The Coolidge effect, individual recognition and selection for distinctive cuticular signatures in a burying beetle

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The ability to recognize individuals is an important aspect of social interactions, but it can also be useful to avoid repeated matings with the same individual. The Coolidge effect is the progressive decline in a male’s propensity to mate with the same female combined with a heightened sexual interest in new females. Although males that recognize previous partners and show a preference for novel females should have a selective advantage as they can distribute sperm evenly among the females they encounter, there are few invertebrate examples of the Coolidge effect. Here we present evidence for this effect in the burying beetle Nicrophorus vespilloides and examine the mechanism underlying the discrimination between familiar and novel mates. Burying beetles feed and reproduce on vertebrate carcasses, where they regularly encounter conspecifics. Males showed greater sexual interest in novel females (virgin or mated) than in females they had inseminated before. The application of identical cuticular extracts allowed us to experimentally create females with similar odours, and male responses to such females demonstrated that they use female cuticular patterns for discrimination. The chemical analysis of the cuticular profile revealed greater inter-individual variation in female than in male cuticular patterns, which might be due to greater selection on females to signal their individual identity.

Keywords: burying beetles; Nicrophorus; Coolidge effect; individual recognition; cuticular lipids; identity signals

1. INTRODUCTION

 Although the cost of mating to males has long been regarded as negligible, recent evidence shows that mating and sperm production may generate non-trivial costs (Dewsbury 1982; Van Voorhies 1992; Olsson et al. 1997; Preston et al. 2001), promoting the evolution of prudent ejaculate allocation or even male reluctance to mate (Wedell et al. 2002). If the value of a female to a male decreases with his increasing mating investment to that individual female, males are expected to avoid re-mating with the female in favour of other reproductive opportunities (Wedell et al. 2002; Pizzari et al. 2003). The ‘Coolidge effect’, defined as a decline in the propensity of a male to copulate repeatedly with the same female combined with a heightened sexual interest in novel females (Wilson et al. 1963; Dewsbury 1981), can be a mechanism to distribute sperm more evenly. The Coolidge effect was first observed in rats (Beach & Jordan 1956) and has since been demonstrated in a number of other mammals (see Dewsbury 1981) and birds (Pizzari et al. 2003). Similarly, animals of many different groups (bees: Barrows 1975, Wcislo 1992; amphibians: Donovan & Verrell 1991; reptiles: Tokarz 1992; flies: Ödeen & Moray 2008; beetles: Arnaud & Haubruege 1999; fishes: Kelley et al. 1999) have been shown to avoid mating or re-mating with familiar individuals.

The Coolidge effect per se has received no attention in invertebrates, with the exception of one recent study on snails (Koene & Ter Maat 2007). Given the number of studies that have examined mate choice in insects, it is surprising that there have been no investigations of the Coolidge effect per se in this group. Researchers may frequently have assumed that the Coolidge effect requires more complex neural processing than invertebrates are capable of (Koene & Ter Maat 2007). However, some social insects have proven capable of individual recognition (Tibbetts 2002, 2004; D’Ettorre & Heinze 2005), and avoiding matings with one’s previous mate may not necessarily require individual recognition. Female crickets Gryllus sigillatus use self-referencing (marking males with their individual specific chemical signature) to avoid re-mating with previous mates (Ivy et al. 2005). Numerous insects have evolved other mechanisms of discrimination against previously mated individuals that have typically been interpreted as adaptations to sperm competition. So-called ‘antiaphrodisiacs’, male-produced chemicals that are transferred to females during mating and discourage further matings by these females (e.g. Happ 1969; Kukuk 1985; Peschke 1987; Carlson & Schlein...
may also help the original male to avoid re-mating with the same female. This study investigated the occurrence and underlying mechanism of the Coolidge effect in the burying beetle *Nicrophorus vespilloides*. Males of this species show clear evidence of sperm depletion after several days spent with multiple females (Eggert 1990). To a male of this species, re-mating with a female may thus entail a twofold cost: time that could have been spent seeking out other females and sperm that could have been more efficiently used to inseminate another female. Burying beetles reproduce on vertebrate carcasses, frequently in groups with several females (Pukowski 1933; Müller & Eggert 1990, 2007). When females leave the carcass early, or have only limited time on a carcass, males may benefit from distributing sperm evenly among the available females. Even without reproductive resources, males mate more frequently when held with multiple females than with a single one (Eggert 1990). This effect could arise if males respond more readily to females other than their last mate.

