



Original Article

Multiple mating predicts intensity but not mechanism of kin recognition

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Understanding how animals recognize their kin has been a major challenge in biology. Most animals use one of 2 mechanisms: “familiarity” whereby kin are remembered from interactions early in life, such as in a nest, or “phenotype matching” whereby putative kin are compared with a template of what kin should look, smell, or sound like. Cross-species studies suggest that there is a link between which of these 2 mechanisms are used and the degree of female promiscuity (multiple mating). Phenotype matching is more likely to be used by promiscuous species because these species have lower average brood relatedness than monogamous species and familiarity is thus an unreliable cue of relatedness. However, it is unclear if this relationship holds within species, across populations that differ in their degree of promiscuity. Here, we take advantage of variation in brood relatedness across populations of guppies (*Poecilia reticulata*) to examine the relationship between kin recognition mechanisms and multiple mating within a single species. Contrary to the established hypothesis, we show that variation in recognition mechanism across populations is not governed by multiple mating. Instead, our data show that kin recognition, quantified as association preferences for shoalmates, is strongest when brood relatedness is high, consistent with Hamilton’s rule, but multiple mating does not otherwise influence the specific recognition mechanism used.

Key words: fish, guppies, kin recognition, multiple mating, *Poecilia reticulata*, promiscuity, relatedness, social behavior.

INTRODUCTION

The formalization of kin selection theory (Hamilton 1964) has greatly increased the understanding of social behavior. Kin selection has been used to explain behavioral phenomena such as cooperative breeding (Hatchwell et al. 2014), alarm calls (Sherman 1977), and gregarious association behavior (Viblanc et al. 2010). These behaviors appear to be costly to the individual performing the behavior because of reduced reproductive output, increased vulnerability to predators, or increased competition for resources, but these behaviors can indirectly benefit the individual by increasing the reproductive output, survivorship, or growth of relatives. Kin-directed social behaviors can arise through passive mechanisms, such as directing the behaviors to conspecifics found in or close to a natal nest, but there are also many examples of direct discrimination between related and unrelated individuals (Greenberg 1979; Wu et al. 1980; Grosberg and Quinn 1986). The ways that animals recognize their kin has thus become a major area of research for psychologists and behavioral ecologists.

Direct kin recognition is typically done using one of 2 mechanisms: familiarity (also known as prior association) and phenotype matching (Mateo 2004). With familiarity, individuals remember

other conspecifics encountered early in life, particularly in the vicinity of the natal area (e.g., the nest), and later treat those individuals as related. With phenotype matching, individuals instead use aspects of their own phenotype or those of conspecifics encountered early in life, such as odor, appearance, or sound, to build a “kin template.” Later, putative kin are compared with the kin template and treated as related or unrelated based on the similarity to the template (Holmes and Sherman 1982; Mateo 2004). So, for familiarity, specific individuals are remembered as kin, whereas for phenotype matching, putative kin are not remembered but instead compared with a template of what kin should look, smell, or sound like. Some species, such as humans and some apes, can use both mechanisms (e.g., Olsson et al. 2006; Alvergne et al. 2009).

Generally, it is hypothesized that animals will use familiarity when it is a reliable form of kin recognition. When familiarity is unreliable, phenotype matching should be used instead (Holmes and Sherman 1982). Familiarity is most commonly an unreliable mechanism when certain reproductive behaviors, such as brood parasitism or multiple mating, lead to unrelated individuals being born together or when dispersal or overlapping generations lead to unfamiliar kin encountering each other later in life (Holmes and Sherman 1982; Hauber and Sherman 2001). This hypothesis implies that variation among species or populations in their ecology, life histories, or mating systems should lead to variation in recognition mechanisms. In particular, when multiple mating

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leads to a low average relatedness of broodmates, phenotype matching should evolve over familiarity as the mechanism of kin recognition. Indeed, several studies have revealed variation in recognition mechanism that relates to the degree of multiple mating. For example, bluegill (*Lepomis macrochirus*) is a fish with alternative mating tactics and high levels of multiple mating: 92% of broods studied were multiply mated, with 77% of the larvae in a nest sired by the “parental” male guarding that nest and 23% of the larvae sired by intruding “cuckolder” males (Neff 2001). Consequently, offspring of cuckolder males were less related to their nestmates than the offspring of parental males. As predicted, cuckolders’ offspring used phenotype matching to recognize and shoal with their kin, whereas parentals’ offspring did not use phenotype matching (Hain and Neff 2006). In birds, Indian peafowl (*Pavo cristatus*) have high levels of multiple mating and use phenotype matching to recognize kin (Petrie et al. 1999). In contrast, other bird species characterized by low-multiple mating and relative monogamy, such as cliff swallows (*Hirundo pyrrhonota*) and barnacle geese (*Branta leucopsis*), use familiarity through prior association to recognize kin (Beecher et al. 1985; van der Jeugd et al. 2002). However, it is not clear if this pattern holds within a single species that is able to use both familiarity and phenotype matching. Here, we provide the first such test by examining the link between multiple mating and the mechanism of kin recognition in the guppy (*Poecilia reticulata*), a species that is known to use both familiarity and phenotype matching (Griffiths and Magurran 1999; Hain and Neff 2007).

