Nestmate recognition in burying beetles: the "breeder's badge" as a cue used by females to distinguish their mates from male intruders

Josef K. Müller,[a] Anne-Katrin Eggert,[a,b] and Tobias Elsner[a]

[a]Institut für Zoologie der Universität Freiburg, Hauptstrasse 1, D-79104 Freiburg, Germany, and [b]Behavior, Ecology, Evolution and Systematics Section, Department of Biological Sciences, Illinois State University, Normal, IL 61790-4120, USA

Burying beetles use small vertebrate carcasses as food for their larvae and defend these carcasses against intra- and interspecific competitors. Breeding associations on carcasses can consist of single females, heterosexual pairs, or various combinations of males and females. When a heterosexual pair collaborate in a breeding attempt, they do not typically exhibit aggressive behavior toward each other, but do attack newly arrived conspecifics that attempt to usurp the carcass. We investigated the cues involved in discrimination between breeding partners and intruders by female burying beetles. We found that resident females tolerate males that have cared for a brood, as well as males that have not cared for a brood but have been on a carcass for a day or two. Males that have had no prior contact with a carcass are attacked. Females appear to use a chemical cue, the "breeder's badge," an apolar substance on the male's cuticle that can be removed by washing with pentane. This cue is reliably correlated with recent male experience with a carcass that is suitable for reproduction. The breeder's badge develops as a result of prolonged contact with such a carcass, and disappears on removal from the carcass; its presence does not require contact with a female or with larvae. Female recognition of their male partners in burying beetles thus does not involve individual recognition, but rather recognition of reproductive condition. *Key words:* aggressive behavior, burying beetles, chemical cues, intraspecific competition, mate recognition, *Nicrophorus*, parental care, recognition of reproductive status. *[Behav Ecol 14:212–220 (2003)]*

The ability to discriminate among conspecifics is a prerequisite for most kinds of social behavior, including territorial behavior, care of young, maintenance of hierarchies, pair bonding, and colony defense (Fletcher and Michener, 1987). To discriminate among different classes of conspecifics, an animal must first be able to "recognize" them. Although we are aware that recognition describes an underlying neural process that need not result in detectable discrimination (Byers and Bekoff, 1986; Lacy and Sherman, 1983; Sherman et al., 1996), we will use the terms recognition and discrimination interchangeably.

One of the most common forms of discrimination, the ability to distinguish between related and unrelated individuals, provides obvious benefits to parental adults, dependent young, social insects, and communal breeders. However, the ability to discriminate among different types of unrelated individuals may also, under certain circumstances, provide important fitness benefits. When a pair of animals provide biparental care to their offspring, they have similar evolutionary interests in the well-being of the brood (paternity issues notwithstanding) that are not shared by other conspecifics. Resources required for the successful completion of a breeding attempt may be coveted by intra- and interspecific competitors and defended by the breeding pair. Joint nesting or communal breeding of several conspecific females creates a similar situation in which the cobreeders, but not other conspecifics, share a similar interest in the survival and growth of the brood. Breeding pairs, as well as members of a cobreeding group (males or females), should

thus benefit from an ability to distinguish between their breeding partner(s) and other conspecifics. In many vertebrates, such an ability exists in the form of individual recognition, manifest as vocal recognition in birds (e.g., Lengagne et al., 2000; Riters and Balthazart, 1998; Wanker et al., 1998; Wiley et al., 1991), and vocal or chemical recognition in mammals (e.g., Gheusi et al., 1997). Nestmate recognition in social insects, on the other hand, is an example of group recognition and is mediated by chemical cues that are typical of the entire colony (for references, see Breed, 1998; Clément and Bagnères, 1998; Singer et al., 1998; Vander Meer and Morel, 1998).

In the present study, we investigate a unique case of discrimination among unrelated conspecifics in insects, the discrimination between breeding partners and intruders in burying beetles, *Nicrophorus vespilloides* Herbst (Coleoptera: Silphidae). Burying beetles fly in search of small vertebrate carcasses that they inter and subsequently use as the sole larval food source. Usually, a pair of beetles cooperate in burying a carcass and rearing a brood on it. Both the male and the female feed and defend the larvae that undergo their development on the buried carcass (Pukowski, 1933). However, even buried carcasses can still be discovered by competitors, placing the young at risk (Hoferer, 1996). Especially in the early stages of parental care, carcasses buried by *Nicrophorus* remain susceptible to takeovers by conspecifics and individuals of other congeneric species (Robertson, 1993; Trumbo, 1990a; Wilson et al., 1984). Successful intruders kill and eat the residents' brood on the carcass (Scott, 1990; Trumbo, 1990a,b). When a resident pair encounter intruders, violent fights ensue that frequently result in injury or death of the contestants (Eggert and Sakaluk, 2000; Scott, 1990; Scott and Gladstein, 1993; Trumbo, 1990a,b). Residents thus require a mechanism that allows them to discriminate between their original mate and an intruder.

Cobreeding female *N. tomentosus* attack unfamiliar, nonbreeding conspecific females presented to them, but they do not attack their cobreeder (Trumbo and Wilson, 1993). In *N. vespilloides*, aggressive behavior is the norm when breeding females encounter nonbreeding conspecific females, but when females have been cobreeding with a conspecific female and their female cobreeder is replaced with a breeding female from another two-female association, aggression is rare (Beck, 1995; Eggert and Müller, 2000). Similarly, females from breeding male–female pairs do not attack breeding replacement males but do attack nonbreeding males (Beck, 1995). We thus hypothesized that there are certain features typical of breeding individuals that make them acceptable to other breeding individuals. We focused on the features of breeding males that make them acceptable to breeding females.

We conducted laboratory experiments to identify the recognition cues used by resident females in this context, evaluating the effects of male breeding status, behavior, contact with females, oral and anal secretions, and contact with or ingestion of carrion on female behavior. By testing the response of females to dead males that had been washed with water and pentane, respectively, we were able to separately assess the importance of nonpolar and polar substances for recognition. We termed the cuticular chemical that appears to render males acceptable to breeding females the "breeder's badge." We studied the acquisition of this badge with increasing time spent on a carcass, as well as its loss over time spent away from a carcass.

