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PREREGISTERED REPORT

The Effects of Marking Methodology on Mate Choice in *Drosophila melanogaster*

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Abstract – Mate choice is an important source of sexual selection and a key driver of evolutionary processes including adaptation and speciation. *Drosophila* species have become an important model system for studying mate choice in the lab. Mate choice experiments often require the marking of individual flies to make those flies identifiable to experimenters, and several marking methods have been developed. All of these methods have the potential to affect mating behavior, but the effects of different marking methods have not been systematically quantified and compared. In this experiment, we quantified and compared the effects of five marking methods commonly used for *Drosophila melanogaster*: wing clipping, applying paint to the thorax, applying marker pen to the wing, dusting flies with fluorescent powder, and dyeing flies by allowing them to ingest food coloring. Females mated with unmarked males more often than they mated with marked males, but we could not detect significant differences among marking methods. Latency to mate differed among marking methods, and also with the time of day and the time within the trial. We discuss how our results can help researchers plan studies that require the marking of *Drosophila*.

Keywords – Mate choice, *Drosophila*, Marking, Methods

Mate choice is a process in which traits expressed by one sex lead to non-random mating by the other (Edward, 2015; Halliday, 1983; Kokko et al., 2003; Rosenthal, 2017). The causes and consequences of mate choice are central to our understanding of sexual selection and reproductive isolation (Rosenthal, 2017). Thus, understanding mate choice is an important goal in behavioral ecology and evolutionary biology.

The ease with which *Drosophila* spp. can be reared and manipulated in the lab, along with their complex mating behavior, have made them a model organism for studying mate choice (O'Dell, 2003). Simple mate choice experiments using *Drosophila* spp. are a powerful way to investigate evolutionary processes in a lab setting, and have allowed researchers to understand how factors including relatedness, experience, and changes in the microbiome can influence mate choice (e.g., Avent et al., 2008; Dagaeff et

al., 2016; Dukas, 2010; Loyau, Blanchet et al., 2012; Loyau, Cornuau et al., 2012; Mery et al., 2009; Sharon et al., 2010; Tan et al., 2012).

A common feature in mate choice experiments are 'mate choice trials,' in which a varying number of individuals are put together in a chamber and their mating displays, mate preference and latency to mate are recorded. Conducting mate choice trials requires a way to distinguish the individuals in those trials. The demand for easy, cheap and long-lasting methods of distinguishing individual *Drosophila* has resulted in the use of a variety of marking methods. However, these marking methods have the potential to influence the results of mate choice experiments. Despite their common use, no study has systematically investigated the effects of a range of marking methods on *Drosophila* mate choice and mating behavior.

There are several ways in which marking has the potential to interfere with *Drosophila* mate choice. Courtship behavior in *Drosophila* involves a complex series of multimodal signals that individuals use to identify and assess potential mates (Bastock & Manning, 1955; Griffith & Ejima, 2009). Chemosensory, auditory, visual, and mechanosensory signals are all employed in the courting process (Griffith & Ejima, 2009). Production and reception of such signals presents multiple opportunities for marking procedures to impact on mate choice. Commonly used marking methods include alterations of the wing via clipping or marking with pen or powder. These techniques hold the potential for mechanical disruption of male wing extension and vibration, which may influence the production of courtship song (Hoikkala & Aspi, 1993). Moreover, with the exception of wing clipping, most marking methods involve the application of paints or dyes that may interfere with pheromone release and/or reception and associated courting behaviors. In this way, marking may interfere with the courtship process itself, leading to reduced recognition and therefore reduced mating with marked individuals. Furthermore, if females assess the quality of potential mates through courtship signaling, then small injuries to flies incurred during the marking process or as a result of the mark itself may be perceived as signals of low mate quality, and so may influence mate choice.

Marking methods differ in both the level of handling required to apply the mark and in the nature of the mark itself. This may have implications for the relative impacts of different marking methods on mate choice. The processes involved in commonly used marking methods are as follows. Fluorescent powder is used to dust the exterior of flies, where it attaches to hairs and covers the body (e.g., Arbuthnott et al., 2017; Mery et al., 2009). Preening can lead to some loss of the marking powder, but most is retained on the thorax. Fluorescent powders can be used to quickly mark multiple flies simultaneously with limited handling (Crumpacker, 1974). Wing clipping involves the removal of a small area of one or both wings (e.g., Dodd, 1989; Dukas, 2010; Partridge & Farquhar, 1983). The process requires anesthesia and individual handling to amputate the wings using a scalpel or similar, and results in a permanent alteration of the wing. Acrylic body paint and wing marking with permanent marker also require anesthesia and individual handling to apply the mark. Body paint involves the application of a small dot of acrylic paint on the thorax (e.g., Dukas, 2010; Tan et al., 2013), whereas wing marking uses a permanent marker to dot one or both wings (current authors, unpublished data). Food coloring can be dropped directly onto standard food media that is subsequently ingested by *Drosophila* (e.g., Avent et al., 2008; Verspoor, Heys et al., 2015). Flies can be placed on dyed food at eclosion or moved to this diet prior to mate choice trials, and color becomes visible in the abdomen within ~24 h of ingestion. This technique allows simultaneous marking of multiple flies with no disturbance or extra handling required, though some concerns about the effect of food coloring on longevity have been raised (Kalaw et al., 2002).

