How prior pair-bonding experience affects future bonding behavior in the monogamous male prairie vole

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Defense Date: April 13th, 2020

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Abstract

Prairie voles are an ideal model for studying the neurobiological underpinnings of pair-bonding and monogamy. While previous studies have shown that voles can form sequential partner preferences, little is known about how prior bonds alter the trajectory and display of new ones. We performed an experiment in which we disrupted an initial pair bond and then varied the amount of time before a new partner was introduced. We assessed how prior bonding experience and time since separation from a first partner affected the stability of partner preference over time and influenced decision-making in voles performing a head-to-head partner preference test in which they chose between the first and second partner. We found that the ability to consistently display a partner preference for the second partner depended on how long the test animal was separated from their first partner; only animals separated for at least 4 weeks showed a consistent preference for the second partner. Mirroring this result, four weeks of separation was also required for the bond with the second partner to consistently supplant the first preference in a head-to-head test. Finally, we also found that partner preference strength was sensitive to latency to mate with the second partner but not the first partner, irrespective of separation time. These results suggest that the ability to form a consistent sequential partner preference depends upon the time between separation from their first partner and introduction to their new partner. Understanding how prior bonds impact future bonds may provide valuable insights into the ways in which human bonds are dynamically shaped by prior social experience and new perspectives on the variables that contribute to successfully overcoming partner loss.

1. Introduction

Romantic relationships are dynamic over time. It is not uncommon for humans to sequentially form more than one romantic relationship or pair bond. The formation and dissolution of these bonds can have profound effects on emotional well-being and health. Given the importance of social bonds to life, understanding how previous relationships and other experiential factors impact subsequent bonds has the potential to elucidate the complex interplay between bonding and health.

Monogamous prairie voles provide a tractable model to begin to explore the factors that contribute to the ability to form a new bond that supplants the old. Voles form life-long pair bonds, which can be measured in the laboratory using a Partner Preference Test (PPT). The PPT tracks how much time the test animal spends with a novel female, compared to their partner and is considered the touchstone for measuring preference behavior in prairie voles (Williams et al., 1992). The two main metrics of partner preference are huddling and average distance. A recent study (Ahern et al., 2019) suggests huddling lacks stability across partner preference tests. However, as our study demonstrates, this is not necessarily the case.

In the wild, prairie voles occasionally re-pair; 20% will take a new partner, but it is unclear how partner availability and high mortality rates limit re-pairing (Carter and Getz, 1993). Recent laboratory studies suggest that most male voles will form a subsequent partner preference after the loss of a partner and can do so multiple times throughout their life (Kenkel et al., 2019). While we have established that voles can form more than one sequential partner preference, we still do not know how the second bond compares to the first bond, nor do we know how time between separation from the first partner and introduction to the second partner affects partner preference.

To further define the effects of previous pair bond experience on the subsequent formation of new bonds, we designed an experiment in which we varied the time between separation from the first partner and introduction to the second partner. Males were allowed to cohabitate with their first partner for 12 days before being separated for 48 hours, 2 weeks, or 4 weeks prior to the introduction of the second partner. In order to assess whether bonds mature with time, we performed a PPT at 5 days and 12 days post-introduction of each partner. Finally, we performed a head-to-head partner preference test to determine whether the second bond had supplanted the first.

We hypothesized that voles in all separation conditions would form subsequent bonds, as seen in previous research; however, we postulated that length of separation time would predict whether the new bond successfully supplanted the first.