METHODS

Origin and maintenance of experimental animals

Experimental animals were the first-generation offspring of beetles collected from carrion-baited pitfall traps in the field in May and June 2000. The field site was a deciduous forest (the "Mooswald") near Freiburg in southwestern Germany (48°00′ N, 07°51′ E). Beetles were maintained in temperature-controlled chambers at 20°C on a 16-h light : 8-h dark cycle. Groups of up to six adults of the same sex were kept in small transparent plastic containers (10 × 10 × 6 cm) filled with moist peat, and were fed freshly killed mealworms twice a week. All experimental specimens were between 20 and 60 days of adult age. The size of experimental individuals was not controlled, but they had been reared under standardized laboratory conditions (see Eggert et al., 1998) and were thus relatively uniform in size. Repeated-measures designs involving repeat observations of females were used in experiment 1; in experiments 2 and 5, both males and females were observed repeatedly. In the remaining experiments, each individual was used for a single observation only. In total, 251 females and 276 males were used in the observations (Table 1); a total of 203 additional males and 119 additional females served as breeding partners of discriminating females or as partners of parental cue-bearing males, and were not used in the observations.

General experimental design

The experiments were designed to test the ability of the female member of a breeding pair to discriminate between her original mate and a male she had not encountered previously. During their activity phase, pairs of one female and one male were placed in small (8 × 6 × 5 cm) transparent plastic containers with a piece of moist paper towel (day 0). About 24 h later (day 1), each pair (or, for part of experiment 1, the female alone) was presented with a 10-g mouse carcass in its container. After 15 min, when the beetles had begun to

inspect the carcass, the carcass with the beetles was carefully transferred to a larger container (10 × 10 × 6 cm) with moist peat, where the beetles began to bury the carcass. These containers were placed inside an environmental chamber and transferred to a dark chamber on day 2, at which time all carcasses were completely buried. From this time on, the beetles were not exposed to any light except during experimental manipulations, which were performed under dim red light.

In the course of a breeding attempt, burying beetles undergo major physiological changes associated with oviposition and parental care, and individual females vary in the time after arrival on a carcass at which they begin to oviposit and, consequently, in the onset of parental care. At a certain time after their first contact with the carcass, females are thus likely to vary in their physiological (hormonal) condition. We therefore attempted to standardize the condition of our experimental females by testing them after a constant period of parental care (7–10 h) rather than at a constant time after the females' first contact with the carcass. To further reduce variation in the condition of the breeding females, brood size was kept constant by adding a certain number of larvae to the carcass.

To facilitate observations of behavior on and around the carcass, the beetles with their carcasses were gently transferred to fresh containers with compacted peat that was only 1–2 cm deep. This was done in the morning of day 3 before larval hatching. The peat in the old container was leveled out, and a small piece of mouse carrion was placed on top. Because larvae that hatch in the soil are attracted to carrion, this procedure enabled us to determine larval hatching times by visual inspection of the piece of carrion without disturbing the breeding adults. We checked the old containers every 4 h, beginning 67 h after the resident pair had received the carcass. Two hours after we first observed larvae in a pair's old container, we placed 10–15 first-instar larvae on the carcass with this pair. Some of the larvae added were not the pair's own offspring. The use of unrelated larvae is of no consequence to the present study because parental beetles do not discriminate between their own and unrelated larvae, provided they appear on the carcass at the right time (Müller and Eggert, 1990).

Terminology and design of tests

Unless otherwise mentioned, pairs or females were left to care for the larvae for 7–10 h after the larvae had been added (standard treatment of females, Table 1). We then removed the original male from the container, placed a new male in the container with the female and the carcass, and observed the response of the resident female to the new male. When unfamiliar "parental" males were presented to the female, these males had also provided care to larvae on a carcass for 7–10 h at the time of the trial. We will refer to the resident female as the discriminating female, and to the novel males as the cue bearer or cue-bearing male, following the terminology of Fletcher (1987). Whenever live males were transferred to a different location, great care was taken to not disturb them. The males were first placed in a small *Drosophila* tube and were then gently poured into their new container. We never touched the beetles by hand, and we grasped them with forceps only when all other methods failed. In most instances, the beetles walked into the *Drosophila* tube themselves when it was held in front of them or were pushed into the tube with forceps.

In the first five encounters between resident females and cue-bearing males, we observed the female's behavior and classified it as aggressive or tolerant. These five encounters always occurred within 5 min or less. When no encounters had

Table 1

Hypothesis tested, treatment, controls, and number of males, females, and trials for each of the experiments (Exp)

Exp	Hypothesis tested	Female treatment (lowercase letters)	Male treatment (uppercase letters)	Male control	No. female	No. male	No. trials
1	Female behavior toward unfamiliar male depends on male reproductive status and on female pairing history	a. Standard (paired): on carcass with male, 7–10 h care, each used in three trials b. Single mother (inseminated): on carcass, 7–10 h care, each used in two trials	A1. Parental: on carcass with female, 7–10 h parental care A2. Inexperienced: no contact with carcass Used once	A0. Female's original mate	a. 15 b. 14	A0. 15 A1. 29 A2. 29	A0. 15 A1. 29 A2. 29
2	Female behavior toward unfamiliar male depends on male behavior	Standard Each used in two trials with the same male (alive/dead)	A. Parental, dead B. Inexperienced, dead, Each used twice (alive/dead)	A. Parental, live B. Inexperienced, live	A. 15 B. 14	A. 15 B. 14	A. 30 B. 28
3	Breeder's badge is hydrophobic or hydrophilic substance on male cuticle	Standard Used once	A1. Parental, dead, washed in water A2. Parental, dead, washed in pentane B1. Inexperienced, dead, washed in water B2. Inexperienced, dead, washed in pentane Used once	A0. Parental, dead, not washed B0. Inexperienced, dead, not washed Used once	72	A0. 12 A1. 12 A2. 12 B0. 12 B1. 12 B2. 12	72
4a	Badge is transferred to male from breeding female	Standard Used once	A1. Single male, 6 days on carcass without female, used once	A0. 6 days on carcass with female, used once	24	A0. 12 A1. 12	24
4b	Badge is acquired from fresh or prepared carrion by contact or ingestion, or through contact with oral and anal secretion	Standard Used once	All inexperienced, dead, used once A1. Rubbed with prepared carcass A2. Fed prepared carrion A3. Fed fresh carrion A4. Treated with breeder's secretions	A0. Parental, dead, used once	43	A0. 11 A1. 5 A2. 9 A3. 11 A4. 7	43
5a	Breeder's badge develops as a result of prolonged stay on carcass	Standard at first trial, used four times with same male	Male on carcass with female for less than 70 h, no larvae; male tested after 28, 45, 55, and 68 h on carcass	None	16	16	58[a]
5b	Breeder's badge is lost as result of prolonged male absence from the carcass	Standard, mate replaced after 7–10 h of care, used four times with former mate Standard, used twice with same male	Parental male, removed from carcass tested with former mate after 12, 24, 36, and 48 h isolation Same male, tested with new female after 60 and 84 h isolation	None	19 19	19	114[b]
Total					251	276	442