One of the biggest problems associated with marking methods such as wing clipping, permanent marker, and acrylic body paint is the requirement for anesthesia to allow application of the mark (Barron, 2000; Verspoor, Heys et al., 2015). In *D. melanogaster*, CO_2 anesthesia has been shown to lead to a considerable increase in latency to mate, even following a 20-hour recovery period (Barron, 2000; Verspoor, Heys et al., 2015). This has led some researchers to suggest that CO_2 anesthesia should be avoided when using *Drosophila* in behavioral studies (Barron, 2000). Despite the caution shown towards CO_2 use, the effect of the mark itself in these cases has rarely been assessed. Due to concerns about the

impacts of anesthesia, methods that do not require anesthesia and speed up the marking process are becoming increasingly popular. These include the use of fluorescent powder to externally dust flies and food coloring to visibly mark drosophila internally (Crumpacker, 1974; Verspoor, Heys et al., 2015). These methods avoid the use of anesthesia, but it is not known whether they impact mating behavior in other ways.

Where studies have specifically investigated marking methods, they have often focused on the survivorship of marked flies rather than on their mating behavior (Crumpacker, 1974; Moth & Barker, 1975). The effect of marking with powders has been tested on *D. pseudoobscura* (Crumpacker, 1974) and *D. simulans* (Moth & Barker, 1975). No effect on survivorship or behavior was found. In *D. pseudoobscura*, mating trials were conducted with the primary goal of ensuring no transfer of powder occurred during copulation, though mate choice was also recorded (Crumpacker, 1974). Marked flies were not chosen more or less frequently than unmarked flies, but there was a significant difference in mating frequency depending on the color of the marking powder (green or yellow). These differences, although indicating some influence of marking powder on mate choice, remain unexplained. Verspoor, Heys et al. (2015) studied the effects of CO_2 anesthesia and food coloring on mate choice in *D. melanogaster*. Males that had been exposed to CO_2 were less likely to mate, but this effect disappeared after two days. Males were also less likely to mate if they were marked with food coloring, but this only became apparent after the effect of CO_2 had worn off.

Many early studies of mate choice in *Drosophila* assumed that wing clipping does not affect mate choice (e.g., Dodd, 1989, and references therein; Partridge & Farquhar, 1983), but more recently researchers have questioned this assumption and suggested that wing clipping may have affected some past results (Dukas, 2010; Partridge, 1988). Ewing (1964) used wing amputation in a study of the effect of wing size on male mating success in *D. melanogaster*. Three different sized areas were amputated, and the mating success of treated flies was compared to that of non-amputated controls in mass mating trials. As the area of wing amputated increased, male mating success decreased linearly. Even the smallest amputated area considered (amputation of ~20% of the wing) resulted in decreased mating success compared to flies with unaltered wings. Wing clipping for marking purposes may remove less than the smallest area amputated in Ewing's (1964) experiment, but the fact that wing size is important may be of concern when wing clipping is used as a marking method. In an extension of Ewing's (1964) study, Hoikkala and Aspi (1993) tested mating success in no-choice as well as competitive choice trials and quantified the effect of wing amputations on male courtship song in several *Drosophila* species. Wing amputations led to changes in both the frequency and amplitude of courtship song. When the mating success of wing-amputated males was tested in no-choice trials, no effect was found on the probability of mating, though latency to mate sometimes increased. However, in competitive choice trials where both the unaltered and wing-amputated males courted the choosing female, unaltered males were chosen over altered males regardless of amputation size. In addition, it has been suggested that wing shape may influence male mating success by altering male courtship song (Menezes et al., 2013). Taken together these findings suggest that courtship and mating in *Drosophila* may be sensitive to changes caused by marking via wing clipping, and possibly to other forms of marking that alter the wings.

In some studies, understanding the effect of marking has not been the primary goal, but effects of marking have nonetheless been detected. We found no literature designed specifically to investigate the effect of acrylic paint on mating, but in a study of the effect of familiarity on male mate choice in *D. melanogaster*, Tan and colleagues (2013, 2014) found that acrylic body paint affected choice. The researchers used acrylic paint to distinguish familiar and non-familiar females in two-choice trials with male choosers, and males courted painted females significantly more often than non-painted females. In a study with female choosers and males marked with a dot of white fabric paint, the opposite effect was found: unmarked males had significantly higher mating frequencies than marked males (Dukas, 2010). No effect of marking on latency to mate was reported.