In burying beetles, cuticular contact pheromones contain information about breeding and nutritional status, sex, and species (Whitlow 2003; Steiger et al. 2007) and could play an important role in recognition processes like nestmate recognition (Müller et al. 2003). To our knowledge, there is no information to date about the role of cuticular substances in interactions between the sexes of burying beetles. We tried to address whether males are capable of discriminating between their last mate and other females on the basis of cuticular substances. Following the experimental protocol of Ivy et al. (2005), we tested whether males transfer their own chemical substances to females or learn cues from the chemical profile of their females to recognize previous mates. If males transfer substances, they should treat the mate of a close male relative like they would treat their own previous mate, provided that there is genetic variation in chemical composition and the cuticular patterns of close relatives are similar to each other. If males rely on female-specific cues, they should instead exhibit reduced sexual interest when presented with a close female relative of their own previous mate. To confirm the importance of cuticular extracts in male discrimination of females, we observed male responses to females whose cuticular patterns we had manipulated to be similar by applying identical cuticular extracts. We examined the similarity of the cuticular pattern among relatives to seek evidence that genetic similarity between individuals is correlated with similarity of cuticular patterns.

2. MATERIAL AND METHODS

(a) 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 June and July 2006. The field site was a deciduous forest near Freiburg in southwestern Germany (48°00′N, 07°51′E). Beetles were maintained in temperature-controlled chambers at 20°C under a 16 L : 8 D photoperiod. Groups of up to six same-sex siblings were kept in small transparent plastic containers (10 × 10 × 6 cm) with moist peat and were fed freshly killed mealworms twice a week. All experimental specimens were between 20 and 60 days of age.

(b) General procedures of behavioural experiments

Experiments that involved observation of copulatory behaviour were conducted during the last 2 hours before the end of the light phase. Males emit pheromones and mate with attracted females during this period both in our own (J. K. Müller 2006, unpublished data) and a northern German population near Bielefeld (Müller & Eggert 1987; Eggert & Müller 1989). Matings were observed in small plastic containers (8 × 5 × 6 cm) with a moistened plaster bottom. To minimize disturbance immediately prior to an observation, males were transferred to observation chambers 3 hours before the onset of an experiment. Over the course of an experiment, males remained in the same chamber while females were introduced and removed. In each trial, a male was presented with a female and either mated with her or did not mate (see below), then the female was removed and the male remained alone for 5 min before the next female (either the same one or a different one) was introduced into the chamber. For each encounter, we recorded the time to mating, defined as the time from the beetles’ first physical contact to the actual coupling of genitalia. Females were removed from the chamber as soon as the first mating ended. If the pair did not mate within 5 min (300 s), we removed the female, scoring a time to mating of 300 s.

Our study was designed to determine the readiness of males to mate, and we interpret a long time to mating as low male sexual interest, since matings are initiated by males, who approach and mount females prior to mating. In order to avoid the potentially confounding effects of the females’ response to male attempts, we excluded trials in which females clearly tried to avoid copulations by struggling and attempting to leave during male mating attempts (17 out of 198 trials).

(c) The effect of female novelty on male mating behaviour

Our first experiment was designed to test for a Coolidge effect in *N. vespilloides* males. Each experimental male (n = 20) was presented with the same female four times. The females were chosen randomly; they were virgin females that had not encountered any males prior to the experiment. In his fifth encounter, each male was presented with a novel unmated female to test for increased sexual interest. To assess possible effects of overall physical exhaustion of males from the effort of mating on time to mating in successive encounters, we established a control group in which males (n = 10) were presented with novel unmated females in all five successive encounters. In an additional experiment, males (n = 19) were presented with the same female in all five successive encounters.

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(ii) Self-referent cues versus female-specific cues
Based on our previous knowledge about the role of cuticular hydrocarbons in social interactions between individuals, it is reasonable to assume that the recognition of mating partners is also mediated by these chemical cues. Therefore, we carried out a second experiment to learn whether males transfer chemical substances from their own cuticle during mating and use self-referent cues to recognize females (as appears to be the case in crickets, where females initiate matings and transfer cuticular chemicals; Ivy et al. 2005) or whether they learn their mates’ chemical signature (their individual hydrocarbon pattern). To obtain individuals that were more similar genetically than full sibs (‘inbred’ individuals, inbreeding coefficient $F_{st}=0.25$), we paired $F_1$ offspring of field-collected beetles with their full sibs and allowed them to rear offspring. Individuals in the resulting $F_2$ were thus the product of brother–sister pairings, and siblings in this population were more closely related than full sibs produced by unrelated parents (coefficient of relatedness, $r=0.6$ for our individuals and $r=0.5$ for ordinary full sibs). As in the first experiment, each experimental male was presented with the same female in four successive encounters. In the fifth encounter, one of the following types of females was introduced to the male: (i) familiar females ($n=20$) that had previously mated with the same male, (ii) novel females ($n=20$) that had previously mated with a different unrelated male, (iii) novel females ($n=16$) that had previously mated with the male’s inbred brother, and (iv) novel females ($n=21$) that were inbred sisters of the male’s original mate. To avoid any confounding effects of inbreeding avoidance, we never presented females to related males. If males use self-referent cues, their response to females inseminated by their inbred brother should resemble their response to their own previous mate. If males learn their mate’s chemical features, their response to their previous mate’s inbred sister should resemble their response to the previous mate herself.