Guppies are a small freshwater fish species that has emerged as a model system for the study of evolutionary ecology because populations have repeatedly experienced convergent evolution across river systems in response to similar selection pressures (Reznick et al. 1997). For example, differences in the intensity of predation affect the frequency of forced copulations (Magurran and Seghers 1994), brood size (Reznick and Endler 1982), and male coloration (Endler 1980). Guppies from different rivers also vary in the degree of multiple mating, which ranges from 70% to 100% of broods in the rivers examined to date (Hain and Neff 2007; Neff et al. 2008; Elgee et al. 2012). Guppy populations are known to show variation in their kin recognition mechanisms (Griffiths and Magurran 1999; Hain and Neff 2007), but these differences in mechanism have never been related to population-level differences in ecology. Guppies thus offer an exceptional opportunity to examine if recognition mechanisms differ across populations, and if the pattern of recognition mechanisms among populations can be explained by differences in multiple mating or other ecological or life-history factors.

In this study, we selected 6 guppy populations from Trinidad that captured variation in mating system, predation pressure, and life history (Hain and Neff 2007; Neff et al. 2008). We tested if individuals from each population used familiarity to associate with familiar rather than unfamiliar individuals as shoaling partners and phenotype matching to associate with related rather than unrelated individuals as shoaling partners. In fish, some potential benefits that arise from associating with kin include improved predator defense arising from group members having similar phenotypes, which enhances the confusion effect on predators (Ruxton et al. 2007; Croft et al. 2009), and leads to a more coordinated response against predators (Hain and Neff 2009), or reduced aggression among related shoalmates, leading to increased growth rates of individuals (Brown GE and Brown JA 1996). In order to gain these potential benefits, we

expected juvenile guppies to prefer to associate with familiar or related individuals over unfamiliar or unrelated individuals. We then asked if the observed variation in kin recognition could be explained by multiple mating, predation regime, or brood size. We predicted that multiple mating would affect recognition mechanism because it reduces the reliability that being born together (familiarity) is a cue of full-sibling relatedness (Hauber and Sherman 2001). Second, we predicted that predation regime would affect recognition mechanism because guppies from low-predation populations tend to live longer than guppies from high-predation populations (Reznick et al. 1996), increasing the likelihood of low-predation guppies meeting relatives from earlier or later broods, in which case phenotype matching would be required for kin recognition. Finally, we predicted that mean brood size in a population would be negatively correlated with the use or accuracy of familiarity. The recognition of individuals is thought to be cognitively demanding (Brosnan et al. 2010), and the ability to remember individuals from large broods may therefore be more difficult than remembering individuals from small broods.

MATERIALS AND METHODS

Field collections and trials

We collected guppies from 6 populations in Trinidad that varied in mating system, predation pressure, and life history (Lower Oropouche: 10°40′N, 61°08′W; Tunapuna: 10°42′N, 61°21′W; Upper Yarra: 10°47′N, 61°21′W; Upper Aripo: 10°42′N, 61°12′W; Lower Guanapo: 10°39′N, 61°12′W; Paria: 10°45′N, 61°16′W). As in other studies with guppies (e.g., Rodd and Reznick 1997), populations were classified as being from a high-predation regime if they were sympatric with the piscivore *Crenicichla* sp. (Lower Oropouche and Lower Guanapo) and from a low-predation regime if the rivers did not contain *Crenicichla* sp. (Tunapuna, Upper Yarra, Upper Aripo, Paria). All fish were collected with dip nets or a 2-m seine net and transported to the University of the West Indies within 3 h of capture (St. Augustine, Trinidad and Tobago). Guppies from 2 populations (Paria, Upper Yarra) were further transported to the University of Western Ontario (London, Ontario, Canada) before conducting the kin recognition trials. All captive guppies were maintained at 24–26 °C on a 12:12 h light:dark cycle (Houde 1997) and fed *ad libitum* twice daily, once with brine shrimp (Brine Shrimp Direct, Ogden, UT) and once with flakes (Tetra Werke, Melle, Germany).