In an unpublished study of the effects of two marking methods on *D. melanogaster* mate choice, the current authors found that female choosers mated with marked males less frequently than with unmarked males. Our first method of marking involved anesthetizing adult flies with CO_2 and marking

one wing with a small dot using a red permanent marker. In the second method, we marked flies with pink fluorescent powder without anesthesia following Mery and colleagues (2009). Powder was applied by transferring flies to an empty vial with a small amount of fluorescent powder and lightly shaking the vial to ensure a light covering. We carried out mate choice experiments using three-fly competitive choice trials. A 'chooser' female and two males, one marked and one unmarked, were present in each mating trial. Unmarked males were 1.71 times more likely to be chosen than males marked with pen, and this effect was statistically significant (logistic regression; $p < .01$). The same trend was seen for males marked with fluorescent powder, with unmarked males being 1.44 times more likely to be chosen, but this trend was not significant (logistic regression; $p = .12$). In multiple choice trials with male choosers, unmarked females were 1.21 times more likely to be chosen over flies marked with either marking method and this trend was not also significant (logistic regression; $p = .15$). Both male and female *Drosophila* are reported to be choosy in mate choice scenarios (Avent et al., 2008; Byrne & Rice, 2006; Friberg, 2006; Hoikkala & Aspi, 1993; Long et al., 2009; Ödeen & Moray, 2008). However, females use male courtship displays to assess prospective mates (Hoikkala & Aspi, 1993; Kyriacou & Hall, 1982), whereas males may choose females based on static criteria such as body size (Byrne & Rice, 2006). Thus, male courtship signaling, and therefore female mate choice, has the potential to be more vulnerable to potential effects of marking. Our results indicate a strong effect of permanent marker on female mate choice and a trend in the same direction with florescent powder with females avoiding marked males.

Here, we report the first systematic attempt to assess the effects of the commonly used marking methods on mate choice in *D. melanogaster*. We studied the effects of marking with marker pen, fluorescent powder, acrylic body paint, wing clipping and food coloring. Because females are believed to be the choosier sex in *Drosophila*, we focused on female mate choice. We conducted both no-choice and competitive choice trials. No-choice trials studied how different marking methods affect latency to mate. Competitive choice trials studied differences in the mating success when marked and unmarked males compete for mates. Our results will help researchers to select the most effective methods for marking *Drosophila* in future mate choice experiments.

Method

Stocks and Rearing Conditions

We studied a wild-type Oregon-R strain of *D. melanogaster* from laboratory stock maintained by the Manchester Fly Facility. Oregon-R has frequently been used in studies of mating behavior (Champion de Crespigny & Wedell, 2007; Leftwich et al., 2017; Sharon et al., 2010). Fly stocks were maintained in 225 ml culture bottles with standard agar medium at 25°C under a 12:12 hour light:dark cycle and a 2-week generation time.

We collected late-stage pupae from stock populations, sorted the pupae by sex, and reared male and female pupae in separate 25 x 95 mm pupation vials at a standard density of 10 flies per vial (Figure 1). This ensured that all flies were virgins at the time of the mating trails. Sexing the flies in the pupal stage allowed us to avoid anesthetizing juveniles for sexing, which has been shown to negatively impact mating success (Verspoor, Heys et al., 2015).

Marking

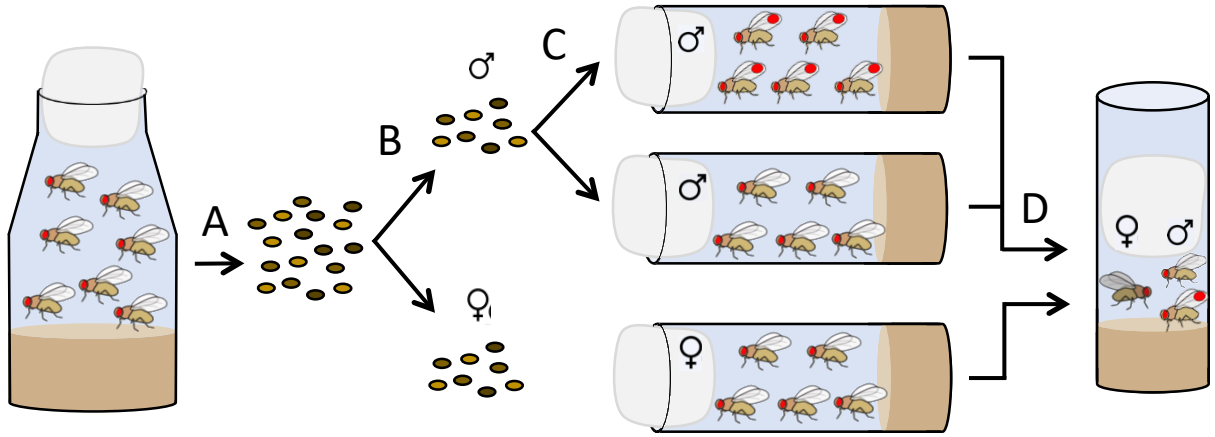
We collected flies from pupation vials upon eclosure and maintained flies collected on each day separately. Flies entered mating trials 7 days after eclosure. Standardizing the age of flies in mating trials is important because mate preference, choosiness and latency to mate can depend on age since eclosure (Avent et al., 2008; Somashekar et al., 2011; Verspoor, Cuss et al., 2015). Only males were marked. For mark types that required anesthesia (i.e., permanent marker, acrylic body paint, and wing clipping), we conducted marking 4 days after eclosure. This allowed flies to recover for 3 days between anesthesia and mate choice trials (Verspoor, Heys et al., 2015). Marking was conducted as follows:

