INQUIRY & INVESTIGATION

An Experimental Test of Kin Recognition in Harvester Ants

RECOMMENDED FOR AP Biology

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Abstract

Many animals direct assistance selectively toward relatives and/or aggression toward non-relatives; the ability to differentiate between kin and non-kin should evolve when doing so incurs fitness benefits. We detail a field-based experiment that tests whether workers of a large-bodied, abundant, and hardy seed-harvester ant are capable of kin recognition. We use this exercise in an undergraduate animal-behavior class to introduce concepts associated with eusocial insects and the study of kin recognition, as well as to reinforce principles of hypothesis testing, experimental design, and scientific writing. Students collect data, analyze and interpret results, and write a formal report; this experiment is one of several we use as models to prepare students for designing and performing their own follow-up studies.

Key Words: *Evolution; animal behavior; kin recognition; harvester ants; scientific writing; experimental design; hypothesis testing.*

Kin selection provides insight into how social behavior evolves. Recognizing kin can benefit animals in a variety of contexts – for example, during care and feeding of young and interactions within and among eusocial colonies. We describe a simple experiment that tests for the presence of kin recognition in a common, hardy ant species. Students discuss the evolution of kin recognition as well as considerations relevant to interpreting studies of kin recognition; they then test the hypothesis that harvester ants recognize kin and predict that, when ants are introduced near a colony's entrance, workers will behave more aggressively toward non-nestmates (ants from another colony) than toward nestmates. Our students collect and analyze data, read relevant primary literature, and write a report in the style of a scientific journal article. This exercise satisfies the Science as Inquiry, Life Sciences (Behavior of Organisms, Biological Evolution), and History and Nature of Science (Nature of Scientific Knowledge) content categories of the *National Science Education Standards* (National Research Council, 1996).

> *Kin selection provides insight into how social behavior evolves.*

We use this exercise in an animal-behavior laboratory course for upper-division undergraduates, which meets once a week for 4 hours. Our students have already taken (or are concurrently taking) a lecture course on animal behavior; most have also taken a course on evolution. This project is one of several we use as models to demonstrate hypothesis testing, experimental design, data collection and analysis, and reading and writing scientifically. The course culminates with final projects: each student conducts a literature review, designs his or her own experiment, collects and analyzes the data, and writes a formal report in the style of a scientific journal. We think that the level of student involvement for this project could easily be altered for introductory college or advanced high school courses. Although data collection usually takes 2–3 hours, depending on transit time and the number of ant colonies available, data could be collected over several shorter periods and combined.

○ Background

Students may be familiar with the idea that animals treat conspecifics differently on the basis of group membership, but they may not be aware that such treatment is often based on relatedness or that this function is important for understanding the evolution of social behavior. Kin selection theory was developed to explain variation in behavior toward conspecifics; differential treatment of relatives evolves when distinguishing between kin and non-kin increases fitness. For example, Belding's ground squirrels (*Urocitellus beldingi*) warn others of an approaching predator more often when their neighbors are relatives (Sherman, 1977). In many social species in which dependent young from different families commingle, parents preferentially feed their own offspring (e.g., Beecher et al., 1981; Jouventin, 1982; McCracken & Gustin, 1991). Aggressiveness may also be reduced toward relatives. For example, adult salmonid fish – Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) – defend territories more aggressively from non-relatives than from relatives (Brown & Brown, 1993). Many social insects use colony-specific odors (e.g., sweat bees [*Lasioglossum zephyrum*]; Greenburg, 1988) or visual cues (e.g., paper wasps [*Polistes fuscatus*]; Tibbetts, 2002) to identify nestmates; non-nestmates often receive increased aggression. This experiment investigates kin recognition in red harvester ants (*Pogonomymex barbatus*).

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○ Study Animal

Red harvester ants belong to a widespread and diverse group of ~75 species in North America, South America, and Australia known as "seed harvester ants" (Johnson, 2001). Workers of these species specialize in gathering plant seeds, which are stored underground and used to feed the colony (Johnson, 2001). Colonies compete for foraging area and maintain foraging territories; border disputes result in fights and, rarely, mortality (Gordon, 1995). Colonies adjust foraging strategies in response to encounters with foragers from neighboring colonies, presumably to reduce competition (Gordon, 1989). Such interactions between colonies may be mediated by the ability to distinguish between colony-mates and ants from other colonies (Gordon, 1995) using colony-specific cuticular hydrocarbons; when exposed to the extracted cuticular hydrocarbons of nestmates and non-nestmates, red harvester ants respond more aggressively to the non-nestmate extract (Wagner et al., 2000). Because each colony is founded by a single queen (Hölldobler, 1976), within-colony relatedness is higher than in species with multiple foundresses. Neighboring colonies should be unrelated because new colonies are not founded near their parent colonies (Gordon, 1989). That members of a colony share high relatedness but are unrelated to neighboring colonies supports the prediction that kin recognition would be adaptive in red harvester ants.