[a] One failed.

[b] Four failed.

occurred within 10 min, the trial was aborted. However, this happened in only five of 442 trials (Table 1). In burying beetles, most fights are easily recognizable because the opponents grasp each other in a venter-to-venter position and attempt to bite each other's hind legs. However, when fights are initiated on the surface of the carrion ball, the aggressor may also bite other body parts, and may do so while both beetles are standing or walking on the surface of the carrion ball. If the discriminating female initiated a fight with the cue bearer, her behavior was considered aggressive. If she did not initiate a fight in any of her encounters with the cue bearer, her behavior was considered tolerant; in those instances, discriminating females responded to the cue bearer by producing a brief low-intensity sound effected via elytral-abdominal stridulation (Bredohl, 1984; Schumacher, 1973) or did not respond at all. Such tolerant interactions are also typical of encounters between the members of undisturbed breeding pairs. In less than 5% of all observations, the females' behavior was equivocal: in the first five encounters, we observed one short fight but also repeated tolerant encounters. We scored these instances as aggressive because we never observed fights between the members of a breeding pair.

Experiment 1: pairing history of discriminating females and reproductive status of cue-bearing males

In our first experiment (Table 1), we tested the response of caring females to novel males and, as a control, to their own mates. Half of the discriminating females were paired females whose mate had been removed just before introduction of the cue bearer ($N = 15$), and the other half were solitary females that had been previously inseminated and had initiated a breeding attempt on their own (single mothers); one of these died before her eggs hatched ($N = 14$). All the cue-bearing males we used were of the same age; however, they belonged to three treatment categories. Some males (inexperienced males, $N = 29$) had never been on a carcass (except, of course, during their own larval development), while others had been on a carcass with a brood and a female for the same time as the discriminating female; that is, they had provided parental care to larvae for 7–10 h (parental males, $N = 29$). Paired females were also tested with their own mates (original mates, $N = 15$). Each paired female was thus tested with three different males, and each solitary female with two. Different males were presented to females a minimum of 4 h apart; after each observation, the female and the carcass were returned to their breeding container with their original mate.

In trials involving the discriminating females' original mates, the male was removed, placed in an empty plastic tube, and immediately placed back with the female. In trials involving parental males, both the cue-bearers and the females were isolated for less than 1 min before observations, just long enough to allow for their transfer. We varied the order in which the different types of cue-bearing males were presented to females: Of the paired females, five encountered inexperienced males (I) first, parental males (P) second, and their original mates (O) last (sequence I–P–O), five others encountered them in the sequence P–O–I, and the last five in the sequence O–I–P; of the single mothers, seven encountered males in the order P–I, and seven in the order I–P. For this and the following experiment, the carcass with the female was transferred to a small transparent observation container, where she then encountered the cue-bearer.

Experiment 2: behavioral cues

An obvious candidate for a cue that females might use to discriminate against intruders is the cue-bearing male's own behavior. We attempted to assess the importance of the cue bearer's behavior by comparing the response of discriminating paired females to live and dead novel males; each female ($N = 29$) was tested twice with the same male, once when he was alive and once when he was dead (Table 1). Four hours after encountering a live male, the female was presented with the dead male. Dead males had been killed by freezing (30 min at less than $-28°C$) and had been thawed for 30 min at room temperature before testing. Half of the cue-bearing males had had no previous contact with a carcass (inexperienced males, $N = 14$), and half had been on a carcass with a female and a brood for as long as the females had (parental males, $N = 15$). For observations of encounters with dead males, the dead beetle was placed on the peat near the carcass or on the carcass itself in its natural position (dorsal side up). Observations continued until the female had encountered the dead male five times.

Experiment 3: cuticular lipids as chemical cues

Another candidate for recognition cues are substances adhering to the male's cuticle, the presence or concentration of which is correlated with his recent history with a carcass, mate, and/or brood. We attempted to assess the importance of such cues by comparing the response of females to unfamiliar dead males that had not been manipulated, had been washed with water, or had been washed with n-pentane (Table 1). Washing was performed by continuously stirring the male in 2 ml of pentane (or water) for 15 min after he had been frozen and thawed as described above. After washing, the males were left to dry for 15 min at room temperature before being presented to resident females. Again, we used both inexperienced and parental males. In this and the subsequent experiments, novel males were presented to resident females in their breeding containers, from which their original partners had been removed. Each discriminating female, and each cue-bearing male, was only used in a single observation ($N = 72$ females and 72 males).

Experiment 4: the origin of the breeder's badge—external sources

Part a: the role of female presence
The chemical cue that distinguishes parental males from inexperienced ones might actually be produced by females and transferred to parental males through repeated contact. To test for this possibility, we compared the response of discriminating females to cue-bearing males that had been on a carcass with or without a female (Table 1). Each discriminating female was only used in a single observation ($N = 24$ females). Males without a female ($N = 12$) had been alone on a carcass for 6 days and had cared for larvae for 7 h; males with a female ($N = 12$) had been on a carcass with a mate for 6 days and had also cared for larvae for 7 h. All cue-bearing males were frozen and thawed before being presented to females to prevent complications arising from the possible aggressive behavior of solitary males towards discriminating females.