Relatedness within broods was measured using females that were pregnant at the time of collection. Each female was placed in an individual tank that contained a small clipping of filamentous algae to act as a refuge for her offspring. Tanks were checked daily for the presence of newborn guppies, and 24 h after the first juvenile was observed the mother and all her offspring were euthanized with an overdose of clove oil and preserved in 95% ethanol for genetic analysis. Broods of fewer than 3 juveniles were not analyzed for paternity.

DNA was extracted from a subset of females and each their offspring using a proteinase K digestion (Neff et al. 2000). Three microsatellite loci (previously described in Becher et al. 2002; Paterson et al. 2005) were then polymerase chain reaction (PCR) amplified for the broods from each population (Lower Oropouche, Tunapuna, Upper Aripo: *Pre8*, *Pre9*, *Pre39*; Paria: *Pre1*, *Pre13*, *Pre15*; Lower Guanapo, Upper Yarra: *Pre13*, *Pre15*, *Pre80*). The PCR

products were visualized on a CEQ 8000 (Beckman Coulter) and the allele sizes determined using a reference size standard. Full- and half-sibling relationships within broods were reconstructed using the maximum likelihood approach implemented in the program COLONY (Wang 2004). Brood relatedness was then calculated as the mean of all pairwise relatedness comparisons within a brood. The average brood relatedness value for guppies could theoretically range from 0.25 (all individuals in a brood sired by different fathers) to 0.50 (all individuals in a brood sired by the same father).

Next, we conducted kin recognition trials to ascertain which, if either, mechanism was used by each population. For each population, approximately 60 juveniles were collected from the wild. These juveniles were checked daily for signs of male sexual dimorphism (appearance of a dark lateral spot or constriction of the anal fin, which typically occurs at about 5 weeks of age: Evans et al. 2002). The immature males were then isolated from females to prevent mating and ensure accurate pedigrees. Once fish had matured (after 7 weeks of age; Houde 1997), we mated each virgin female to a single male by placing the pair in a tank together for 7 days (Supplementary Figure 1). The male was then removed from the tank and was not mated again. Females were monitored twice daily for births, and newborn guppies were visually and chemically isolated from their broodmates as soon as they were discovered, which was always within 24 h of birth to prevent familiarity among broodmates (familiarity preferences develop after 12 days of association in guppies and are undeveloped at 8 days or less of association; Griffiths and Magurran 1997). This methodology of isolating or cross-fostering newborns within 24 h of birth is commonly performed in kin recognition studies that use internally brooded animals (Mateo and Johnston 2003). Because the female guppies were virgins, each brood was composed entirely of full-siblings, and because each mature guppy was mated only once, all full-sibling families were unrelated to each other.

Familiarity among individuals was manipulated by rearing 2 full-sibling families from the same population that were unrelated to each other together in a single tank. Families were divided into up to 3 groups of 3 or 4 fish, anaesthetized with MS-222, and given tail clippings (a notch in either the top or bottom part of the caudal fin) to allow identification to the family level. Familiarity was then developed by taking 2 such groups from 2 different families (born within 7 days of each other) and placing them together in a single rearing tank for 12–15 days in a group size of 6–8 individuals, which is within the shoal size range observed in nature (Magurran and Seghers 1991). Thus, individuals in these tanks were reared with familiar full-siblings and familiar nonkin. Individuals reared in separate tanks were treated as unfamiliar to each other.

At the end of the familiarization period, dichotomous choice trials were used to measure association preferences in sexually immature juvenile guppies for familiar versus unfamiliar and related versus unrelated individuals. The test arena was a 34-cm long tank (total volume = 10 L) divided into 3 compartments by 2 transparent, porous barriers that allowed visual and chemical communication between compartments, as both visual and chemical cues have previously been shown to be used in kin recognition in guppies (Griffiths and Magurran 1999). The center compartment was 18 cm in length and contained a focal fish. Each of the outer compartments was 8 cm in length and contained a single juvenile stimulus fish. Each trial presented focal fish with the choice of associating with either of 2 stimulus fish that differed from each other in either familiarity or relatedness (but not both). That is, in trials that manipulated familiarity, one stimulus fish was familiar and one

stimulus fish was unfamiliar to the focal fish, with both stimulus fish sharing the same relatedness to the focal fish (both related or both unrelated). In trials that manipulated relatedness, one stimulus fish was related and one stimulus fish was unrelated to the focal fish, with both stimulus fish sharing the same familiarity to the focal fish (both familiar or both unfamiliar). The side of the tank occupied by the more familiar (or more related) stimulus fish was assigned randomly and stimulus fish were matched for similarity in age and size.

