (approx. $120 \times 60 \times 60$ cm) containing 250 l of fresh lake water. Simultaneously, groups of fry were placed into 1 l of fresh lake water inside 5 l plastic carboys. One set of fry had been conceived in vitro with sperm from the focal individual and the other had been conceived in vitro with sperm from another male from the same breeding colony. The test individual had no prior exposure to either group of fry. To avoid experimental artifacts associated with handling and injuring fry, samples were paired by eye to have roughly equal numbers; fry numbers were counted at the end of each experiment in 2002.

Once everything was in place, the fish were left alone to acclimatize for 1 h. Then water from the carboy containing offspring and the carboy containing non-kin fry was simultaneously dripped into opposite sides of the test aquarium at a rate of 10 ml/min. A video camera with a field of view that included the entire tank was switched on and behaviors of the focal male were recorded for 1 h. The sides of the test tank were alternated in successive trials and the tank was drained and rinsed between each trial. Each adult bluegill was tested once, after which it was released near the site where it had been collected. In total we tested 24 parental males and 13 cuckolder males.

Fry samples were used at most twice as stimuli, once for their genetic parent and again as an alternative, unrelated sample for a non-parent. In some instances, the unrelated sample was sired by a parental male that was not used in the present study (because the male was used in another study not reported here). All fry samples were paired to be equal in age (i.e. they were produced by in vitro fertilization on the same day and following the same methods). After each test in 2002, numbers of fry in each sample were counted on a gridded tray, and fry were released back into the lake. Samples of fry comprised 130–1575 individuals ($\bar{x} = 454 \pm 53$ SE), and differences in paired fry samples averaged 95 ± 75 individuals (i.e. $< 20\%$ of the total). Most importantly, numbers of fry did not differ significantly between stimulus groups (i.e. offspring vs. non-offspring) in the discrimination tests (paired t-test: $t = 1.26$, df $= 19$, $p = 0.22$).

Dye-Tracer Trials

To visualize how olfactory cues might circulate and be distributed during the course of our kin recognition trials, we added Methylene Blue (1.0 g/l; Sigma-Aldrich, St. Louis, MO, USA) to lake water in one of the carboys that we normally used for fry, and paired it with a carboy containing untreated lake water. A parental male that was not involved in the recognition trials was placed into the test tank and after a 1 h acclimation period the water from each carboy

was dripped into the tank at a rate of 10 ml/min. Every 10 min for 60 min, a 25 ml water sample was collected from the center of the tank. A spectrophotometer (Spectronic 20; Milton Roy, Conroe, Texas, USA) was used to quantify absorption of each sample in the blue–green wavelength (500 nm). Values at a given time were averaged across trials and expressed as a percentage of the absorption of the stock solution in the carboy. Four dye trails were conducted, each with a different parental male. After a trial the fish was released near the site where it had been collected.

Analyses

Video tapes of male behaviors were replayed to a 'blind' observer who recorded the time the test individual spent on each half of the tank and the number of times it crossed the centerline. The number of centerline crosses was used to gauge each individual's activity level and to ensure that each male assessed both sides (i.e. odors) of the tank in each trial (see Griffiths & Magurran 1999). Data from the first and second 30 min of each trial were analyzed separately because it takes time for odor cues to accumulate in the test tank and for the test individuals to assess those odors (see below; also Hauber et al. 2000). We used Wilcoxon signed-rank tests for two related samples to determine if test individuals spent more time on the side of the aquarium with dripping water that had been conditioned by offspring vs. unrelated fry, and also to compare the number of times test individuals crossed the centerline in the first vs. second 30 min of each trial. Although the data did not deviate from normality (Shapiro–Wilk test: $p > 0.49$ for all) we relied on non-parametric statistics because they yield more conservative tests than parametric tests (Zar 1999).

We used linear regression to explore the relationships between time males spent on the side of the tank with offspring and each male's length, condition (calculated from weight divided by cube of length, which is referred to as Fulton's condition factor), time of day when the trial was conducted, fry numbers in each stimulus sample, and the side of the tank on which the related fry sample was located. Total body length was used instead of weight because, although the two variables are highly correlated, length is less prone to short-term fluctuations. Moreover, weight was captured within the condition index, a measure that correlates with non-polar lipid density in parental male bluegill and other fish (Sutton et al. 2000; Neff & Cargnelli 2004). Both length and condition previously have been shown to correlate with a male's association behavior with nestlings (Neff & Sherman 2003). We also used linear regression to explore the relationship between the mean absorption values and the collection time in the dye trials. All statistics were performed using SPSS (version 12.0) and are expressed as $\bar{x} \pm 1$ SE.