2. Methods

2.1 Animals

Sexually naive adult prairie voles (Microtus ochrogaster, n = 66: 22M, 44F) were bred in-house in a colony originating from a cross between voles obtained from colonies at Emory University and University of California Davis, both of which were established from wild animals collected in Illinois. Animals were weaned at 21 days and housed in same-sex groups of 2 – 4 animals in standard static rodent cages (7.5 x 11.75 x 5 in.) with ad-lib water, rabbit chow (5326-3 by PMI Lab Diet) supplemented with alfalfa cubes, sunflower seeds, cotton nestlets, and igloos for enrichment until initiation of the experiment. In order to eliminate confounds of pregnancy, females were tubally ligated and given at least two weeks to recover prior to the start of the experiment (details below). All voles were between the ages of 8 and 16 weeks at the start of the experiment. Throughout the experiment, animals were housed in smaller static rodent cages (11.0 in. x 8.0 in. x 6.5 in.) with ad-lib water, rabbit chow (5326-3 by PMI Lab Diet), and cotton nestlets. They were kept at 23–26°C with a 10:14 dark: light cycle to facilitate breeding. All procedures were approved by the University of Colorado Institutional Animal Care and Use Committee.

2.2 Tubal ligation

Tubal ligation surgeries were performed using a dorsal approach and isoflurane as an anesthetic (Souza et al., 2019). Iodine was used to disinfect the skin and fur around the cut site. An electric razor was used to clear the immediate area of fur and iodine was applied again to the exposed skin. A horizontal cut was made through the first layer of epithelial tissue in the lumbar region. An internal cut was made through the next layer of tissue, near each ovary. A cauterizing tool was applied to each tube until separation. The internal and external cuts were sutured using vicryl-coated sutures, size 4-0. The external cut was then sealed with surgical staples, which were removed within one-week post-surgery. All females were given at least 2 weeks to heal before their first pairing.

2.3 Experimental Design

All animals were paired with an initial partner (Partner 1) and placed on opposite sides of a custom transparent, ventilated, divider for 48 hours to reduce aggression and induce sexual receptivity in the female voles (Roberts et al., 1998). Dividers were removed after 48 hours and sexual behavior was recorded on Sony Handycams (DCR-SX85) with four cages captured per frame, for the first 3 hours following divider removal. Partner preference tests (PPT) were performed at one short-term (3 days post-divider removal) and one long-term timepoint (10 days post-divider removal). The decision to test partner preference at two timepoints was based on previous work in our lab that indicated pair bonds strengthen with time (Scribner et al., 2019). For all PPT tests, except for the final head-to-head test, novel females consisted of partners from other pairings. Test animals were never re-exposed to the same novel animals nor were they paired with or exposed to a sibling during PPT. Immediately following the long-term PPT, the partners were separated and singly housed for 48 hours, 2 weeks, or 4 weeks, according to the experimental condition to which they were randomly assigned (Fig. S2B). This was done to test the presence of an effect of separation time between partners on subsequent partner preference. After the respective periods of isolation, the experiment was repeated with a new sexually naive partner (Partner 2). Following the long-term PPT, all voles were singly housed for 48 hours. A final PPT was performed in which the test animal chose between Partner 1 and Partner 2 which were placed in the adjacent chambers. This final head-to-head PPT enabled us to determine whether the second bond had supplanted the first.

2.4 Partner Preference Test

We performed a partner preference test (PPT) to assess selective partner affiliation at various timepoints. Each PPT apparatus consisted of a box (75.0 cm. x 20.0 cm. x 30.0 cm.) sectioned into three equal size chambers separated by removable dividers. To start, dividers were placed with the male untethered in the center chamber. His Partner and a novel age-matched conspecific (Novel) were tethered to bolts located on opposite sides of the apparatus using fishing swivels and zip ties with a water bottle affixed to the same wall. One cup of bedding and two alfalfa pellets were placed in each chamber containing a tethered animal. Overhead cameras (Panasonic WVCP304) were used to film two boxes simultaneously. The test animal freely explored the apparatus for 3 hours. In each round of PPT, the novel female was picked from a different pair to ensure novelty was preserved between different testing timepoints. The movement of the test animal was recorded and tracked post-hoc using Topscan software (Cleversys Inc.). Previous studies demonstrated that Cleversys is a reliable and efficient method to obtain data from PPTs (Ahern et al., 2009). We used TopScan 3.0 with some minor customizations. Frame by frame behavioral analysis was analyzed using a custom Matlab script created in our lab to generate average distance between the test animal and tethered animal when in the same chamber, time spent huddling with the tethered animal, and total distance traveled. The partner preference score was calculated using huddle time (Partner Huddle/Partner + Novel Huddle).