Part b: the role of cues associated with the carcass
We hypothesized that males might acquire the pertinent cues through contact with or ingestion of carrion. Alternatively, the breeder's badge might originate from anal or oral secretions that the beetles spread over the carcass (Pukowski, 1933). Therefore, we tested the response of 43 resident females to inexperienced males that had undergone four different experimental treatments ($N = 32$), or to a control group of untreated parental males ($N = 11$; Table 1). Each male and female was used once. The inexperienced males used had been subjected to the following treatments: (1) after being killed, they were rubbed with a carcass that had been treated by a pair of beetles for 72 h ($N = 5$); (2) for 3 days before being killed, they were fed small pieces (less than 0.5 g) of a carcass prepared by a pair of beetles for 72 h ($N = 9$); (3) for 3 days before being killed, they were fed small pieces (less than 0.5 g) of fresh mouse carrion ($N = 11$); or (4) after being killed, they were treated with oral and anal secretions of caring males ($N = 7$). We collected secretions by gently squeezing caring males and collecting the droplets produced from mouth and anus with a pipette. Secretions from two males were spread over the cuticle of inexperienced cue-bearing males 10 min before they were presented to females.

Experiment 5: development and loss of the breeder's badge with time spent on or away from a carcass

Part a: development of the breeder's badge over time
In this experiment, the development of the breeder's badge in the course of a breeding attempt was investigated by

presenting discriminating females with cue-bearing males that had initiated a breeding attempt on a carcass with another female but had spent less time on a carcass than had the discriminating females. The experiment involved repeat observations of the same discriminating females and cue-bearing males at different points in time (Table 1). A total of 16 pairs were established on carcasses as described above. The first larvae usually hatch in the morning of day 4. In the afternoon of day 3, 20 h before the first larvae of resident pairs began to hatch, 16 cue-bearing males were provided with carcasses and females of their own. They were first presented to discriminating females after these females had provided about 7–10 h of parental care.

Before observing the female's response to a male, we tested whether the male himself was ready to care for larvae, that is, whether he had entered the parental state. Males and females are ready to care for larvae close to the time their own larvae hatch, but they kill larvae that appear on the carcass long before this time (Müller and Eggert, 1990). The male was placed in a small container with 15 first-instar larvae, and his response to the larvae was observed for 5 min; he was considered to be in a parental state if he did not kill any larvae during the observation. After interacting with the discriminating female, the cue bearer was returned to the container with his original mate until he was tested again with the same female.

Testing was repeated three times, approximately every 12 h, until all males had entered the parental state and had been accepted by females. Two males were severely injured in their first trial with a female and were not tested again. An additional deviation of the sample size from 16 was caused by a female that remained hidden under the carcass during one trial and thus did not encounter the male.

Part b: loss of the breeder's badge
In this experiment, females ($N = 19$) were left on a carcass with their original mate until the pair had provided 7–10 h of parental care, after which time the male was removed from the carcass and replaced with another parental male. From then on, the original males ($N = 19$) were kept singly in plastic containers with moist peat. We used a repeated-measures design for this experiment: after being isolated from the carcass for 12, 24, 36, 48, 60, and 84 h, the males were presented to discriminating females (Table 1). Each male was thus observed in six trials at different times. For the tests after 12–48 h of isolation, the males' original mates served as discriminating females (these females had been given replacement parental males when their original mates were removed in order not to affect the females' behavior). For the observations after 60 and 84 h, each male was tested with a second female ($N = 19$) that had been caring for a brood for about 7–10 h at the time of the first encounter. This was done because males typically abandon the carcass after 5–9 days under laboratory conditions and are frequently attacked by females at this time, and we wanted to ensure that it was not simply the females' behavior that changed. Using different females should not have affected the results, because the response of females to novel parental males is not different from their response to their own original mates (see Results of Experiment 1). In a total of four trials, the discriminating female remained hidden under the carcass and thus did not encounter the cue-bearing male, leading to deviations of the sample size from 19.

Statistical treatment of results

The alpha level for statistical significance was set at 0.05 for all comparisons. Statistical analyses were performed by using SAS 8.1 for personal computers (SAS Institute Inc., 1998). We used Fisher's exact tests for frequency tables containing cells with values less than two. For the analysis of repeated measures on the same individuals, we adopted a Mantel-Haenszel approach. The Mantel-Haenszel statistic is identical to McNemar's test for the significance of changes for two repeated measurements, and to Cochran's Q for three or more repeated measurements (Stokes et al., 2000). Whenever we employed McNemar's test, we calculated the χ^2 statistic following the method of Sachs (1992). The Mantel-Haenszel statistic can address the hypothesis of no association between a repeated-measurement factor, such as time, and a response variable, adjusting for the effect of subject (Stokes et al., 2000). For the analysis of our results with washed males, we used logistic regression (Proc Genmod in SAS, Stokes et al., 2000) to test for effects of washing and male reproductive status.

RESULTS

Behavior of females during tests

Females responded to males only if they came into direct physical contact with males in such a manner that their antennae, or mouth parts, touched the male's body. When males walked by females without making contact with female antennae or mouth parts, they were consistently ignored by the females, even when other parts of their body touched or when they walked in front of the female at a distance of less than 5 mm. When inexperienced males approached discriminating females from behind, they frequently succeeded in mounting, and mating with, these females without any apparent resistance on the part of the female.

Experiment 1: pairing history of discriminating females and reproductive status of cue-bearing males

The response of care-providing resident females to novel males was plainly contingent on the reproductive condition of these males (Table 2). Parental males were attacked less frequently than were inexperienced males, both by single mothers (McNemar's test for the significance of changes, $Q_M = 7.36$, $p < .01$) and by paired females (McNemar's test for the significance of changes, $Q_M = 11.27$, $p < .001$). Paired females, as well as single mothers, attacked all inexperienced males (males that had never been on a carcass before), with only one exception. Unfamiliar parental males (males that had been on a carcass with a female and larvae for as long as the female had) were not attacked by any of the paired females, and single mothers attacked parental males in only four out of 14 tests. Paired females were equally tolerant to novel parental males as they were to their original mates (zero out of 15 males were attacked in either situation).