(e) Masking of cuticular substances with concentrated extracts
The experiment was designed to test the hypothesis that cuticular substances are involved in the recognition process. To this end, we attempted to experimentally manipulate female surface chemicals to create pairs of females with similar odour. We first extracted females individually in pentane for 15 min. These females came from our inbred group that resulted from full-sib matings (see previous experiment). The extracts of four inbred sisters were combined and their combined extract was completely reduced by evaporation under a stream of gaseous nitrogen and dissolved in $40 \mu l$ pentane. We applied $20 \mu l$ of the extract to each of two live virgin females by spreading small droplets of it evenly over the elytra, pronotum and the exposed part of the dorsal side of the abdomen. In effect, we applied a concentration of substances that should have been equivalent to amounts found on two females rather than one to increase the probability that the experimental odour would conceal the females’ actual individual chemical pattern. Live females were used because males give up mating attempts when they experience difficulties with intromission of their aedeagus into the genital tract of dead females. To control for any effects of the solvent, a separate group of females was treated with $20 \mu l$ of pentane only. We started a trial 20 min after applying the extracts to allow the experimental females some time to recover from the pentane application. In the experimental group, each male ($n=12$) was presented with the same extract-treated female four times. In the fifth encounter, a new unfamiliar female was introduced that had been treated with the same extract. In the control group, males ($n=10$) were presented with the same pentane-treated female in the first four encounters and with a novel unfamiliar pentane-treated female in the fifth.

(f) Cuticular patterns of inbred families: chemical analysis
Five brothers from each of five different inbred families (families A, B, C, D and G, each of which resulted from a different brother–sister pairing) and five sisters from each of five different inbred families (families B, D, E, F and G) were killed by freezing at $-27^\circ C$ for 15 min. They were then thawed for 30 min at room temperature, placed individually in flasks with 3 ml $n$-pentane ($>99\%$, Fluka, Switzerland), and shaken for 15 min on an orbital shaker for extraction. The extract was then transferred to a clean vial and reduced by evaporation using a stream of gaseous nitrogen until approximately 0.1 ml remained. Samples were quantified on an HP 6890 gas chromatograph with a split/splitless injector (300°C, automatic sampling, injection of 1 µl). We used a fused silica column (DB-1, 30 m × 0.25 mm ID, 0.25 µm, J&W Scientific, Folsom, Canada) with a helium flow of 1 ml min$^{-1}$. The oven temperature was programmed as follows: 2 min at $35^\circ C$, to $100^\circ C$ at a rate of 20°C min$^{-1}$, to $300^\circ C$ at 6°C min$^{-1}$, 25 min at $300^\circ C$. The flame ionization detector was run with 40 ml H$_2$ min$^{-1}$ and 450 ml air min$^{-1}$. In a previous study (Steiger et al. 2007), 88 peaks out of 91 regularly occurring peaks were identified. For the current study, the 40 peaks with the largest area were chosen for integration (see electronic supplementary material for chemical identity of the 40 peaks). One of the males (MC3) had to be excluded from the analysis because the extract was contaminated.

(g) Statistical analyses
Statistical analyses were performed using SPSS v. 15. We used a repeated measures ANOVA to compare the same male’s behaviour in successive matings and a one-way ANOVA to compare the behaviour of males from different treatment groups in their fifth encounter with a female. To meet the criterion of homogeneity of variances, we log transformed all data prior to analysis.