2.5 Statistics

Data were analyzed using SPSS version 25. Details of all statistical tests are provided in Supplementary Table 2. As a behavioral test, comparison of time spent with the partner versus the novel animal violates the assumptions of a traditional T-test because time with each tethered vole is not truly independent. To address this, partner preference was assessed using the percent of time spent huddling with the partner where huddle time with both tethered animals was used as the denominator. This was compared to a null value of 50% (no preference) in a two-tailed one-sample T-test. Differences in preference across groups and/or testing timepoints were analyzed using an RM-ANOVA with Timepoint as a within-subject factor and Treatment as a between-subject factor. To gain further insight into the underlying behavioral changes that contributed to differences in partner preference over time, total partner huddle or total novel huddle across timepoints were analyzed separately using a paired T-test. To determine behavioral consistency across timepoints, we examined correlations between the total partner huddle time, novel huddle time, and percent partner huddle (as above) between short-term and long-term timepoints.

To strengthen our interpretation, we also examined the average distance between the test animal and the tethered animals when the test animal was in the chamber with the tethered animal. We have previously shown that this behavioral metric serves as a proxy for partner preference (Scribner et al., 2019). Because the distance from the partner while in the partner chamber does not influence the distance from the novel in the novel chamber, these variables can be considered independent, and we performed a paired T-test and/or RM-ANOVA to determine whether these metrics differed within and across tests. In addition, we calculated a ratio of novel distance/partner distance to create a within-animal preference score based on distance and asked whether this score correlated with percent partner huddle.

Finally, we examined the effects of mating latency on partner preference. We performed a Kaplan Meyer survival analysis with Log Rank for overall comparison to examine potential group differences in mating latency. This approach provides an ideal non-parametric test that takes into account failure to complete the task (e.g. failure to mate).

3. Results

3.1 Excluded Animals

Excluded animals and the reason for their exclusion are available in Figure S3A. One animal from the 48 Hour condition, two from the 2 Week condition, and two from the 4 Week condition were excluded when their partner was killed by aggressive males during partner preference tests in which their partners were acting as the novel female. Four animals were excluded from the 48 Hour condition due to camera failure during the partner preference test.

3.2 Partner Preference for Partner One

Partner preference was measured at two timepoints to assess stability of the bond with Partner 1. Average Distance was evaluated as a potential estimate of partner preference.

The duration of pairing predicts decreased novel interaction without altering partner interaction.

Relative to a null hypothesis of no preference, sexually naive male voles paired with a female partner demonstrated a partner preference at short-term ($p = 0.017$) and long-term ($p < 0.001$) timepoints (Fig. 1A). Time spent with the partner did not change over time ($p = 0.849$), thus, the strengthening of the preference for Partner 1 was reflected exclusively in a decrease in novel huddle time at the long-term timepoint (Fig. 1B; $p = 0.025$). Time spent huddling with the partner or with the novel animal were positively correlated across the two tests (partner: $p = 0.018$; novel: $p =0.032$), suggesting intra-animal behavioral consistency across tests. Average distance was measured only while the test animal occupied the same chamber as the tethered animal (Fig. S2A). Preference resulted in a decreased distance between the test animal and partner compared to the test animal and novel (Fig. 1C; main effect of tethered animal; $p < 0.001$). These two metrics, time spent huddling and average distance, were correlated strongly at both testing timepoints (Fig. 1D; short-term: $p < 0.001$; long-term: $p < 0.001$), indicating that they measured overlapping aspects of preference behavior and can both be used as proxies for inferring partner preference.