Wing Clipping

Flies were anesthetized with CO_2 and the tip of one wing was removed using a scalpel. Wing clipping was alternated equally between the left and right wings. We removed the smallest proportion of the wing that allowed us to accurately distinguish marked and unmarked flies, rather than attempting to remove a fixed proportion of the wing. This was to ensure that wing amputation in our treatment is consistent with marking in experiments, rather than representing an arbitrary proportion of the wing that is not used in practice.

Figure 1

Schematic of the Protocol for Mate Choice Trials



Note. *Drosophila* were collected from rearing stock (A) and sexed as pupae (B). The sexes were reared to adulthood in separate vials. A subset of males was marked with one of five marker types (C). For each competitive choice trial (D), one marked and one unmarked male were placed in the mating arena, and then one female was placed in the arena. Trials were observed for 2 h or until the first mating occurred, and the successful male was recorded. No-choice trials (not shown) followed the same procedure, but only one male (either treated or unmarked control) was placed in the arena, and the time to until mating occurred was recorded.

Permanent Marker Pen

Flies were anesthetized with CO_2 and one wing was marked with a small dot using a Staedtler Lumocolor Fine Art. Nr. 318-2 red permanent marker pen (Staedtler Mars GmbH & Co. KG, Nuernberg, Germany). The mark was alternated equally between the left and right wings.

Acrylic Body Paint

Flies were anesthetized with CO_2 and a small dot of Amsterdam Standard Series Acrylic Reflex Orange paint (Royal Talens, Apeldoorn, The Netherlands) was placed on the center of the thorax.

Fluorescent Powder

One hour before mate choice trials, flies were transferred to an empty vial containing a small amount of fluorescent pink powder (Dalton Manor LTD, Blaydon-on-Tyne, UK) and lightly shaken so the powder adhered to the body. Flies were then transferred to an empty vial and left for 1 hour to allow

any excess powder to be preened before the mating trials. The fluorescent powder is visible under ultraviolet (UV) light, so a handheld UV lamp was used to allow easy identification of marked flies.

Food Coloring

Two drops of Dr. Oetker Extra Strong Red Food Colour Gel (Dr. Oetker, Bielefeld, Germany) were added to the surface of standard food medium. Food coloring was left to dry and excess liquid was removed. Flies were moved to the colored food 48 hr before the mating trials. This allowed enough time for the food coloring to become visible in the fly abdomen (Verspoor, Heys et al., 2015). In pilot tests we observed no mortality in the 48 hr after flies were exposed to food coloring.

Unmarked Flies

No-choice trials in our study included two unmarked treatments and one unmarked control group. The two unmarked treatments were anesthetized at the same time and for the same length of time (i.e., 10 min) as flies to be marked, but remained unmarked. In one treatment, anesthesia was achieved with CO_2, and in the other with ice. The unmarked control group was neither anesthetized nor marked, and thus represented the behavior of the study population in the absence of manipulation.

No-Choice Trials

Empty standard 25 x 95 mm vials were used for mating trials. The bottom of each vial was lined with Whatman filter paper moistened with a solution of warm water (50 ml), dried yeast (2 g), and table sugar (2 g). Trials were carried out at 27°C. We used an aspirator to transfer one male fly and then one female fly into the vial. Each male was either an unmarked control or had experienced one of the seven treatments described (i.e., marking with one of the five marking methods, anesthesized with CO_2 but not marked, or anesthesized with ice but not marked). A cotton wool stopper was pushed down the vial to create a 25 x 25 mm mating arena (Figure 1). We watched flies continuously and recorded the time at which mating occurred. We classified a mounting attempt as a mating if it lasted for at least 30 s. Trials were terminated after mating occurred, or after 2 hr if no mating occurred.