For the analysis of chemical data, the total peak area of the 40 peaks of each individual was standardized to 100% and multivariate analyses were performed. Because peak areas represent compositional data, they were transformed to logcontrasts (Aitchinson 1986). To assess the similarity of the pattern of individuals within and between families, a cluster analysis was performed using the PAM procedure (partitioning around medoids; Kaufman & Rousseeuw 1990) of the R package with the chord distance as the distance index. The average silhouette width, which provides an evaluation of clustering validity, was used to select an appropriate number of clusters (Kaufman & Rousseeuw 1990). In addition, a discriminant analysis (DA) was performed to determine whether females of different families could be discriminated on the basis of their cuticular profile. To reduce the number of variables prior to the DA, we first performed a principal component analysis (PCA).
3. RESULTS

(a) The effect of female novelty on male mating behaviour

In the first experiment, time to mating varied significantly with the number of encounters (repeated measures ANOVA of log-transformed times, \(n=18\), d.f. = 4, \(F=6.31, p<0.001\); figure 1). Time to mating increased continuously over the course of the first four encounters (with the same female), but only the pairwise comparisons between the first and third or fourth exposure were significant. When males were exposed to a novel virgin female in the fifth encounter, they mated significantly faster than in the third or fourth exposure to the first female and as fast as in their first encounter with a female (figure 1).

Our results provided no indication that physical exhaustion caused males to increase their time to mating after multiple copulations. When males were exposed to a new virgin female each time, time to mating remained short and did not differ between the encounters (\(n=9\); back-transformed mean and mean ± s.e. for time to mating: 17.06, 20.96 and 13.89 s; repeated measures ANOVA, d.f. = 4, \(F=0.88, p=0.49\)). Most of these males mated with all five females (mean ± s.e.: 4.4 ± 0.2), whereas males encountering the same female five times copulated an average of three times (\(n=19\); mean ± s.e.: 2.9 ± 0.1), which is significantly less (\(t\)-test, \(t=6.23, p<0.001\)).

(b) Recognition of familiar females

(i) Female mating status

Female mating status had no influence on male mating behaviour. Time to mating did not differ significantly between trials in which the novel female was a virgin or a mated female (back-transformed mean and mean ± s.e. for time to mating: virgin female (\(n=16\)): 32.13, 45.22, 22.82 s; mated female (\(n=19\)): 23.64, 31.31, 17.85 s; \(t\)-test, \(t=0.70, p=0.49\)).

(ii) Self-referent cues versus female-specific cues

Time to mating in a male’s fifth encounter was significantly affected by features of the females he encountered.
took significantly longer to mate than males of the control group (t-test, \( t = 2.77, p = 0.016; \) figure 3). This difference was not due to an overall reduction in the attractiveness of females treated with female cuticular extract: in the first mating encounter, there was no difference between the time to mating in the treatment and control (back-transformed mean and mean ± s.e. for time to mating: female with surface extract \((n=12)\): 25.74, 33.98, 19.50 s; female with pentane \((n=10)\): 31.60, 46.09, 21.67 s; t-test, \( t = -0.45, p = 0.66 \)).

**4. DISCUSSION**

The results of our study provide unambiguous evidence for the Coolidge effect in burying beetles *N. vespilloides*. Male sexual interest declined over the course of several repeat encounters with the same female and was renewed when males encountered a novel female. This effect was not caused by a preference for virgin females: male responses to novel mated and novel virgin females were similar.

Our experimental manipulation of female chemical features documents the role of cuticular substances in the discrimination between familiar and novel mates. When a male encountered a novel female that had been coated with the same cuticular substances as his previous mate, he acted as if this female was his previous mate.

We could also show that males do not simply leave some of their own substances on the female to mark them as previous mates. Males treated the mates of their inbred brothers like they treated the mates of unrelated males. The application of extracts suggested that males instead learn their mates' individual specific cues during mating and discriminate against similar scents in their subsequent mating behaviour. Consistent with this interpretation, male responses to their previous mate's close relative (inbred sister) were intermediate to their response to their previous mate and novel females. This indicates that inbred sisters were similar but still somewhat different from each other in

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Figure 4. Visual representation of the cluster analysis (PAM) based on 40 cuticular substances of individuals from different families. Different colours represent different clusters. (M, males; F, females; A–G, different families; 1–5, different individuals of a family).