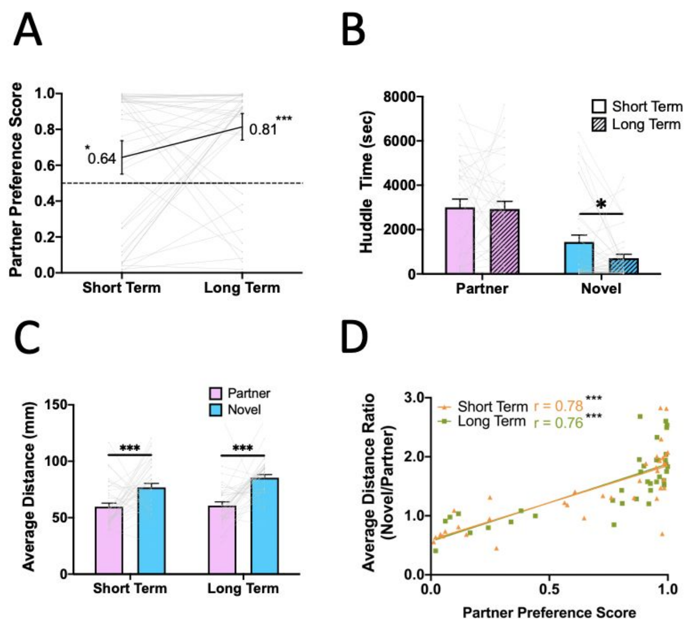

Figure 1. Metrics of partner preference for Partner 1. Male prairie voles housed with a sexually receptive tubally-ligated female prairie vole exhibited a partner preference following 3 days (short-term) and 10 days (long-term) of cohabitation post divider removal. **A)** Partner preference score (proportion of time spent huddling with the partner) was significantly greater than chance (0.5) at both timepoints (short-term: $p = 0.017$, long-term: $p < 0.001$). **B)** Time spent huddling with the partner did not change over time ($p = 0.849$) while time spent huddling with the novel decreased over time ($p = 0.025$). The increased partner preference score in (A) is explained by a significant decrease in the time spent huddling with the novel at the long-term timepoint compared with the short-term. **C)** Average distance from the tethered animal while in the same chamber also reflects partner preference. At both timepoints, the test animal was physically closer to their partner when in the partner chamber than they were to the novel animal when they were in the novel chamber (main effect of tethered animal: $p < 0.001$). **D)** To examine the consistency between preference score and distance metrics, we calculated a distance ratio (novel distance/partner distance) from (C). There was a strong correlation between the preference score and the distance ratio at both timepoints (short-term: $p < 0.001$; long-term: $p < 0.001$), suggesting that both metrics provide valid estimates of partner preference. Significance notated as: *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

3.3 Partner Preference for Partner Two

Following separation from Partner 1, males were singly housed according to their assigned period of separation before being introduced to Partner 2. The effect of separation time was analyzed from partner preference data taken at two timepoints.

Stability of preference for a new partner is sensitive to the time since separation from the first partner.

Confirming previous work, we found that voles in all conditions were capable of showing a preference for their second partner shortly after pairing. However, when re-tested following a longer cohabitation, only animals in the 4 Week condition showed an initial trend towards preference for Partner 2 which manifested fully at the long-term timepoint. This suggests that preference was no longer intact at the long-term timepoint except in the 4 Week condition, indicating an effect of pairing time that depends on the duration of the separation period. Average distance from the tethered animals revealed similar patterns to those of partner preference. Males from the 48 Hour and 2 Week conditions were closer to the partner than the novel animals at the short-term timepoint, but not at the long-term timepoint. In contrast, the 4

Week condition was closer to the partner than the novel at both timepoints. As with the first pairing, partner huddle and novel huddle were tightly correlated across tests for Partner 2 (partner: $p < 0.001$, novel: $p = 0.032$).