○ Experimental Procedure

To test the hypothesis that red harvester ants recognize kin, students introduce nestmates (control trials) and non-nestmates (experimental trials) near the colony entrance and observe behavioral responses. Aggressive behaviors (biting and dragging the introduced ant) are predicted to be more frequently directed at non-nestmates, whereas exploratory behaviors (antennation) might not differ between treatments. We like working with harvester ants because they are common in our region, large, diurnal, and hardy; these ants are easy to locate and work with, and they are active through early fall, when we conduct this experiment. Additionally, the mating system and foraging habits of red harvester ants are well understood (e.g., Hölldobler, 1976; Gordon, 1995; Johnson, 2001), which is especially helpful for students who wish to further investigate this species.

Several days before the experiment, we locate red harvester ant colonies and mark them with survey flags so that we can find them again rapidly. Red harvester ants prefer open fields with clay soils (Johnson, 2001). Searching for bare patches in a field is often the quickest way to find colonies because harvester ants typically clear vegetation around the nest entrance and along major foraging paths ("trunk trails"; Hölldobler, 1976). In some regions, such cleared foraging trails are less common (Gordon, 1995) but colonies can be located by following workers back to the nest. Harvester ant workers are reddish brown and about 5 mm long (Cole, 1968); workers returning to the nest usually carry seeds. We recommend using one "focal" colony per four or five students plus one "donor" colony, which should be outside the foraging range of the other colonies (i.e., ≥40 m away; Hölldobler, 1976). Red harvester ants occur from Mexico north to Kansas and west to Arizona (Cole, 1968); outside this range, this study could be conducted with other species (Table 1). Many ants discriminate against non-nestmates (Jaisson, 1991); however, invasive ants (e.g., Argentine ants [*Linepithema humile*] and red imported fire ants [*Solenopsis invicta*]) are often not aggressive toward non-nestmates outside of their native range (temperate South America; Tsutsui & Suarez, 2003). Excellent field guides are available (e.g., Fisher & Cover, 2007), and photographs of common species, including harvester ants, can be found online at the AntWeb and Ants of the Southwest websites (Anonymous, 2011; Ward, 2011).

Before class, students read a handout with background information on kin recognition and an article that describes considerations for studying it (Waldman et al., 1988). We begin by discussing circumstances that promote the evolution of kin recognition, the distinction between recognition and discrimination, and the difference between direct and indirect recognition (Waldman et al., 1988). We briefly explain the experimental goal (to test the hypothesis that harvester ants are capable of kin recognition) and protocol (below), and ask students to provide the rationale for key features of the experimental design (e.g., observers remaining blind to treatment, randomizing order of control and experimental trials, etc.). There are several alternative ways to introduce this project. Once students are aware that neighboring colonies of ants are competitors, for example, they are likely to be able to predict that workers from different colonies will behave more aggressively toward each other than workers from the same colony. Instructors may also wish to have students read a general overview of kin recognition, such as Pfennig and Sherman's excellent article, which is available both in print (Pfennig & Sherman, 1995) and online (2011).

Each group of four or five students needs two small glass jars for holding ants, one small cooler with ice (to cool ants prior to their release so that they move slowly and are easier to observe), a stopwatch (or other timekeeping device), a 25-cm piece of string or a ruler, and data sheets. Harvester ants can sting, so we ask all students to wear long pants and closed-toed shoes on the day of the project, and we caution them to avoid standing in areas of high ant traffic (e.g., near nest entrances or on foraging trails). We also recommend that students who are allergic to insect stings record data rather than working directly with the ants. Students who follow these simple

Table 1. Additional large-bodied ant species with which this study could be conducted; most ant species show some aggression toward non-nestmates, but see text for exceptions.

Species	Location	Notes
Harvester ants (*Pogonomyrmex* spp., *Aphaenogaster* spp., *Messor* spp.)	Open areas, deserts; most common from southern California west to Texas	All genera are known as harvester ants, share similar life histories, and often co-occur
Carpenter ants (*Camponotus* spp.)	Widespread in North America	Nests in dead trees and rotten wood; also nests in rotten wood on buildings
Pavement ants (*Tetramorium caespitum*)	Urban North America	Common on sidewalks; nests often have multiple entrances, so "donor" colonies should be far from "focal" colonies
Wood ants (*Formica* spp.)	Widespread in North America	Common in forests; builds mounds of vegetation (≤1 m) around nest entrances

directions are rarely stung while observing ant behaviors, but if they are, applying ice to the sting alleviates pain. As a precaution, teachers may wish to consult with the school nurse for first aid protocol.