In our pre-registration, we proposed to conduct 100 no-choice trials for each marking or unmarked anesthesia method, and 200 no-choice trials for unmarked control flies. The larger sample size for unmarked control flies was important because unmarked control flies form the baseline against which other treatments can be compared, and it was necessary to invest extra effort to ensure that this baseline could be estimated accurately. In practice, we conducted more trials than we had planned. We did this because we had to mark more flies than we needed in case of mortality or escapes, and once flies were marked it was important to test them all. Testing all of the marked flies allowed us to avoid selection bias. For example, the first flies selected for trials may have been captured from their vials because they were less active and thus easier to catch, and less active flies may have different latencies to mate.

Competitive Choice Trials

Competitive choice trials were conducted under the same conditions as no-choice trials. One marked and one unmarked control male were transferred to each vial, followed by a female 'chooser.' We watched flies continuously and recorded the first mating in each vial. We terminated trials after 2 hr if no mating had occurred. We conducted competitive choice trials until we had observed 200 matings per marking method. In mate choice experiments, observers are often blind to treatments. We could not do this, because the purpose of the marks is to be readily visible to observers. However, mounting attempts that lasted for at least 30 s generally continued for several minutes, so we believe there was little ambiguity in whether a mounting attempt was a mating, and thus little opportunity for observer bias.

Scheduling of Trials

Because of the large number of mating trials in our study, it was necessary to conduct trials over the course of several weeks. Because each trial had to be set up separately and began as soon as it was set up, it was impossible to run trials synchronously on each day. To balance any effects of day across marking methods, we conducted mate choice trials for multiple marking methods on each day. To balance any effects of time of day across marking methods, we rotated through marking methods when setting up trials. We accounted for the effects of day and time of day in our analysis. Due to an error in the standardization of the treatments, some of the no-choice trials we conducted with CO_2-anesthetised unmarked males had to be discarded, and we had to conduct additional trials with this treatment group after testing of the other groups was complete. So that we could disentangle the effects of CO_2-anesthesia from any effects of day, we continued to conduct no-choice trials with control flies and other treatment groups. All decisions about trials in excess of the number that we had planned were made before any data were examined.

Analysis

To test whether the latency to mate differed among treatments in our no-choice trials, we used Cox proportional hazards models (Cox, 1972) implemented in the R package coxme (Therneau, 2018). The models included a random effect of day and fixed effects of marking method, time of day, and time of day squared. Because all flies tested on a given day had eclosed exactly 7 days earlier, the random effect of day accounted for variability in latency to mate due to conditions on the day of the trial and also due to differences among groups of flies that eclosed on particular days. The fixed effect of time of day allowed for the possibility that flies mate more quickly at some times of day than at others, and the fixed effect of time of day squared allowed for the possibility that this relationship might be nonlinear (e.g., Hardeland, 1972). Practically, including an effect of time of day squared in the model requires breaking trials into one-minute blocks, each of which can be thought of as occurring at a specific point in time (Therneau et al., 2019). When this is done, it becomes necessary to account for the time within the trial and to include a random effect of the individual trial in the analysis. The effect of time within the trial allows for the possibility that the mating rate may change over the course of the trial, and the random effect of individual trial allows for the possibility that flies in some trials may be more eager to mate than flies in other trials. In keeping with our decision to study the effects of time of day and time of day squared, we included time within the trial and time within the trial squared as fixed effects in the model, even though these predictors were not of direct interest in this study. We used backwards selection to remove non-significant predictors from the model. We used an omnibus likelihood ratio test to ask whether latency to mate differed among treatments, and we used pairwise comparisons between treatments to ask which treatments differ from which others, using the Holm-Šidák approach (Ryan, 1960) to correct for multiple comparisons.

There are at least two ways in which anesthesia or marking might affect latency to mate. In the simplest case, treatment might make males less attractive, so treated males might have a reduced probability of mating in every minute of the mating trial. In this case, the effect of the treatment would not change over time. If a treated male were half as likely as a control male to mate in the first minute of the trial, he would be half as likely to mate in the last minute. Alternatively, treatment might render some males unable to mate at all. In this case, the effect of the treatment would change over time. As more treated males mate, males that are unable to mate would comprise a larger proportion of the unmated population. Therefore, the rate at which the remaining treated males mate would decline relative to the rate at which untreated males mate. This pattern would appear as an interaction between the treatment and the time within the trial on the probability of mating. We did not propose to study the interaction between treatment and time within the trial when we registered our report. However, a visual inspection of the latency to mate data suggested that the effect of some treatments may have changed over the course of our no-choice trials. Therefore, we added a *post hoc* analysis to our study to assess the strength of this pattern.

This analysis consisted of the same proportional hazards model we proposed in our registration, with the addition of an interaction between treatment and time within the trial.