48 Hour separation: Partner preference was evident at the short-term test ($p = 0.022$) but not at the long-term test ($p = 0.285$) (Fig. 2A). There were no changes in total time huddling with the partner or the novel between the two timepoints (partner: $p = 0.092$; novel: $p = 0.172$) (Fig. 2B). Confirming a lack of partner preference, in-chamber distance showed that test animals were closer to their partner than the novel animal during the short-term ($p = 0.002$) but not the long-term test ($p = 0.196$) (Fig. 2C).

2 Week separation: Partner preference was evident at the short-term test ($p = 0.003$) but not at the long-term test ($p = 0.850$) (Fig. 2D). There was no change in the time huddling with the partner between tests ($p = 0.131$) but there was an increase in the time spent with the novel in the long-term test relative to the short-term ($p = 0.032$) (Fig. 2E). Mirroring partner preference results, test animals were closer to their partner than the novel animal during the short-term ($p = 0.005$) but not the long-term test ($p = 0.549$) (Fig. 2F).

4 Week separation: Unlike shorter separation timepoints, partner preference was significant at the long-term timepoint ($p = 0.016$; short-term: $p = 0.075$) as indicated by two metrics: average distance and proportion huddle (Fig. 2G). Overall, there was no difference in how long the test animal huddled with the partner ($p = 0.577$) or the novel ($p = 0.571$) across the two tests (Fig. 2H). Test animals were significantly closer to their partner than the novel at both timepoints (Fig. 2I; short-term: $p = 0.042$, long-term: $p = 0.002$).