Results

Parental males spent more time on the side of the tank with dripping water conditioned by offspring than on the side of the tank with dripping water

Table 1: Summary of results (times spent on the side of the test tank with kin or non-kin) from the kin recognition trials over the entire 60 min and over the first or second 30 min

	Entire 60 min		First 30 min		Second 30 min	
	Kin	Non-kin	Kin	Non-kin	Kin	Non-kin
Parentals (n = 24)	34.4 ± 2.4 Z = 1.7, p = 0.092	25.6 ± 2.4	16.2 ± 1.3 Z = 0.70, p = 0.48	13.8 ± 1.3	18.2 ± 1.3 Z = 2.3, p = 0.022	11.8 ± 1.3
Cuckolders (n = 13)	29.7 ± 4.2 Z = 0.28, p = 0.78	30.3 ± 4.2	14.5 ± 2.4 Z = 0.35, p = 0.73	15.5 ± 2.4	15.2 ± 2.1 Z = 0.07, p = 0.94	14.8 ± 2.1

Values are $\bar{x}$ ± 1 SE. The Z statistics are from Wilcoxon signed-rank tests for two related samples.

conditioned by unrelated fry during entire trials (60 min) and during the first and second 30 min of trials (Table 1). The latter difference was significant, and it remained so after correction for multiple comparisons (corrected $\alpha = 0.05/2 = 0.025$). This correction is for only two comparisons because the 60-min data are simply the sum of the two, 30-min data and thus not a third, independent variable as assumed by the Bonferroni method (Sokal & Rohlf 1995, p. 239; also see Nakagawa 2004). In contrast to parental males, cuckolder males did not prefer the side of the tank with dripping water conditioned by offspring during entire 60-min trials or either the first or second 30 min of trials.

For both parental and cuckolder males there were positive correlations between the time each individual spent on the side of the tank with dripping water that had been conditioned by offspring during the first and second 30 min of each trial (parentals: $r_s = 0.63$, n = 24, p = 0.003; cuckolders: $r_s = 0.62$, n = 13, p = 0.024), indicating some consistency in their preference between the two halves of the trial.

In all trials, focal males darted back and forth between the two ends of the test tank. Parentals crossed the centerline more often during the first than the second 30 min of each trial (first 30 min: 25.6 ± 5.1 times, range: 4–102; second 30 min: 20.0 ± 4.4 times, range: 2–79), although this difference was not significant (Z = 1.9, n = 24, p = 0.059). Cuckolders crossed the centerline a similar number of times during the two halves of trials (first 30 min: 25.1 ± 6.3 times, range: 0–83; second 30 min: 28.9 ± 6.5 times, range: 0–78; Z = 0.36, n = 13, p = 0.72).