Figure 5. DA based on the relative amount of cuticular hydrocarbons of five *N. vespilloides* families (each with 5 females). Envelopes represent 95% confidence ellipses. Filled squares, family B; filled circles, family D; triangles, family E; open squares, family F; open circles, family G.
unequivocal evidence of the role of cuticular substances. As in this study, can be a useful method to provide between individuals, direct application of concentrated substances in mate or colony member recognition requires experimental manipulations, such as stripping dead individuals of cuticular substances and reapplying extracts to abolish and restore specific response behaviours (e.g. Wedell & Tregenza 1997), treating mummies with extracts (Akino et al. 2004) or applying extracts, fractions of extracts or solid-phase cuticular hydrocarbons from one individual to another live individual to manipulate the response to the second individual (Lahav et al. 1999; Torres et al. 2007). In the context of discrimination between individuals, direct application of concentrated cuticular extracts in order to mask cuticular compositions (as in this study) can be a useful method to provide unequivocal evidence of the role of cuticular substances.

A study of the responsiveness of male bees (Lasiosglossum zephyrum) to novel females after an initial 10 min encounter with a first female showed that as the relationship between two females increased, males increasingly failed to distinguish them, indicating that the cues produced by females have a genetic basis (Smith 1983). Similarly, this study suggests that males may be confused about female identity when encountering close relatives (inbred sisters) of their original mate, which also suggests that genetic effects are important. Diet and other environmental factors can affect chemical cues produced by animals (Thom & Hurst 2004), but in our study, there was little to no variation in environment or diet, since all females in the study had been reared under identical laboratory conditions, kept in the same containers and substrate and fed the same diet.

Our results provide, to our knowledge, the first clear demonstration of the Coolidge effect and definitive evidence of its underlying mechanism in an insect. In burying beetles, their complex social lives may have been selected for the ability to discriminate between individuals, which may have facilitated the Coolidge effect. Generally, the risk of re-mating with a previous mate will increase if a male has continued access to the same female, and preferring alternative mates will only benefit a male if such alternatives are actually available. Thus, we would expect the Coolidge effect only if male–female associations are somewhat stable in time and space, and if there is some clumping of females. The carcass as an essential, but rare, ephemeral and unpredictable resource required for feeding and reproduction can temporarily cause such clumping (Pukowski 1933). On any carcass, suitable for reproduction or not, potential mates may be available for a limited time only. The losers of aggressive interactions on carcasses, subordinate males (Bartlett 1988) and females (Müller et al. 1990), leave the carcass early (Bartlett 1988; Müller et al. 1990, 2007; Scott & Williams 1993). Male or female intruders may leave quickly after unsuccessful attempts to take over the carcass from the original residents (Trumbo 1990). In both situations, males may have limited opportunities to mate with particular females and may benefit from spreading sperm evenly.

The Coolidge effect wears off quickly, and is non-existent approximately 30 min after an initial mating (J. K. Müller 2006, unpublished data). Our experiments were not affected by this short duration because males re-encountering the same female always did so within 5–10 min after their previous contact. If the loss of sexual interest was of longer duration, it could potentially interfere with frequent matings used by males to maximize their parentage on carcasses. Dominant males increase their parentage with the dominant female through repeated matings during carcass burial and preparation, approximately 70 during the first 24 hours (Müller & Egger 1989). The short-term loss of male sexual interest in his previous mate may actually function in part to allow him to space out matings evenly during the oviposition period to allow for optimal fertilization success.

Male N. vespilloides perceive individual differences in the cuticular signatures of individual females and use them to discriminate between familiar and unfamiliar females. This constitutes individual recognition sensu Breed & Bekoff (1981) and Dale et al. (2001) because each female individual in our population can be discriminated from every other individual on the basis of a unique set of cues. However, although males use individual specific cuticular information, they do so simply to discriminate between two ‘classes’ (Tibbetts & Dale 2007) or ‘heterogeneous subgroups’ (Barrows et al. 1975) of individuals, familiar females and unfamiliar females. This has led some authors to classify systems like this as cases of ‘binary discrimination’ (Gherardi & Tiedemann 2004) instead of true individual recognition.

Interestingly, our study revealed higher variation in the chemical composition of the female than the male cuticle. This suggests that females may be under stronger selection for individual distinctiveness. In Polistes wasps, there is evidence that complex social behaviour can select for variation in traits used in individual recognition (Tibbetts 2004) and similarly, signals of individual identity may facilitate stable joint-breeding associations of burying beetles on carcasses, which are more common in females.
than males (Müller et al. 2007). Being individually recognizable may also benefit female burying beetles in the context of mating. Female reproduction depends on the amount of fertile sperm they have available for fertilization, and sperm degenerate after prolonged storage in the spermatheca (Eggert 1992). When encounters between a particular male and female are brief or infrequent, an even distribution of male sperm through the Coolidge effect may benefit females because it increases the probability that they receive sufficient fertile sperm to ensure fertilization of their egg clutch.

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