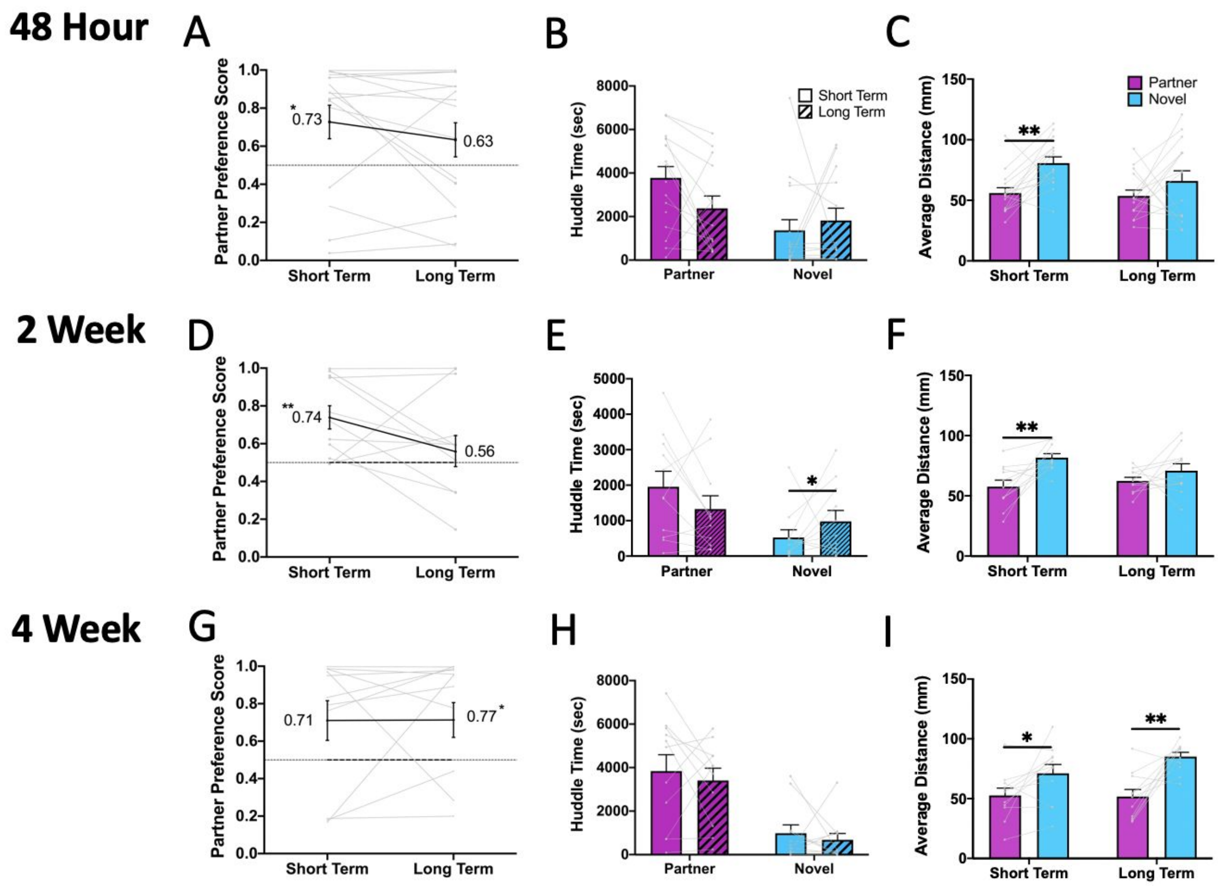

Figure 2. Metrics of partner preference for Partner 2. Male prairie voles spent 48 hours, 2 weeks, or 4 weeks singly housed between separation from their first partner and introduction to their second partner. As with Partner 1, animals were tested for partner preference at 3 days (short-term) and 10 days (long-term) post cage divider removal.

48 Hour condition: **A)** Male voles showed a clear partner preference at the short-term test (p = 0.022) but not at the long-term test (p = 0.285). **B)** There were no changes in partner huddle time (p = 0.092) across tests, nor in novel huddle time (p = 0.172) across tests. **C)** Males were closer to their partner than the novel animal during the short-term (p = 0.002) but not the long-term test (p = 0.196).

2 Week condition: **D)** Partner preference was evident at the short-term (p = 0.003) but not the long-term (p = 0.850) test. **E)** Time spent huddling with the partner did not change between tests (p = 0.131) while time spent huddling with the novel increased (p = 0.032). **F)** Males were closer with their partner than the novel at the short-term test (p = 0.005) but not at the long-term test (p = 0.549).

4 Week condition: **G)** Partner preference was evident at the long-term (0.016) test but only slightly present (p = 0.075) at the short-term test. **H)** Neither time spent huddling with the partner (p = 0.577) nor time spent huddling with the novel (p = 0.571) varied over time. **I)** Test animals were closer in proximity to the partner than the novel at both the short (p = 0.042) and long-term (p = 0.002) tests. Notably, males in the 4 week separation condition were the only males to show a partner preference at the long-term test, which is reflected in a consistent decreased distance from the partner compared with the novel.

3.4 Mating Latency Comparison Across Pairings

Previous work suggests that mating facilitates partner preference. Thus, we separated animals into early and late mating groups based on whether they mated within 3 hours of divider removal.

Latency to mate did not vary significantly between the first and second pairings or between separation conditions.

There were no significant differences in mating latency across pairings or between conditions, indicating that likelihood to mate within the first 3 hours after divider removal is not influenced by prior pairing or by separation time. There were no differences between first and second pairings (Fig. 3A:C; p = 0.452). In both instances, a similar proportion of animals (62% in the first pairing and 49% in the second pairing) mated within the first 180 minutes (Fig. 3A:A,B; p = 0.187). When analyzed by separation condition (48 Hour, 2 Week, 4 Week) there was no significant difference in latency to mate between conditions at either pairing (Fig. S1:A, B; first pairing p = 0.505, second pairing p = 0.653).