Experiment 2: behavioral cues

When we presented live and dead cue-bearing males to discriminating females, the females attacked inexperienced males significantly more frequently than they attacked parental males (Table 3; Fisher's exact tests, $p < .0001$ for live males; $p = .0007$ for dead males), and this was true regardless of whether the males were alive or dead (Table 3; McNemar's test, for parental males: $Q_M = 1.0$, $p > .25$; for inexperienced males: $Q_M = 0.33$, $p > .5$).

Experiment 3: cuticular lipids

When discriminating females were presented with parental or inexperienced cue-bearing males that had been either left

Table 2

Number of original mates, parental males, and inexperienced males attacked or tolerated by paired resident females and single mothers

Female status	Male reproductive status	Attack	Tolerate
Paired	Parental[a]	0	15
	Inexperienced[b]	14	1
	Original mate	0	15
Single	Parental[a]	4	10
	Inexperienced[b]	14	0

[a] Parental males had provided 7–10 h of parental care on a carcass with a female.
[b] Inexperienced males had never been in contact with a carcass.

untreated or washed with water or *n*-pentane, the females attacked inexperienced males significantly more frequently than they attacked parental males (logistic regression, $\chi^2 = 49.22$, $p < .0001$). The overall analysis shows that washing the males also affected the females' response (logistic regression, $\chi^2 = 18.21$, $p < .0001$), but specific contrasts reveal that only washing with pentane resulted in an increased frequency of attacks against parental males (pentane versus untreated: $\chi^2 = 15.17$, $p < .0001$; water versus untreated: $\chi^2 = 0.37$, $p = .5440$; Table 4). The behavior of discriminating females toward nonbreeding males that were washed with pentane or water, or not washed, was almost identical; all but one of these males were attacked by the female.

Experiment 4: the origin of the breeder's badge—external sources

Part a: the role of female presence
When discriminating females encountered paired or single males that had each spent 6 days on a carcass, their response was unaffected by the cue-bearing male's pairing status (Table 5, Fisher's exact test, $p > .99$). All of the males tested proved to be in the parental state; that is, they did not eat any first-instar larvae they encountered. Nonetheless, not all of these males were accepted by the discriminating females.

Part b: the role of cues associated with the carcass
When females were presented with inexperienced males that were manipulated in various ways or with parental males as a control, inexperienced males were attacked without exception, although they had been rubbed with a treated carcass, had been fed vertebrate carrion for 3 days (either fresh carrion or carrion prepared by a pair of beetles), or had been treated with the oral and anal secretions of brood-caring males (Table 6). The females' response to these males was

Table 3

Behavior of resident females (attack/tolerate) toward live or dead cue-bearing males

	Male reproductive status			
	Parental[a]		Inexperienced[b]	
Male condition	Attacked	Tolerated	Attacked	Tolerated
Live	0	15	13	1
Dead	3	12	12	2

[a] Parental males had provided 7–10 h of parental care on a carcass with a female.
[b] Inexperienced males had never been in contact with a carcass.

Table 4

Behavior of discriminating females (attack/tolerate) toward washed and untreated dead cue-bearing males

	Male reproductive status			
	Parental[a]		Inexperienced[b]	
Manipulation	Attacked	Tolerated	Attacked	Tolerated
Untreated	0	12	12	0
Washed with water	1	11	12	0
Washed with pentane	10	2	11	1

[a] Parental males had provided 7–10 h of parental care on a carcass with a female.
[b] Inexperienced males had never been in contact with a carcass.

significantly different from their behavior towards parental males (Fisher's exact test of general association for the 5×2 table, $p < .0001$; and Fisher's exact test of all inexperienced males versus parental males, $p < .0001$).

Experiment 5: development and loss of the breeder's badge with time spent on or away from a carcass

Part a: development of the breeder's badge
When we replaced the original mates of discriminating females with preparental cue-bearing males (i.e., males that had spent some time with a female and a carcass, but not long enough to encounter their own larvae), the females' response was contingent on the time the cue bearer had spent with a carcass and a female (Figure 1). The longer the cue-bearing males had been on a carcass, the greater the proportion of males that was accepted by discriminating females. However, resident females frequently accepted cue-bearing males that were not ready to provide care. The females we tested tolerated most males that had been on a carcass for 45 h, even though most of these males were not in a parental state (Figure 1). In the course of this experiment, we directly observed three different females sitting calmly beside a cue-bearing male that was eating their larvae.

Part b: loss of the breeder's badge
When we tested the response of discriminating females to males that had been isolated for 12–84 h after providing care for 7–10 h on a carcass, the duration of male isolation from the carcass significantly affected the discriminating female's response (Mantel-Haenszel test of general association, df = 5, $Q_{GMH} = 50.74$, $p < .0001$). However, males were attacked more often only after they had been isolated from the carcass for 60 h or more; there was no difference in the frequency of female attacks when males had been isolated from the carcass for 12–48 h (Mantel-Haenszel test of general association, df = 3, $Q_{GMH} = 2.53$, $p = .468$; Figure 2). However, this duration must be regarded as a maximum estimate for the persistence of the badge because our repeated-measures design, which

Table 5

Behavior of discriminating females (attack/tolerate) toward males that had been on a carcass for 6 days, either with or without a female

	Female response	
Male reproductive history	Attack	Tolerate
On carcass with female	4	8
On carcass, no female	4	8

Table 6

Behavior of discriminating females (attack/tolerate) toward unfamiliar inexperienced males (treated) and unfamiliar parental males (control)

Type of unfamiliar male	Female response	
	Attack	Tolerate
Inexperienced males		
Rubbed with prepared carcass	5	0
Fed carrion prepared by beetles	9	0
Fed untreated carrion	11	0
Smeared with oral/anal secretion of parental males	7	0
Parental males	0	11

Figure 1
Proportion of cue-bearing males tolerated by discriminating females, and proportion of males in the parental state (i.e., accepting first-instar larvae), in relation to the length of time the males had spent on a carcass with a female. The time cue-bearing males had spent on a carcass significantly affected their acceptance by discriminating females (Mantel-Haenszel test of general association, df = 3, Q_{GMH} = 9.783, p = .0205)and the males' response to larvae (Q_{GMH} = 27.548, $p < .0001$). Numbers in bars are sample sizes, deviations from 16 are caused by severe injuries incurred by two males in their first trial and by the failure of one female to emerge from under the carcass during the trial period.

gave the male brief access to a carcass at 12-h intervals during the trials, may have caused the badge to persist longer than it might have if the males had had no access to carrion at all.