To understand whether the marking method affects mate choice in our competitive choice trials, we regressed the mate choice (i.e., marked or unmarked) on the marking method in each trial using a mixed logistic regression implemented in the R package lme4 (Bates et al., 2015). The model included a random effect of treatment nested within day. This allowed for the possibility that the strength of preferences varies by day and/or eclosure group and may vary differently for each marking method. Furthermore, we included the effect of the time of day at which each competitive choice trial began, estimated from the latency-to-mate model, as a fixed predictor. We included this predictor because realized mate preferences may depend on how long females spend evaluating potential mates. We used backwards selection to remove non-significant predictors from the model. We tested for differences in the mating success among males marked with different methods using an omnibus likelihood ratio test. We asked which marking methods performed differently than which others using pairwise comparisons, with p-values computed using the Satterthwaite method (Satterthwaite, 1941; Luke, 2017) implemented in the R package lmerTest (Kuznetsova et al., 2017). We used the Holm-Šidák approach to correct for multiple comparisons.

The data and the code used to analyze the data in this paper are available from the Open Science Framework (https://osf.io/d5mvp/) as is the peer-reviewed pre-registered version of this paper (https://osf.io/p37ru/).

Results

No-Choice Trials

Flies mated in 93.2% of 1,162 no-choice trials. In 50% of trials flies mated within 6 min, and in 80% of trials flies mated within 15 min.

Figure 2 shows the latencies to mate for males that were anesthetized but not marked (A) and for marked males (B). The best model for the observed data includes significant effects of time of day (p = .0026), time within the trial (p < .0001), and treatment (p = .0391). The rate of mating decreased by 0.19% min^{-1} between 9:00 and 16:00. Independently, the rate of mating decreased over the course of each trial, but it decreased at a decreasing rate (6.3% min^{-1} in the first minute, 2.6% min^{-1} in minute 119). Males marked with paint mated at 54% the rate of males marked with powder (p = .0001 against Holm-Šidák corrected significance thresholds of .0028 and .0058 for analysis-wide false positive rates of .05 and .10, respectively), and at 67% the rate of unmarked control males (p = .0049 against Holm-Šidák corrected significance thresholds of .0030 and .0062 for analysis-wide false positive rates of .05 and .10, respectively). No other pairwise differences could be detected with an analysis-wide false positive rate of .10.

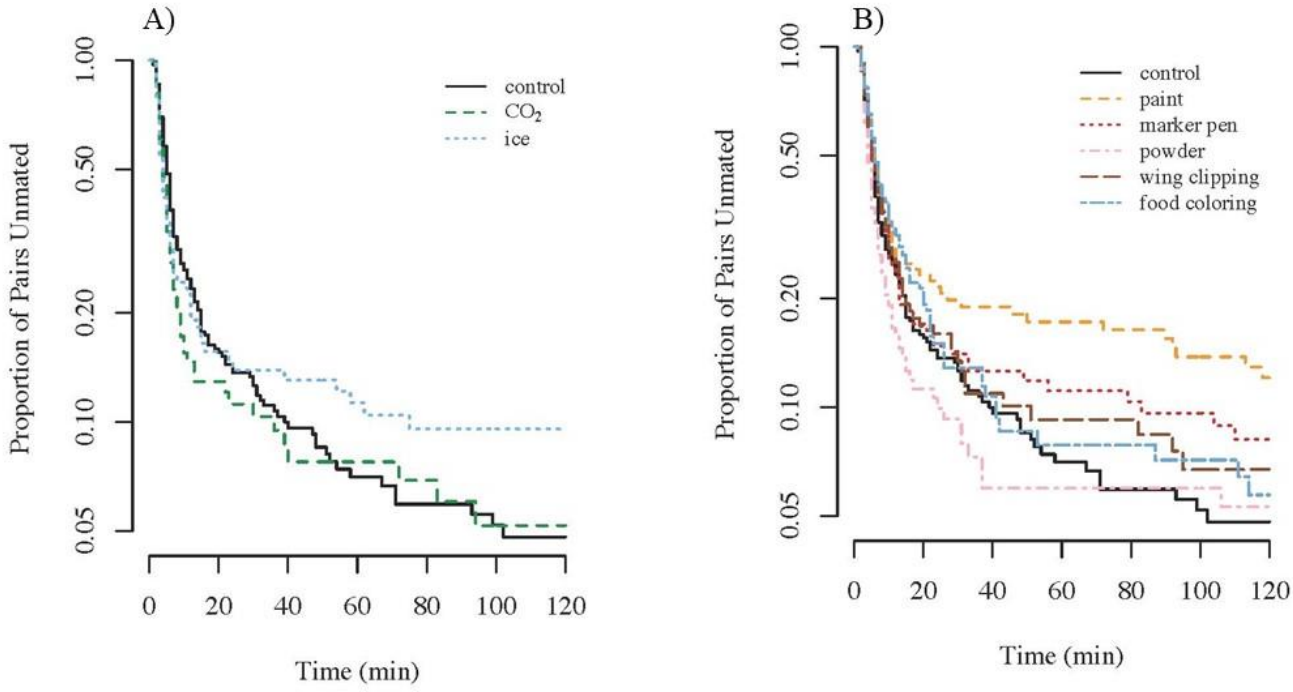
When an interaction between treatment and time within the trial was included in the model, the mating rate declined more rapidly for males that had been anesthetized with ice than for unmarked control males (p = .018). At the start of trials, males that had been anesthetized with ice mated at the same rate as unmarked control males (p = .81), but by minute 119 they mated at only 6.3% of the rate of unmarked control males.