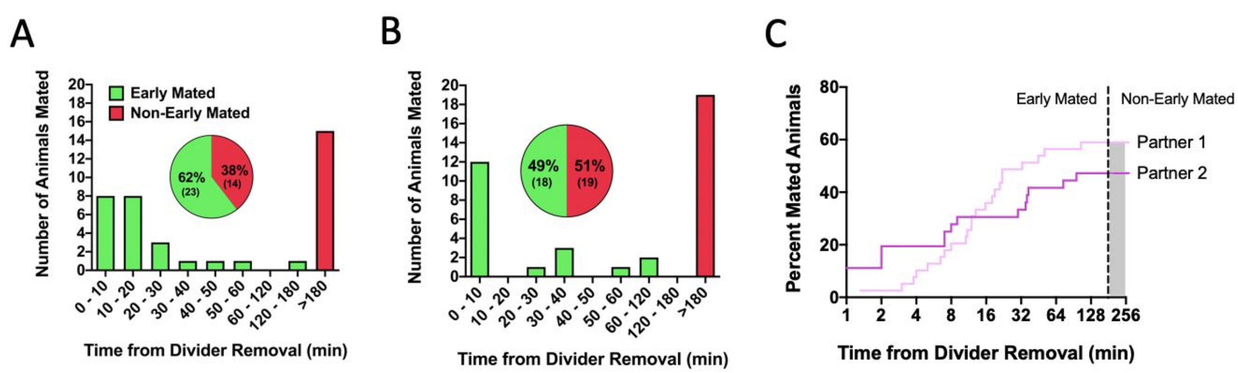

Figure 3A. Mating Latency for both partners. Dividers were removed from cages 48 hours post pairing and mating behavior was filmed and scored for the first 180 minutes of interactions. **A, B)** For the first and second pairings, approximately half of the test animals mated within the first 180 minutes. **C)** There were no significant differences in mating latency between the first and second pairings (p = 0.452).

3.5 Early Mated vs. Non-Early Mated Groups

After establishing that mating latency did not vary significantly between pairings or separation conditions, we examined whether mating latency predicted differences in partner preference.

Longer latency to mate was associated with weaker bonds for Partner 2, but not Partner 1.

Early maters only showed a preference at the short-term timepoint for Partner 2 (Fig. 3B:A; Partner 1: $p = 0.094$, Partner 2: $p = 0.002$), while they showed a preference at the long-term timepoints for both Partner 1 ($p < 0.001$) and Partner 2 ($p < 0.001$) (Fig. 3B:C). Preference for Partner 1 for the non-early maters was not evident at the short-term test ($p = 0.099$) but was at the long-term test ($p = 0.039$) (Fig. 3B:B). For Partner 2, partner preference was not evident at either timepoint for non-early maters (short-term $p = 0.309$; long-term $p = 0.623$) (Fig. 3B:D). There was not a significant effect of time ($p = 0.279$) or mating latency group ($p = 0.969$) on partner preference for Partner 1. For Partner 2, there was a significant effect of time ($p = 0.041$) and mating latency group ($p = 0.005$).