For each trial, a focal fish was allowed 15 min to acclimate to the test arena, after which its behavior was recorded for 15 min. The focal fish's compartment was divided into 3 zones: two 5-cm "association zones" next to the barriers separating it from the stimulus fish and one 8-cm "neutral zone" in the center. We recorded the amount of time the focal fish spent in each association zone, counting both the time that the focal fish was entirely within the association zone and the time the focal fish had its gill slits in the association zone with its head oriented toward the stimulus fish on that side. Fish were used only once as focal fish but were used up to 4 times as a stimulus fish across tests of familiarity and phenotype matching. Among fish used 4 times as stimuli, there was no tendency for stimulus fish to be preferred more or less as shoaling partners as they were used in multiple trials ($F_{3,176} = 2.0$, $P = 0.11$). Tanks were cleaned between trials and filled with fresh water to remove olfactory cues. A total of 402 trials were conducted. Five trials were excluded from analysis either because of inactivity of the focal fish ($n = 4$) or because a stimulus fish displayed courtship behavior ($n = 1$). Variation in the number of broods produced by the 6 populations was associated with differences in sample size (25–119 trials per population).

Statistical analysis

For each of the 6 populations, the percentage of time spent associating with the full-sibling (or familiar) stimulus fish was compared with the percentage of time spent with the nonkin (or unfamiliar) stimulus fish using 1-tailed paired-sample *t*-tests (i.e., the time spent in the center "neutral zone" was excluded). We then combined these 2 types of association trials into a single analysis, in which we compared the percentage of time spent associating with putative kin (i.e., either familiar or related) to the percentage of time spent associating with putative nonkin (either unfamiliar or unrelated) using 1-tailed paired-sample *t*-tests. We did this latter comparison to test if populations in general preferred to associate with putative kin over unrelated individuals, independent of mechanism. Next, we used Pearson correlations to test if the time spent associating with the full-sibling (or familiar, or putative kin) stimulus was related to the average brood relatedness in the population. Average brood relatedness is inversely related to multiple mating but also captures reproductive skew among sires, where broods predominantly sired by 1 male have higher brood relatedness than broods equally sired by many males. Thus, it is average brood relatedness that is expected to affect the reliability of familiarity for kin recognition (Hain and Neff 2007). These correlations were based on population averages, so might underestimate the effect of within-population variance on the relationship between these factors. Thus, to better explore these relationships, we generated 10 000 simulated datasets by randomly drawing values for association time and brood relatedness for each population using the observed sample sizes and the total distribution of these parameters across populations. We then calculated the slope of the relationship between these factors for each simulated dataset to generate a null distribution of expected slopes. Finally, we compared the magnitude of our observed slopes

to the random distributions to determine the probability of observing a slope of at least that magnitude given the underlying structure of our data.

We tested the effect of predation regime on time spent associating with the related (or familiar) stimulus using ANOVAs with population nested within predation regime, where predation was coded as either high or low. Finally, we used Pearson correlations to test if the time spent associating with the full-sibling (or familiar) stimulus was related to the average brood size in the population. For all tests, the significance level was set at $\alpha = 0.05$ and performed using SPSS (version 22.0).

RESULTS

The relatedness figures calculated from the COLONY analysis and the proportion of broods that were multiply mated are presented in Table 1. Across the 6 populations, the average allelic richness was 9.7 (range = 6.3–16.7), average brood size was 10.4 (range = 3.8–14.7), average brood relatedness was 0.345 (range = 0.323–0.363), and an average of 95% of broods were multiply mated (range = 83–100%). There was no correlation between allelic richness and brood relatedness across populations ($R = 0.26$, $n = 6$, $P = 0.62$) or between brood size and brood relatedness across populations ($R = 0.37$, $n = 6$, $P = 0.47$).

Among populations, guppies varied in their association preferences for both related and familiar individuals (Table 2). Specifically, guppies from the Tunapuna, Paria, Upper Yarra, and Lower Guanapo populations preferentially associated with related rather than unrelated individuals, indicating their ability to discriminate between individuals using phenotype matching (Figure 1). Guppies from the Upper Aripo and Paria populations preferentially associated with familiar rather than unfamiliar individuals, showing their ability to discriminate between individuals using familiarity

(Figure 1). When all association trials were combined into a single analysis, every population except the Lower Oropouche preferred to associate with the putative kin stimulus rather than the nonkin stimulus (Table 2).