Competitive Choice Trials

Females were less likely to choose marked than unmarked males (462 of 1022 trials, p = .0256) (Figure 3), but we found no significant effect of mark type on the probability that marked males were chosen (p = .4119). We cannot determine which mark types were chosen less often that unmarked control males while maintaining an analysis-wide false positive rate of .10.

Figure 2

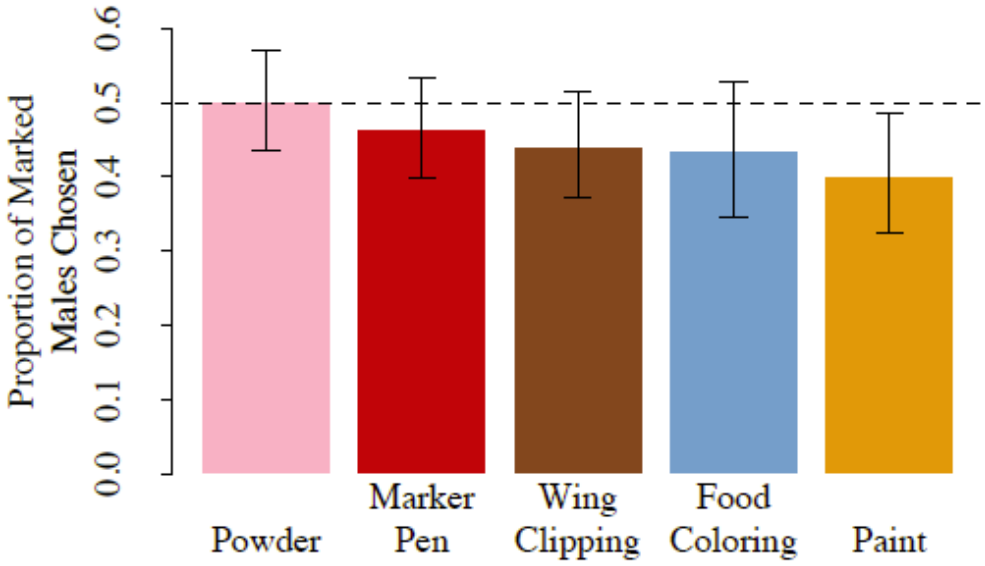
Proportion of D. melanogaster Males Remaining Unmated over Time in 1162 No-Choice Mate Trials



Note. (A) shows results for males that had been anesthetized but not marked, and (B) shows results for marked males. Results for unmarked control males are shown in both panels for comparison.

Figure 3

Proportion of D. melanogaster Males Marked with Different Methods Chosen by Females in Competitive Mate Choice Trials Against Unmarked Control Males



Discussion

Our results show that the methods commonly used to mark *Drosophila* in mate choice studies can affect both mate preferences and latencies to mate. Mating rates changed during the day and during individual mate choice trails. We found some evidence that anesthetising males with ice may affect their latency to mate even 3 days after anesthesia is applied. These results can help researchers plan experiments that require anesthetising or marking *Drosophila*.

We found clear evidence that marking affects mating behavior, but we were unable to distinguish between most individual marking methods while maintaining a low analysis-wide false positive rate. The exception was for marking with acrylic paint. In no-choice trials, males marked with acrylic paint had longer latencies to mate than males marked with powder or unmarked control males. In trials where females chose between marked and unmarked males, there was a non-significant trend in the same direction: males marked with acrylic paint were chosen less often and males marked with powder were chosen more often than other marked males. These results are consistent with past work. Dukas (2010) found that females preferred unmarked males over males marked with white fabric paint, and Crumpacker (1974) found no preference for unmarked males over males marked with green or yellow powders. Our results improve on past work by assessing these marking methods under identical conditions so their effects can be directly compared.

Drosophila show increased mating activity near dawn and dusk (Sakai & Ishida, 2001). Our trials began at 9:00, so it is not surprising that the mating rate declined as time moved further from the dawn peak. Trials sometimes continued until 16:00, and it is perhaps more surprising that we did not detect an increase in the mating rate near the end of our trials. In nature, the onset of the evening mating peak is induced in part by a decrease in temperature at the end of the day (Chen et al., 2007). We conducted mating trials at a constant temperature of 27 °C, near the upper end of the preferred temperature range for *D. melanogaster* (Sayeed & Benzer, 1996). This may have delayed or prevented the onset of the evening mating peak. Alternatively, because the flies in our experiment were reared with a 7:00-19:00 light period, the evening mating peak may not have begun until mate choice trials had ended.