Among parental males and cuckolder males, there was no relationship between body length and time spent on the side of the tank with dripping water that had been conditioned by offspring during the entire trial or either the first or second 30 min of trials (p > 0.11 for all). There also was no relationship between the condition of parental males and the time spent on the offspring side during the entire trial or the first 30 min of trials (p > 0.10 for both), but there was a positive relationship during the second 30 min ($r^2 = 0.17$, n = 24, p = 0.045; Fig. 1a). There was no similar relationship for cuckolder males ($r^2 = 0.05$, n = 13, p = 0.48; Fig. 1b).
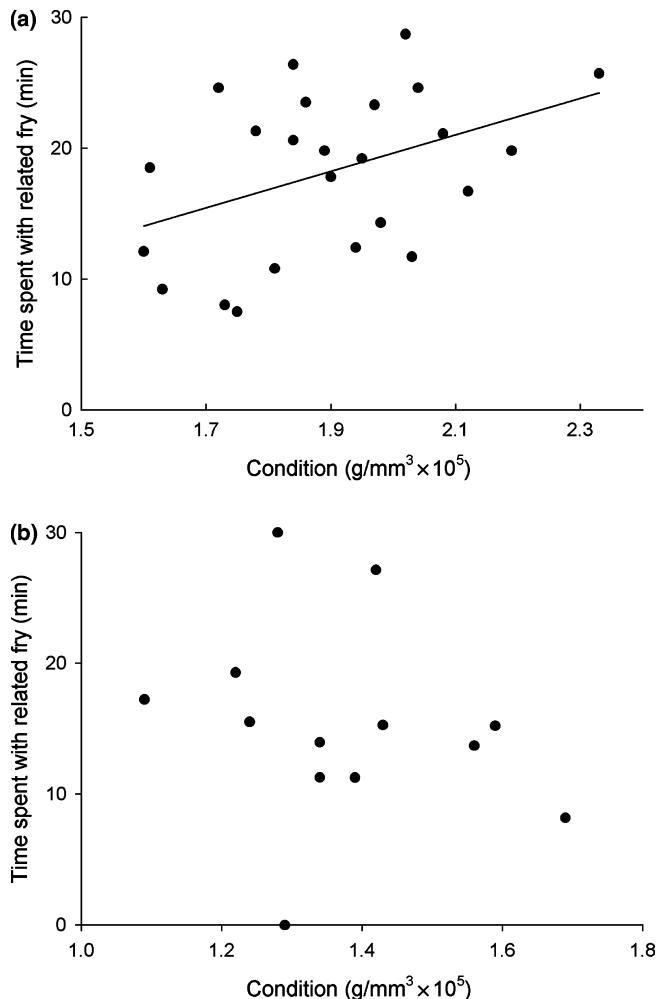
Fig. 1: Relationship between the time bluegill spent associated with related fry and their condition (Fulton's index) during the second 30 min of the recognition trials for (a) parental males or (b) cuckolder males. The solid line is from a significant regression

The positive relationship observed for parental males during the second 30 min of trials was stronger when multiple linear regression was used (overall model: $r^2 = 0.59$, n = 20, p = 0.018) including condition ($\beta = 0.63$, p = 0.003), time of day the trail was conducted ($\beta = 0.54$, p = 0.010), and side of the test tank on which the offspring were located ($\beta = -0.41$, p = 0.041); absolute differences in numbers of fry in the two stimulus samples (offspring minus unrelated) and total numbers of fry used to condition the water (offspring plus unrelated) were excluded from the final model (p > 0.18).

Dye trials revealed that the blue color spread throughout the test tank and increased in concentration linearly over time ($r^2 = 0.89$, n = 7, p = 0.001). It took approx. 30 min for the concentration to double at the centerline; it doubled again 30 min later. The blue color always appeared most intense right under the source carboy and least intense at the far end of the test tank.

Discussion

Our experiment capitalized on the fact that bluegill eggs are fertilized externally. We used in vitro fertilization to generate groups of fry that were either offspring or unrelated to parental or cuckolder males, and with which they had no physical or chemical contact prior to being tested. Results demonstrated that parental males could distinguish water conditioned by fry conceived with their gametes from water conditioned by fry conceived with gametes from a conspecific. In contrast, cuckolder males did not appear to display a similar kin discrimination behavior, although statistical power in this group was low (= 35% assuming an effect size equivalent to that observed during the second 30 min of parental male trials; Zar 1999, p. 107) and one cuckold was inactive during the trial.

Parental males showed heightened tendencies to associate with offspring-conditioned water during the second 30 min of trials (Table 1). Although the overall proportion of choice time is conventionally used to detect an animal's preferences (Wagner 1998), we believe results from the second 30 min of our trials are most revealing for three reasons (also see Hauber et al. 2000). First, videotaping of males' choice behavior began simultaneously with starting the water dripping, and undoubtedly it took some time for water-borne olfactory cues from the fry to reach detectable levels or a critical threshold in the test tank. Our dye trials showed that the blue color continued to diffuse and increase in concentration at the midline throughout each 60-min trial. Secondly, during the first 30 min of a trial males probably were familiarizing themselves with the novel stimulus of dripping water. Indeed, parental males appeared to dart back and forth across the centerline more often during the first than the second 30 min of trials (p = 0.059). Thirdly, cuckolder males did not show a significant preference during the first or second 30 min of trials, whereas parental males discriminated in favor of offspring in the second 30 min, suggesting that choices by parental males likely were not experimental artifacts.