There was a significant positive correlation between association time with full-sibling stimuli and brood relatedness ($R = 0.86$, $n = 6$, $P = 0.028$; Figure 2a). The simulation analysis confirmed that the slope of this regression was greater than expected by chance ($P = 0.025$; for full distributions, see Supplementary Figure 2). There was no significant relationship between association time with familiar fish and brood relatedness ($R = 0.70$, $n = 6$, $P = 0.12$; Figure 2b), but when association times with either familiar or related fish were combined into a single analysis, we found a strong positive correlation between association time with putative kin and brood relatedness ($R = 0.90$, $n = 6$, $P = 0.014$; Figure 2c), and the simulation analysis confirmed that the slope of this regression was significantly greater than expected by chance ($P < 0.001$).

We found no significant effect of predation regime on association time with familiar fish ($F_{1,206} = 1.22$, $P = 0.27$), and no difference among populations in association time with familiar fish when population was nested within predation regime ($F_{4,206} = 1.22$, $P = 0.19$). There was no significant difference in association time with related fish between high- and low-predation populations ($F_{1,179} = 3.39$, $P = 0.067$), but there was a significant difference among populations in association time with related fish, and thus differences among populations in their use of phenotype matching, when population was nested within predation regime ($F_{4,179} = 2.81$, $P = 0.027$). Tukey's post hoc tests revealed that this difference was driven by the Lower Oropouche population associating with related fish less than the Lower Guanapo, Upper Yarra, and Paria populations ($P < 0.05$ for each).

Finally, we found no significant relationship between association time with related fish and brood size ($R = 0.45$, $n = 6$, $P = 0.37$)

Table 1
Summary of genetic and parentage analyses for 6 guppy populations

Population	Predation regime	Allelic richness	Number of broods	Brood size	Brood relatedness	Multiple mating ^a
Lower Oropouche	High	16.7 (12–24)	10	14.3 (8–21)	0.323	100%
Tunapuna	Low	6.7 (6–7)	10	14.7 (4–27)	0.334	100%
Upper Yarra	Low	6.7 (4–10)	10	3.8 (3–5)	0.346	90%
Upper Aripo	Low	6.3 (3–9)	10	7.3 (4–11)	0.348	100%
Lower Guanapo	High	12.7 (7–20)	12	9.3 (4–25)	0.357	83%
Paria	Low	9.7 (9–10)	23	12.8 (3–40)	0.363	96%

Data comprise predation regime (high, low), allelic richness (average and range across loci), number of broods analyzed, brood size (average and range), brood relatedness, and the percentage of broods that had multiple sires.

^aData on multiple mating in the Lower Oropouche, Tunapuna, Upper Aripo, and Paria populations were previously published (Hain and Neff 2007; Neff et al. 2008). One additional brood from the Paria population was included in the current study.

Table 2
Significance of association preferences based on paired *t*-tests in 6 populations of guppies

Population	Phenotype matching			Familiarity			Combined		
	<i>N</i>	<i>t</i>	<i>P</i>	<i>N</i>	<i>t</i>	<i>P</i>	<i>N</i>	<i>t</i>	<i>P</i>
Lower Oropouche	12	0.94	0.18	13	0.27	0.39	25	0.30	0.38
Tunapuna	60	2.66	0.005*	59	0.34	0.37	119	2.03	0.022*
Upper Yarra	12	2.08	0.031*	24	1.56	0.067	36	2.44	0.010*
Upper Aripo	37	1.26	0.11	35	2.38	0.012*	72	2.54	0.007*
Lower Guanapo	40	3.60	<0.001*	43	1.16	0.13	83	3.28	<0.001*
Paria	24	2.85	0.005*	38	2.82	0.004*	62	4.02	<0.001*

Significant preferences for the full-sibling or familiar stimuli are indicated with boldface type and an asterisk (*).