The rate of mating in our study decreased over the course of each trial. This is unsurprising. There is likely to be natural variation among pairs of flies in eagerness and ability to mate, and some pairs may be unable to mate at all. At the beginning of trials we should expect a high mating rate while the most able pairs mate. As these pairs are removed from the population, the remaining pairs will be less eager or able to mate, and the mating rate will decline.

We found no effect of anesthesia with CO_2 on latency to mate 3 days after treatment. In past work, Barron (2000) found that anesthesia with CO_2 increased latency to mate 20 hr after treatment, but Verspoor, Heys et al. (2015) found no effect 2 days after treatment. Barron (2000) reported that anesthesia with ice increases latency to mate 20 hr after treatment, but that the effect was smaller than for anesthesia with CO_2. In contrast, we found some evidence that anesthesia with ice increases latency to mate for at least some flies even 3 days after treatment, by which time the effect of CO_2 anesthesia is no longer detectable. However, it is important to note that this analysis was *post hoc*. Thus, the pattern we report should be treated as a hypothesis rather than a hypothesis test, and new data will be needed to test it.

Recommendations

Researchers who mark *Drosophila* for behavioral studies must ensure that the marking methods they choose do not impact their results. In some studies, researchers have attempted to control for the effects of marking by marking both competitors in mate choice trails (e.g., Loyau, Blanchet et al., 2012; Loyau, Cornuau et al., 2012; Ödeen & Moray, 2008). However, marking both competitors may not fully solve the problems associated with the effects of marking. If a marking method increases latency to mate, then researchers who use that method may collect less data or may spend more time collecting the same amount of data, than if they chose a method with a smaller effect. Moreover, the effects of marking may

alter or swamp the effects that researchers intend to study. For example, Verspoor, Heys et al. (2015) found that the effect of food coloring on mate choice was revealed only after the effect of CO_2 anesthesia had worn off. In other studies, researchers have attempted to control for the effects of marking during analysis by including marking or marker type in their statistical analyses of mate preference (Avent et al., 2008; Loyau, Blanchet et al., 2012; Loyau, Cornuau et al., 2012; Ödeen & Moray, 2008; Tan et al., 2013, 2014). However, these analyses usually assume that the effects of marking are additive with the biological effects the researchers wish to study, and to our knowledge this assumption has not been tested. If the effects are non-additive, then marking could exacerbate, reduce or even reverse the effects of the factors being studied. Thus, when possible, it will be best practice to choose marking methods that have small effects to ensure that the behaviors being studied are as natural as possible. After correcting for multiple comparisons, we are unable to report statistically significant differences among most of the marking methods we studied. Nonetheless, even non-significant trends can help researchers minimize risks and maximize efficiency in experiments that may be expensive or time-consuming.

Currently accepted best practice is to avoid anesthetizing *Drosophila* prior to behavioral experiments (e.g., Avent et al., 2008; Dierick, 2007; Larner et al., 2019). If anesthesia cannot be avoided, we recommend that researchers anesthetize flies with CO_2 and allow at least 3 days of recovery time prior to experimentation. We found weak evidence that anesthesia with ice may have longer-lasting effects on behavior and cannot recommend anesthesia with ice without further study.

Among the marking methods we used, we found florescent acrylic paint to be the easiest to see during experiments, and only slightly more difficult to apply than the other marking methods. Unfortunately, this method also had the largest effect on mating behavior. Thus, we would recommend this method only if systematic changes in the behavior of marked flies, or in the behavior of other flies with respect to marked flies, will not weaken the experiment.

Marking with florescent powder was among the easiest methods to apply, does not require anesthesia, and had the smallest effect on mating behavior in each of our experiments. However, this method was also the shortest-lasting of the methods we studied. Flies remove the powder during grooming, and marks become difficult to distinguish within a few hours of marking. Thus, we recommend this method only for experiments that can be completed quickly after marking takes places.

Differences in the effects of the other marking methods on mating behavior were small, the rank order of those differences differed between our no-choice and competitive choice trials. Therefore, it is difficult to recommend one method over another. Marking with food coloring is easier to apply, does not require anesthesia, and we did not find it more difficult to see during experiments than the other methods. However, we made no attempt to assess how long food coloring remains visible in the abdomens of marked flies after marking. Furthermore, we do not know if it would be difficult to distinguish among different food colorings in abdomens of marked flies. If distinguishing among colors is not possible, then researchers will not be able to mark flies with different treatments in the same mate choice trial.

Finally, it is important to note that we studied only representatives of each marking method. There are other paints, powders, food colorings, and marker pens that we could have chosen, and all of these may have had effects on mating behavior. We believe our results will help researchers plan their experiments, but additional work remains needed to optimize the marking methodology for behavioral studies in *Drosophila*.

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