DISCUSSION

Our results demonstrate that breeding female burying beetles discriminate against intruding males on the basis of the intruding individual's reproductive condition. Inexperienced novel males (i.e., males that had had no prior contact with a carcass) were attacked, whereas parental novel males (i.e., males that had spent sufficient time in a breeding attempt on a carcass) were tolerated. Our experiments show that the cue used by resident females in this discrimination is a hydrophobic substance on the cuticle of parental males, which we termed the breeder's badge. For the acquisition of this badge, the presence of a female is not required. Neither the ingestion of nor contact with treated carrion, nor treatment with the oral and anal secretions of caring individuals, is sufficient for acquisition of the badge. Our results suggest that the badge develops within a day or so during a breeding attempt on a suitable carcass, and that it disappears within about 3 days of the male's departure from the carcass. It is clearly associated with the male's reproductive status. It is possible that the badge, like parental behavior, is affected by an individual's juvenile hormone titer, which undergoes dramatic changes during carcass burial and preparation (Trumbo, 1997; Trumbo et al., 1995). Although access to a carcass appears necessary for its development, contact with a female, or with larvae, is not required.

During the first days on the carcass, the breeding male and female's response to larvae changes from infanticidal to parental (Müller and Eggert, 1990; Figure 1), and the breeder's badge begins to appear. However, the male's badge appears to be present well before he exhibits parental behavior, and thus, the presence of the badge is not perfectly coupled with a male's readiness to exhibit parental care. Females frequently tolerated males that had been on the carcass for a while but were not yet in the parental state (Figure 1). Females even tolerated males that killed larvae in close proximity to the female. The lack of a complete agreement between a male's readiness to provide care and the response of discriminating females was also apparent in another result: one third of males that had been on a carcass for 6 days (either with or without a female) were attacked by discriminating females although all males were in the parental state. Females of undisturbed male–female pairs also some-
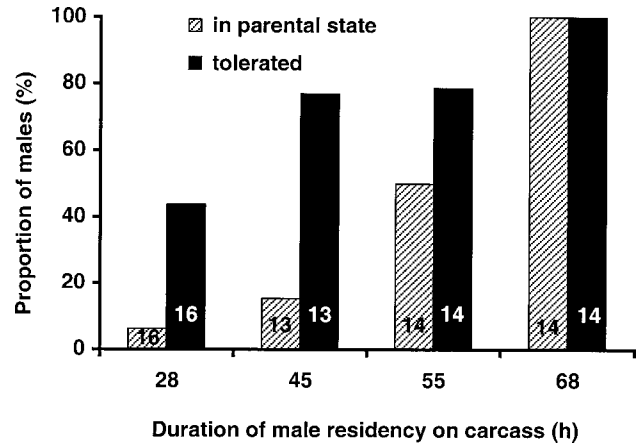
times attack their male nestmates after several days of care, and it is possible that at least some males are beginning to lose their breeder's badge at this time. However, whether the discriminating females acted aggressively for a different reason, and whether the males' own mates had been equally aggressive, is currently unknown.

There is no evidence that the members of a breeding pair of *N. vespilloides* recognize each other individually, as no aggression is triggered when individuals of the same sex are exchanged between breeding pairs once larvae are present on the carcass (Beck, 1995). Like discriminating females, discriminating males treated in the exact same manner and confronted with parental and inexperienced replacement females also tolerated parental females and attacked inexperienced ones (Elsner T, unpublished observations). In *N. tomentosus*, as well as in *N. vespilloides*, swapping females between dyads of female cobreeders does not trigger aggression between females, whereas inexperienced females are usually vigorously attacked by females from such dyads (Beck, 1995; Eggert and Müller, 2000; Trumbo and Wilson, 1993). It thus appears that nestmate recognition in burying beetles (or, alternatively, intruder recognition) is generally achieved via recognition of breeding status, although the data presented here provide evidence for recognition of male *N. vespilloides* by female *N. vespilloides* only. It appears unlikely that recognition of breeding females by breeding male and female conspecifics is achieved by a mechanism that is principally different from the one used in recognition of males by breeding females.

We suggest that in nature, recognition of breeding status virtually ensures that intruders are attacked but cobreeders are not. A breeding pair is very unlikely to encounter an intruder bearing the breeder's badge under field conditions. All the evidence available to date suggests that carcasses—and thus, breeding opportunities for burying beetles—are rare, and that the search for a suitable carcass is normally prolonged (Eggert and Müller, 1997; Scott, 1998). If this is true, the vast majority of intruders (if not all of them) are
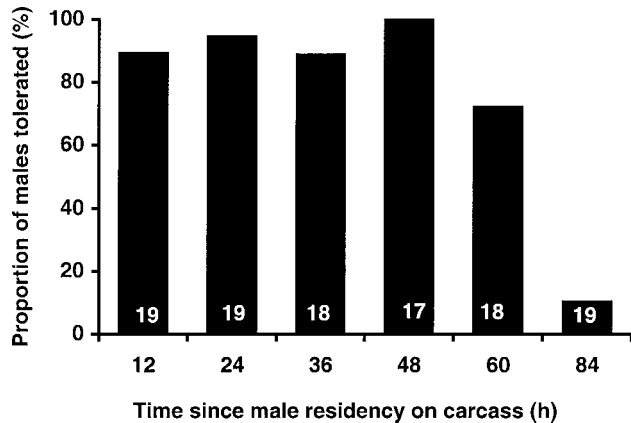
Figure 2
Proportion of cue-bearing males tolerated by discriminating females in relation to the time elapsed since removal of males from their own carcass and brood. The time elapsed since the males' removal from the carcass significantly affected their acceptance by discriminating females (Mantel-Haenszel test of general association, df = 5, $Q_{GMH} = 50.74$, $p < .0001$). Numbers in bars are sample sizes; deviations from 19 are owing to four instances in which females failed to emerge from under the carcass during the trial period.

individuals that have not recently been involved in a breeding attempt. Because the badge is temporary and lost within days of a breeding attempt, the risk of mistakenly tolerating intruders may be trivial. Our results also suggest that feeding on a carcass for 72 h is not sufficient for males to develop the badge, possibly because the hormonal changes accompanying an actual reproductive attempt are not induced. Males that have recently been feeding on a large carcass that is unfit for reproduction are thus unlikely to possess the badge and to be mistaken as a breeder. Although individual recognition might constitute a more perfect mechanism of nestmate recognition, it is likely that individual recognition is more difficult to achieve than recognition of breeding status, and that the selective advantage of individual recognition may be minimal in the vast majority of situations.