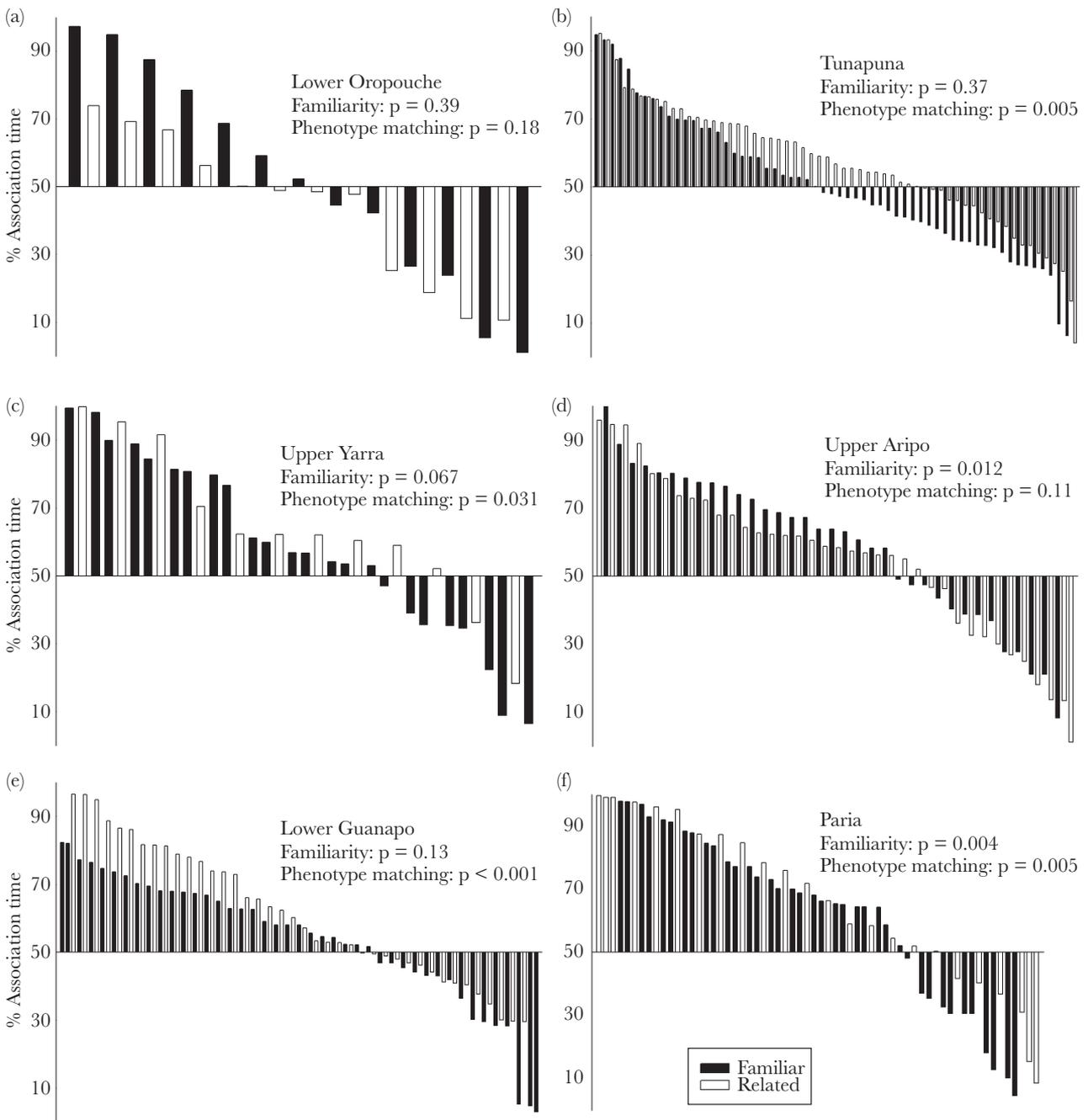


Figure 1

Association preferences of juvenile guppies for familiar versus unfamiliar individuals (black bars) and related versus unrelated individuals (white bars). Data are from 6 populations in Trinidad: (a) Lower Oropouche, (b) Tunapuna, (c) Upper Yarra, (d) Upper Aripo, (e) Lower Guanapo, and (f) Paria. Each bar represents an individual presented in rank order. Percent association time was calculated as time spent associating with either stimulus as a percentage of the total time spent associating with either stimulus in a 15-min trial. The 50% line indicates fish that associated equally with both stimulus fish, with bars above the 50% line representing time spent with related (or familiar) individuals that was greater than expected by chance. The results of paired *t*-tests comparing association time for related versus unrelated stimuli are shown above each panel.

or between association time with familiar fish and brood size ($R = 0.46$, $n = 6$, $P = 0.36$).

DISCUSSION

Here, we have provided the first data showing variation in kin recognition mechanisms across populations within a single species.