We also found evidence that parental male association with related fry was context dependent. First, there was a significant positive relationship between Fulton's condition factor and time parentals spent near the source of water conditioned by fry conceived with their sperm. Fulton's factor is correlated with males' non-polar lipid stores, and therefore reflects energy levels (Neff & Cargnelli 2004; also see Sutton et al. 2000). If parental males in poor condition were especially hungry, they may have avoided offspring to minimize chances of cannibalizing them. Secondly, there was an independent effect of the time of day when trials occurred on the discrimination behavior of parentals. Later in the day, parental males were more likely to associate with dripping water conditioned by

offspring than they were earlier in the day. In nature, bluegill feed voraciously early in the day and feeding rates drop off later on (e.g. Baumann & Kitchell 1974; Collins & Hinch 1993). Because we fed our captive bluegill each morning, we probably reinforced morning foraging behavior. Early in the morning, parental males may have avoided water conditioned by offspring to minimize chances of cannibalizing them. Context-dependent variation in tendencies to avoid associating with close relatives when individuals are hungry also has been documented in cannibalistic tiger salamanders (*Ambystoma tigrinum*) and spadefoot toads (*Scaphiopus bombifrons*; Pfennig et al. 1993, 1994).

How did the offspring-recognition template of our parental male bluegill develop? It is conceivable that parentals use so-called recognition (green beard) alleles (Dawkins 1982) to distinguish offspring from unrelated fry. Although the occurrence of such alleles has been inferred in tunicates (*Botryllus schlosseri*: Grosberg & Quinn 1986) and fire ants (*Solenopsis invicta*: Keller & Ross 1998), in general, green beard alleles are expected to be rare due to suppression resulting from intra-genomic conflict (Alexander 1990; Sherman et al. 1997). Breeding experiments would be required to infer the existence of green beard alleles in bluegill.

Another possibility is that the parental males we studied had already learned their offspring-recognition template prior to our tests. It is conceivable that when parental male bluegill themselves were fry they had learned the chemical signature of their 'father' (the male that tended their nest) or their nestmates, as occurs in Arctic charr (Olsén & Winberg 1996). Or, because parentals may spawn several times in a breeding season and live to spawn in multiple years (Gross & Charnov 1980; Cargnelli & Gross 1996), it is possible that our subjects had imprinted on the chemical characteristics of their first brood to form their template. Such imprinting has been reported in female great reed warblers (*Acrocephalus arundinaceus*) who learn the color patterns of their first clutch, presumably enabling them to recognize as different any parasitic eggs laid by European cuckoos (*Cuculus canorus*) in later clutches (Lotem et al. 1992; Lotem 1995).

However, first clutches of female great reed warblers are parasitized infrequently enough and broods of Arctic charr are multiply sired rarely enough that their visual and chemical characteristics can yield a reliable recognition template. In contrast, the paternity of nest-tending male bluegill is highly variable, and cuckoldry rates appear to be especially high among broods of younger, smaller parental males (i.e. first-time breeders; B. D. Neff, unpubl. data). Furthermore, broods typically comprise offspring from numerous cuckolders and females. Thus, an olfactory signature from a brood would be highly variable (mixed) and not specific to the parental male guarding the brood or to the fry themselves. Finally, parental and cuckolder life histories have low levels of heritability (M. R. Gross, unpubl. data; but see Alcock 1989, p. 412) and therefore, some adult parentals will have been sired by cuckolders. Learning the odor of the nest-tending parental, of an early brood, or of one's nestmates thus would provide an unreliable kin recognition template (Neff & Sherman 2002).

Because all our tests were conducted in an aquarium that was previously unfamiliar to the parental males, our results indicate that parentals do not require physical features of their breeding colony to discriminate chemical cues of offspring from those of non-offspring. Results also indicate that parentals do not require associations with the mother of their fry to enable them to discriminate. In our experiment, females were collected from the school above the colony prior to spawning, and the males we tested had no direct experience with any particular female (i.e. they had not yet spawned). Males also did not know which female we used to produce the artificial crosses, and both samples presented to each parental male were generated using females from the same school.

Taken together, our data imply that parental male bluegill sunfish can use (chemical) cues from their own phenotype in discriminating among offspring and non-kin. Apparently this mechanism is what enables parental males to make the adaptive adjustments in parental care that have been documented in previous studies. Our results bring the number of species in which self-referencing has been shown or legitimately inferred to four comprising golden hamsters, brown-headed cowbirds, savannah baboons, and bluegill. The search for the chemical cues that facilitate self-referent phenotype matching in bluegill now is underway.

Acknowledgements

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