For any intruding individual, it would be highly advantageous if he or she were able to pretend to be a breeder. That the breeder's badge appears to be a reliable signal of breeding status strongly suggests that its production and/or maintenance is costly to its bearer. The cost may be tied to its close association with the reproductive situation, which involves profound changes in physiology and behavior.

It appears that the response of conspecifics to the badge is not uniform, but varies depending on the social context. Only breeders will attack nonbreeders in the way described above; nonbreeders of either sex are usually very tolerant of each other, as evidenced by the fact that several individuals can be kept together in small containers for weeks or months (Müller JK, Eggert A-K, unpublished observations). When opposite-sex conspecifics first arrive on a carcass, they treat each other as mates, attacking only individuals of the same sex. The beetles also appear to process information about the number and sex of their current breeding partner(s) in their decision to fight or tolerate. When male and female members of breeding male-female pairs were presented with breeding unfamiliar individuals of the "wrong" sex, they attacked them in about half of the cases (Beck, 1995). In the present study, singly breeding females attacked some breeding males, although they accepted most of them (Table 2).

We do not know the exact chemical nature of the badge. It appears to be a contact pheromone, as females did not respond to cue-bearers unless they contacted the males with their mouth parts or antennae. Washing with pentane removed the substance that rendered males acceptable to females, and because pentane removes all nonpolar cuticular lipids (Vander Meer and Morel, 1998), the breeder's badge in burying beetles could be any nonpolar lipid or a blend of nonpolar lipids. Hydrocarbons are important components of the insect cuticle and have been found to be important in recognition and intraspecific communication in many species of social insects (Singer, 1998; Wagner et al., 2000); however, we do not know if the breeder's badge is a hydrocarbon. Cuticular hydrocarbons have been found to vary with reproductive status in wasps and ants (Liebig et al., 2000; Monnin et al., 1998; Peeters et al., 1999; Sledge et al., 2001), and a similar correlation between cuticular lipids and reproduction is likely to exist in burying beetles as well. The breeder's badge is a substance that appears to be present on the male beetle's cuticle during (and immediately after) a breeding attempt only. The temporary nature of the badge suggests that the substance evaporates or breaks down chemically at a rapid rate, such that a breeding male must produce it continuously if he is to remain recognizable as such. During a breeding attempt in burying beetles, the breeders' juvenile hormone titer undergoes dramatic fluctuations (Scott et al., 2001; Trumbo, 1997; Trumbo et al., 1995). It is possible that these temporary and dramatic changes in juvenile hormone concentration are involved in the regulation of the production of the breeder's badge as well, given that juvenile hormone is the predominant endocrine factor regulating pheromone production in the Coleoptera (Tillman et al., 1999).

REFERENCES

Beck D, 1995. Erkennen Totengräber *Nicrophorus vespilloides* Herbst (Coleoptera: Silphidae) einander am Aas? (Diplomarbeit). Freiburg: Albert-Ludwigs-Universität.

Bredohl R, 1984. Zur Bioakustik mitteleuropäischer Totengräber (Coleoptera: Silphidae: Necrophorus). Entomol Gener (Stuttgart) 10:11–25.

Breed MD, 1998. Chemical cues in kin recognition: criteria for identification, experimental approaches, and the honey bee as an example. In: Pheromone communication in social insects: ants, wasps, bees and termites (Vander Meer RK, Breed MD, Espelie KE, Winston ML, eds). Boulder, Colorado: Westview Press; 57–78.

Byers JA, Bekoff M, 1986. What does "kin recognition" mean? Ethology 72:342–345.

Clément J-L, Bagnères A-G, 1998. Nestmate recognition in termites. In: Pheromone communication in social insects: ants, wasps, bees and termites (Vander Meer RK, Breed MD, Espelie KE, Winston ML, eds). Boulder, Colorado: Westview Press; 126–155.

Eggert A-K, Müller JK, 1997. Biparental care and social evolution in burying beetles: lessons from the larder. In: The evolution of social behavior in insects and arachnids (Choe JC, Crespi BJ, eds). Cambridge: Cambridge University Press; 216–236.

Eggert A-K, Müller JK, 2000. Timing of oviposition and reproductive skew in cobreeding female burying beetles (*Nicrophorus vespilloides*). Behav Ecol 11:357–366.

Eggert A-K, Reinking M, Müller JK, 1998. Parental care improves offspring survival and growth in burying beetles. Anim Behav 55:97–107.

Eggert A-K, Sakaluk SK, 2000. Benefits of communal breeding in burying beetles: a field experiment. Ecol Entomol 25:262–266.

Fletcher DJC, 1987. The behavioral analysis of kin recognition: perspectives on methodology and interpretation. In: Kin recogni-

tion in animals (Fletcher DJC, Michener CD, eds). New York: Wiley; 19–54.

Fletcher DJC, Michener CD, eds, 1987. Kin recognition in animals. New York: Wiley.

Gheusi G, Goodall G, Dantzer R, 1997. Individually distinctive odors represent individual conspecifics in rats. Anim Behav 53:935–944.

Hoferer S, 1996. Variabilität im Fortpflanzungssystem des Totengräbers *Nicrophorus vespilloides* Herbst (Coleoptera: Silphidae) (Diplomarbeit). Freiburg: Albert-Ludwigs-Universität.