Previous studies of guppies suggested the presence of population-level differences in kin recognition mechanisms, as the Lower Tacarigua River population used familiarity to recognize kin (Griffiths and Magurran 1999), whereas the Paria River population used both phenotype matching and familiarity (Hain and Neff 2007). However, these studies differed in methodology, so could not rule out effects of the experimental design. By examining 6

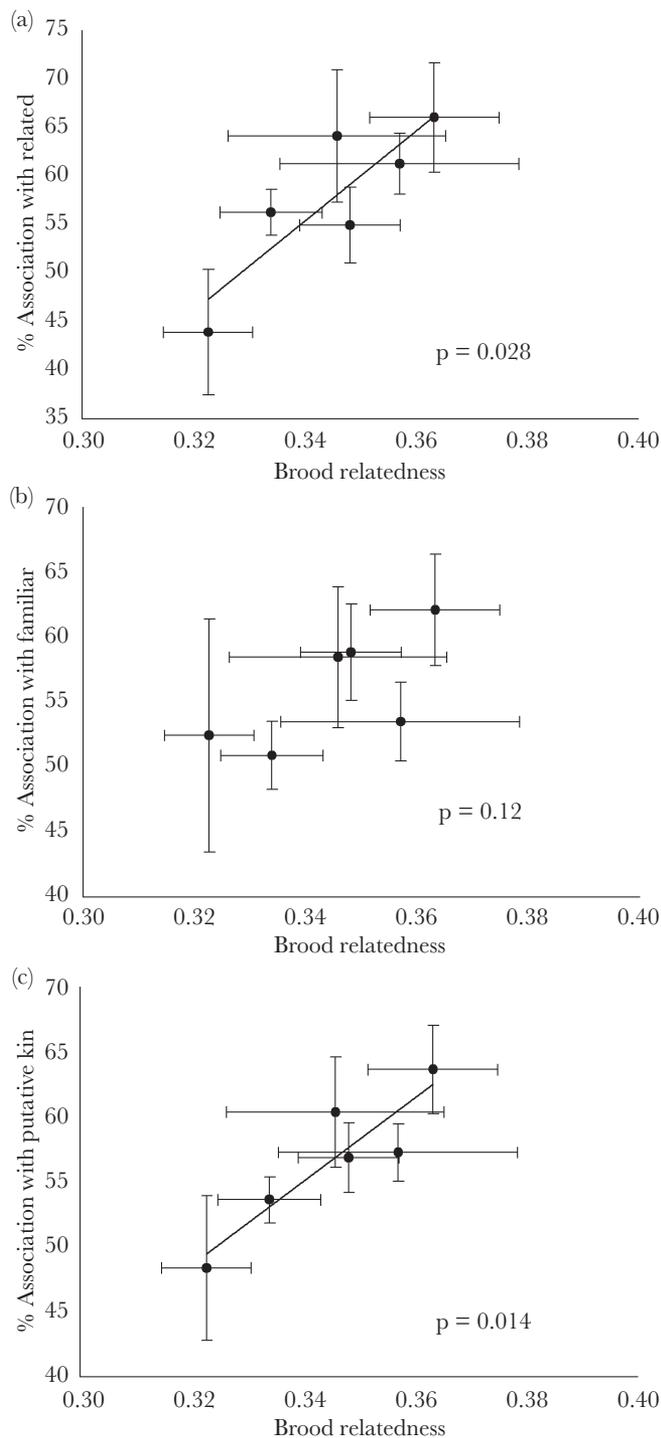


Figure 2

Relationships between association time with related or familiar individuals and within-brood relatedness for 6 guppy populations in Trinidad. Averages \pm standard error of the mean are presented for each population, showing the relationships between within-brood relatedness and association time with (a) related individuals, (b) familiar individuals, or (c) related and familiar individuals combined. *P* values testing the significance of the relationships between these factors were generated by a Pearson correlation, and best fit lines are shown for significant correlations.

populations of Trinidadian guppies using the same methodology, we found that 1 population used familiarity, 3 populations used phenotype matching, 1 population used both familiarity and

phenotype matching, and 1 population did not use either kin recognition mechanism to choose shoalmates. We also confirmed the earlier finding that the Paria River population used both phenotype matching and familiarity. Our data show that kin recognition mechanisms are variable within a species and that there is thus the potential for local conditions to influence the recognition mechanism used by a population.

One variable that has long been thought to influence kin recognition mechanisms is the level of multiple mating within a population (Hauber and Sherman 2001). Previous studies have found a positive association between the use of phenotype matching and multiple mating, both among species (e.g., Petrie et al. 1999; van der Jeugd et al. 2002) and among reproductive tactics within a species (Hain and Neff 2006). Here, we examined the effect of natural variation in multiple mating among guppy populations on the expression of kin recognition behaviors. We found that the strength of kin recognition was related to brood relatedness, despite a relatively narrow range of brood relatedness values across populations. However, this relationship was in the opposite direction from the prevailing hypothesis based on cross-species studies, as populations were more likely to use phenotype matching when brood relatedness was relatively high (i.e., multiple mating was low). There was also a similar trend to use familiarity more when brood relatedness was high, albeit the relationship was marginally nonsignificant. Our data thus reject the hypothesis that the degree of multiple mating affects the specific mechanism of kin recognition in guppies. Further within-species studies that examine the relationship between multiple mating and kin recognitions mechanism would help clarify the generality of this result.