At the "donor colony," we teach students to identify red harvester ants, and each group collects ants for use in experimental trials. Students should target individual ants from the clearing around the nest or along the trails, scoop them onto a sheet of paper, and deposit them into the jar. Each group observes a single focal colony and collects enough ants for control trials; ants from the focal and donor colonies should be kept in separate, labeled jars. The number of ants needed per group depends on how many focal colonies are available; we typically use 10 experimental and 10 control trials at each of 3–5 focal colonies and run 35–50 trials for each treatment.

The experiment consists of experimental and control trials, during which an ant from the donor or focal colony, respectively, is placed near the focal colony's nest entrance; behavioral responses of the focal colony's workers are then observed. We have students choose tasks before beginning data collection. One student records data and monitors the stopwatch, two or three observe ant behavior, and one "ant dispenser" introduces focal or donor colony ants. Students in each group use the string or ruler to identify an area on the ground that is clear of vegetation, trafficked by workers, and located 25 cm from the nest entrance. This site serves as the point where nestmate (or non-nestmate) ants are subsequently introduced.

Once ants have been collected, the ant dispenser decides whether the first trial will be experimental or control, and briefly (about 2–3 minutes) places the appropriate ants, while still in the jar, on ice. Between trials, ants should be allowed to warm up (i.e., do not keep ants on ice all the time). Cooling the ants ensures that they do not simply wander off once introduced, and also controls, to some extent, for differences in the behavior of introduced nestmate and non-nestmate ants. When the ants are sluggish and slow to respond to light stimulation (about 2–3 minutes), the ant dispenser should transfer one ant from the jar onto the ground at the predetermined release site. Observers should monitor the introduced ant and begin timing the trial at the first encounter between a colony member and the introduced ant. Observers should remain blind to ant identity; the ant dispenser and data recorder should avoid influencing observations. This design limits observational bias, since observers will not know whether each trial is experimental or control.

During each 3-minute trial, students should record all instances of antennation (touching the introduced ant's body with the antennae), biting (grasping the introduced ant with the mandibles), and dragging (walking while grasping the introduced ant with the mandibles). Bites and drags are aggressive acts, whereas antennations may represent more exploratory behaviors (Gordon, 1995). If introduced ants enter the nest or are dragged out of sight, or if observers simply lose track of them, students should record trial duration and the reason for terminating the trial (see data sheet, Table 2). We periodically remind observers of the operational definitions of the behaviors and we check that experimental and control trials are being conducted with blind observers and in a random sequence.

○ Data Analysis

Our students combine their data into a pre-prepared SPSS file (Table 2). To adjust for variation in trial duration, the number of antennations, bites, and drags should be converted to rates (e.g., number of antennations divided by length of trial). Students then (1) use descriptive statistics to compare each of the behavioral variables across treatments (e.g., mean ± standard deviation for experimental vs. control trials) and (2) check for differences among colonies with a multivariate analysis in which the dependent variables are the rates of antennations, bites, and drags per trial.

The simplest analysis consists of graphically comparing means and 95% confidence intervals between control and experimental trials (Figure 1); this could be analyzed statistically with t-tests. We usually use a multivariate general linear model (GLM) with treatment as a fixed factor and the rates of antennation, bites, and drags as dependent variables. Ideally, analysis would also account for differences among colonies by including colony as a random factor (Littell et al., 2006), but few undergraduates possess the statistical expertise to interpret such models. Teachers can tailor the analysis to the level of their students.

○ Results

As we guide students through data analysis, we ask them to interpret their results and to evaluate their hypothesis (discussion questions below). Our students typically find that non-nestmates receive more bites and drags than nestmates; differences in biting were significant in 3 of 7 years that we conducted this project, and differences in dragging were statistically significant in all years. Significant differences in antennations, by contrast, were detected in only 2 years, and in both cases, antennation was more frequently directed toward nestmates. Results from the most recent experiment are shown in Figure 1.