Lacy RC, Sherman PW, 1983. Kin recognition by phenotype matching. Am Nat 121:489–512.

Lengagne T, Aubin T, Jouventin P, Lauga J, 2000. Perceptual salience of individually distinctive features in the calls of adult king penguins. J Acoust Soc Amer 107:508–516.

Liebig J, Peeters C, Oldham NJ, Markstädter C, Hölldobler B, 2000. Are variations in cuticular hydrocarbons a reliable signal of fertility in the ant *Harpegnathos saltator*? Proc Nat Acad Sci USA 97:4124–4131.

Monnin T, Malosse C, Peeters C, 1998. Solid-phase microextraction and cuticular hydrocarbon differences related to reproductive activity in queenless ant *Dinoponera quadriceps*. J Chem Ecol 24:473–490.

Müller JK, Eggert A-K, 1990. Time-dependent shifts between infanticidal and parental behavior in female burying beetles: a mechanism of indirect mother-offspring recognition. Behav Ecol Sociobiol 27:11–16.

Peeters C, Monnin T, Malosse C, 1999. Cuticular hydrocarbons correlated with reproductive status in a queenless ant. Proc R Soc London B 266:1323–1327.

Pukowski E, 1933. Ökologische Untersuchungen an *Necrophorus* F. Z Morph Ökol Tiere 27:518–586.

Riters LV, Balthazart J, 1998. Behavioral evidence for individual recognition in Japanese quail. Behaviour 135:551–578.

Robertson IC, 1993. Nest intrusions, infanticide, and parental care in the burying beetle, *Nicrophorus orbicollis* (Coleoptera: Silphidae). J Zool Lond 231:583–593.

SAS Institute Inc., 1998. SAS/STAT user's guide, 8.1 ed. Cary, North Carolina: SAS Institute.

Sachs L, 1992. Angewandte Statistik: Anwendung statistischer Methoden, 7th ed. Berlin: Springer.

Schumacher R, 1973. Beitrag zur Kenntnis der Stridulationsapparate einheimischer *Necrophorus*-Arten (*Necrophorus humator* Ol., *Necrophorus investigator* Zetterst., *Necrophorus vespilloides* Herbst) (Insecta, Coleoptera). Z Morph Tiere 75:65–75.

Scott MP, 1990. Brood guarding and the evolution of male parental care in burying beetles. Behav Ecol Sociobiol 26:31–39.

Scott MP, 1998. The ecology and behavior of burying beetles. Annu Rev Entomol 43:595–618.

Scott MP, Gladstein DS, 1993. Calculating males? An empirical and theoretical examination of the duration of paternal care in burying beetles. Evol Ecol 7:362–378.

Scott MP, Trumbo ST, Neese PA, Bailey WD, Roe RM, 2001. Changes in biosynthesis and degradation of juvenile hormone during breeding by burying beetles: a reproductive or social role? J Insect Physiol 47:295–302.

Sherman PW, Reeve HK, Pfennig DW, 1996. Recognition systems. In: Behavioural ecology: an evolutionary approach (Krebs JR, Davies NB, eds). Oxford: Blackwell; 69–96.

Singer TL, 1998. Roles of hydrocarbons in the recognition systems of insects. Amer Zool 38:394–405.

Singer TL, Espelie KE, Gamboa GJ, 1998. Nest and nestmate discrimination in independent-founding paper wasps. In: Pheromone communication in social insects: ants, wasps, bees and termites (Vander Meer RK, Breed MD, Espelie KE, Winston ML, eds). Boulder, Colorado: Westview Press; 104–125.

Sledge MF, Boscaro F, Turillazzi S, 2001. Cuticular hydrocarbons and reproductive status in the social wasp *Polistes dominulus*. Behav Ecol Sociobiol 49:401–409.

Stokes ME, Davis CS, Koch GG, 2000. Categorical data analysis using the SAS system, 2nd ed. Cary, North Carolina: SAS Institute.

Tillman JA, Seybold SJ, Jurenka RA, Blomquist GA, 1999. Insect pheromones: an overview of biosynthesis and endocrine regulation. Insect Biochem Mol Biol 29:481–541.

Trumbo ST, 1990a. Interference competition among burying beetles (Silphidae, *Nicrophorus*). Ecol Entomol 15:347–355.

Trumbo ST, 1990b. Reproductive benefits of infanticide in a biparental burying beetle *Nicrophorus orbicollis*. Behav Ecol Sociobiol 27:269–273.

Trumbo ST, 1997. Juvenile hormone-mediated reproduction in burying beetles: from behavior to physiology. Arch Insect Biochem Physiol 35:479–490.

Trumbo ST, Borst DW, Robinson GE, 1995. Rapid elevation of juvenile hormone titer during behavioral assessment of the breeding resource by the burying beetle, *Nicrophorus orbicollis*. J Insect Physiol 41:535–543.

Trumbo ST, Wilson DS, 1993. Brood discrimination, nestmate discrimination and determinants of social behavior in facultatively quasisocial beetles (*Nicrophorus* spp.). Behav Ecol 4:332–339.

Vander Meer RK, Morel L, 1998. Nestmate recognition in ants. In: Pheromone communication in social insects: ants, wasps, bees and termites (Vander Meer RK, Breed MD, Espelie KE, Winston ML, eds). Boulder, Colorado: Westview Press; 79–103.

Wagner D, Tissot M, Cuevas W, Gordon DM, 2000. Harvester ants utilize cuticular hydrocarbons in nestmate recognition. J Chem Ecol 26:2245–2257.

Wanker R, Apcin J, Jennerjahn B, Waibel B, 1998. Discrimination of different social companions in spectacled parrotlets (*Forpus conspicillatus*): evidence for individual vocal recognition. Behav Ecol Sociobiol 43:197–202.

Wiley RH, Hatchwell BJ, Davies NB, 1991. Recognition of individual males' songs by female dunnocks: a mechanism increasing the number of copulatory partners and reproductive success. Ethology 88:145–153.

Wilson DS, Knollenberg WG, Fudge J, 1984. Species packing and temperature dependent competition among burying beetles (Silphidae, *Nicrophorus*). Ecol Entomol 9:205–216.