Our observed positive relationship between association behavior and brood relatedness may instead reflect Hamilton's rule whereby a social behavior should be performed when the product of the relatedness coefficient of the individual being helped and the benefits of the behavior exceed the costs (Hamilton 1964). If costs and benefits of associating as a shoal are equal across the populations we studied, then the probability of associating with broodmates, regardless of the specific mechanism used to recognize them, scales directly with relatedness. Indeed, even though the differences among populations in within-brood relatedness were relatively small, there was a strong positive linear relationship between association time with putative kin and the mean within-brood relatedness of the population (in our combined analysis). Hamilton's rule can explain the observed relationship between brood relatedness and the strength of kin recognition, regardless of the actual mechanism used.

Hamilton's rule also offers an alternative explanation for previously observed relationships between multiple mating and kin recognition mechanism. For example, in social insects, promiscuous colonies have low within-colony relatedness and frequently show reduced preferences for kin and less aggression toward intruders than monogamous colonies that have high within-colony relatedness (e.g., Hogendoorn and Velthuis 1988; Pirk et al. 2001; Tsutsui et al. 2003; Adams et al. 2007). This trend has predominantly been interpreted as a breakdown in kin recognition because the greater genetic diversity in colonies with multiple breeders could increase the likelihood of recognition errors. However, the reduced recognition by promiscuous colonies are explained equally well by Hamilton's rule, as monogamous colonies have a greater within-colony relatedness coefficient and would thus gain greater benefits from kin discrimination. Indeed, in our study, we can rule out variation in recognition errors among our populations arising from difference in brood relatedness during development because we raised

test individuals from all populations with the same degree of mixed relatedness. Evolutionary effects driven by Hamilton's rule thus offer a robust explanation for differences in kin recognition across guppy populations and might also be important across a wide range of species.

The contributions of ecological or life-history factors other than multiple mating to the expression of kin recognition are not well understood. Perhaps the best example of a study that linked variation in recognition mechanism to ecological variation was an examination of 3 *Drosophila* species, which showed that recognition mechanisms could be explained by the species' mating system, gregariousness, or diet (Lizé et al. 2014). Here, we investigated the effects of predation regime and brood size on recognition mechanism and found no effect of either factor. We hypothesized that predation regime might affect recognition mechanism because it explains much of the variation in other traits among guppy populations (e.g., Reznick and Endler 1982). In particular, guppies from low-predation populations have significantly lower mortality than guppies from high-predation populations (Reznick et al. 1996; Reznick and Bryant 2007) and consequently also have an increased likelihood of encountering unfamiliar relatives from other broods. We thus expected that phenotype matching would evolve as a recognition mechanism in low-predation populations. However, we found that there was no difference in recognition mechanism between low- and high-predation populations. A previous study has shown that juvenile guppies from both low-predation and high-predation populations have a large number of siblings outside of their natal shoals (Piyapong et al. 2011). This high dispersal from natal shoals, independent of predation regime, may result in juveniles from both population types encountering unfamiliar kin and might explain the absence of any apparent effect of predation regime on kin recognition. Second, we hypothesized that populations with large broods might use phenotype matching because of the increased difficulty associated with remembering many individuals, as would be required with familiarity. Many species with large family sizes use phenotype matching to recognize relatives (e.g., fish: Olsén et al. 1998; Hinz et al. 2013; insects: Getz and Smith 1983; El-Showk et al. 2010). However, although there was almost 4-fold variation in average brood sizes across our populations, we did not observe a relationship between brood size and the use of phenotype matching. It is possible that the brood sizes seen across the populations studied here were too small and cognitively undemanding to have an effect on recognition by familiarity. Indeed, guppies have relatively small broods compared with externally fertilizing fishes and insects known to use phenotype matching (e.g., Whitehouse and Jaffé 1995; Olsén et al. 1998; Power et al. 2005; Ferguson-Gow et al. 2014).

In conclusion, we have provided the first within-species test of the effect of multiple mating on the mechanism of kin recognition. Although we found variation in mechanism across populations, we did not find the expected relationship with multiple mating. Instead, we found that kin recognition, regardless of mechanism, is strongest when multiple mating is low, and hence brood relatedness is high. This result is consistent with Hamilton's rule that the expression of a social behavior is directly related to the relatedness of the individuals.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.beheco.oxfordjournals.org/>

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