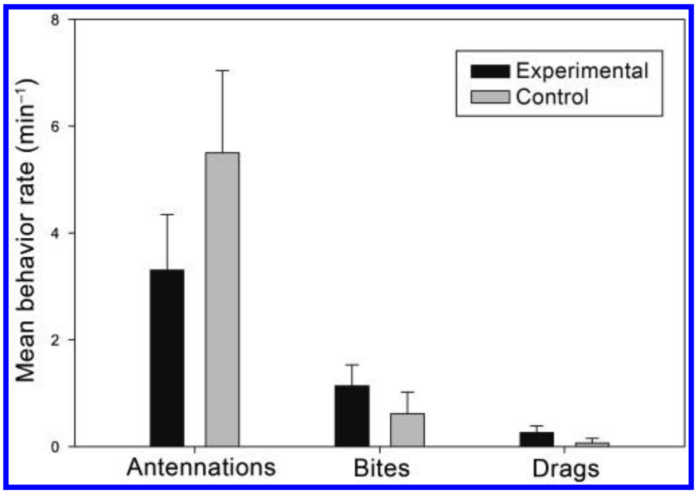

Figure 1. Mean and 95% upper confidence limit for the rate of antennations, bites, and drags over 46 experimental and 46 control trials.

Table 2. Sample data sheet, including data from sample experimental (E) and control (C) trials.

Trial Type	Number of Antennations	Number of Bites	Number of Drags	Trial Duration (seconds)	Notes
E	14	2	1	180	
C	5	0	0	180	
E	4	1	1	150	Dragged out of sight

○ Discussion Questions

Procedure

(1) Why do Waldman et al. (1988) advocate that observers be blind to the actual genetic relatedness of interacting individuals during kin recognition assays?
(2) Why should the order of experimental and control trials be randomized?
(3) What variables were controlled during the trials? (Capture/handling of introduced ants, cooling of ants, etc.) Why are controls necessary? What variables were not controlled? (Differences between colonies, multiple observers, etc.) How might this affect results? Could you improve the experimental design?
(4) Could there be differences in behavior among colonies? What factors might generate such differences? (e.g., size or age differences among colonies, or differences in how student groups defined and scored behaviors.) How might this affect results?

Interpreting Results

(5) Why convert the raw numbers of each behavior to rates (e.g., bites per minute) before further analysis? (Would it be appropriate to compare the number of antennations, bites, etc. for experimental and control trials if they were significantly different in length?)
(6) Were aggressive behaviors (biting and dragging) more common during experimental trials than during control trials?
(7) Were kin and non-kin workers antennated at equal rates? How would you interpret the existence of a difference in antennation rates of nestmates vs. non-nestmates?

Testing the Hypothesis

(8) Can you conclude that workers of this species are able to discriminate between nestmates and non-nestmates? Can you conclude that workers of this species are able to recognize their kin? [We like to emphasize that because kin recognition is an internal process, one must measure evidence of discrimination between kin and non-kin, and then infer whether recognition has occurred (Waldman et al., 1988).]
(9) What proximate mechanism(s) might explain how harvester ants recognize nestmates? [As in many social insects, chemical cues such as colony-specific hydrocarbons seem to mediate recognition (Wagner et al., 2000).]

Extensions

(10) Under what circumstances would you expect kin recognition to evolve? Compare characteristics of species that would be expected to recognize kin with characteristics of species that would be unlikely to do so.
(11) Did you observe any behaviors that you did not understand or would like to examine in more detail? Write hypotheses to explain such behaviors, and predictions you could use to test each hypothesis. After this exercise, many of our students have chosen ants as the subject of their final projects; topics have included alarm communication, recruitment to food in relation to resource quantity, seed preferences, aggression to non-nestmates as a function of distance to nest entrance, and kin recognition (in carpenter ants; see Table 1).
(12) Foraging harvester ants regularly encounter their neighbors and seldom encounter workers from more distant colonies; one could also test whether harvester ants distinguish between ants from neighboring colonies and more distant colonies (for published results of this comparison, see Gordon, 1989).
(13) An extension to invasion ecology can be made by addressing the finding that, in populations of fire ants (*S. invicta*) that lack a "kin recognition allele," colonies are more densely aggregated, a feature that increases their detrimental impact on native ant communities (Keller & Ross, 1998; Tsutsui & Suarez, 2003).

○ Summary

Because "recognition" cannot be observed directly, we have found this experiment particularly useful in illustrating the distinction between hypotheses and predictions (see McPherson, 2001). Participation in both the project and the pre- and post-experiment discussions also reinforces fundamental principles of experimental design, familiarizes students with basic features of eusocial insects, and allows students the opportunity to observe, first-hand, discriminative treatment of kin in a field environment. In the course of preparing their lab reports, students also read primary literature, which demonstrates species diversity in kin-recognition mechanisms and reveals additional features of harvester-ant biology. Judging from their written reports, most students seem to understand circumstances that promote the evolution of kin recognition and why, specifically, recognizing colony mates would be adaptive for harvester ants. Beyond that, the project serves an important role in stimulating students' interest in developing their own hypotheses, which they test at the end of the course. In some years, up to half of the class has opted to use harvester ants as subjects for their final project.

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