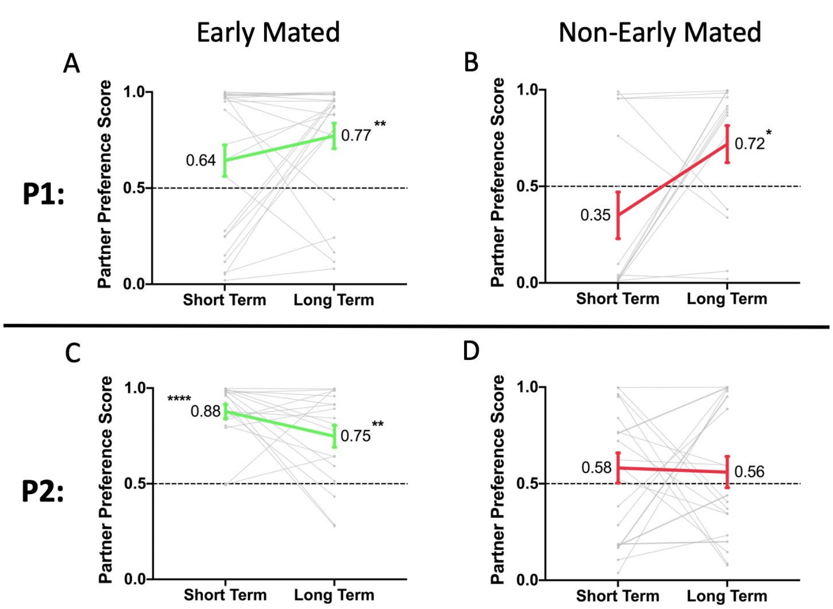

Figure 3B. Partner preference scores of early vs. non-early maters. Voles that mated within the first 180 minutes were classified as "Early Mated" while those who did not were classified as "Non-Early Mated". Their partner preference scores for both partners were analyzed by this grouping. **A)** Partner preference was evident at the long-term ($p = 0.000$) but not the short-term ($p = 0.094$) tests. **B)** Partner preference was not evident at the short-term test ($p = 0.099$) but was at the long-term test ($p = 0.039$). **C)** Partner preference was present at the short-term ($p < 0.001$) and long-term test ($p = 0.002$). **D)** Partner preference was not evident at either timepoint (short-term $p = 0.309$; long-term $p = 0.623$).

3.5 Head-to-Head Partner Preference Test

Previous work had shown that male voles can form multiple sequential pair bonds following partner loss. However, it is not yet known whether the subsequent pair bond formed is sufficient to supplant the first. In order to examine this, we performed a final PPT in which the male test animal chose between Partner 1 and Partner 2.

Four weeks of separation are required to supplant an old bond with a new one.

Males in the 4 Week condition spent more time huddling with Partner 2 than Partner 1 (48 Hour: $p = 0.981$, 2 Week: $p = 0.406$, 4 Week: $p < 0.001$) (Fig. 4A). Similarly, only males in the 4 Week condition stayed significantly closer to Partner 2 than Partner 1 (48 Hour $p = 0.573$; 2 Week $p = 0.315$; 4 Week $p < 0.001$) (Fig. 4B). This suggests an effect of separation duration on the ability of the new bond to supplant the first, even when tested against a previous partner.

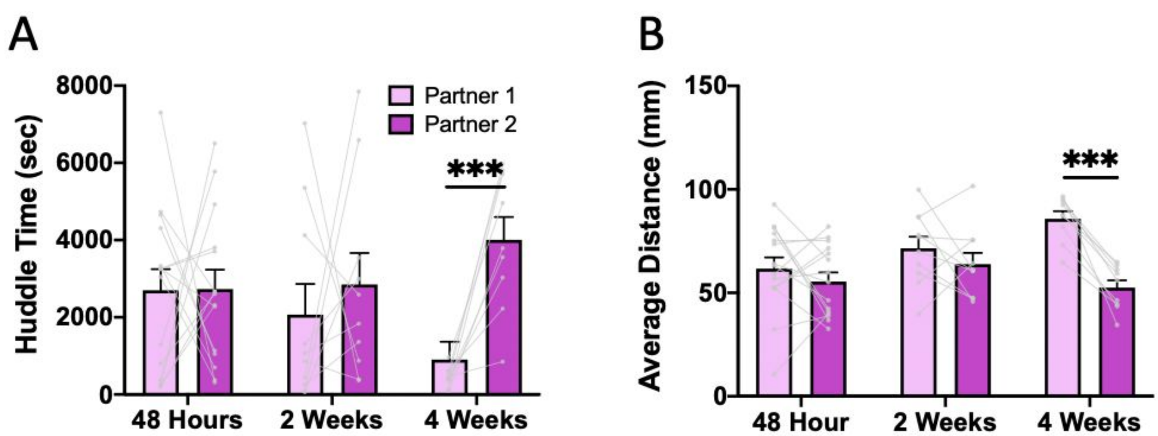

Figure 4. Metrics for the head-to-head partner preference test. Voles were singly housed for 48 hours following the long-term partner preference test with Partner 2. After 48 hours the males were placed in a partner preference test with Partner 1 and Partner 2 as the tethered animals to see which partner they preferred. **A)** Males in the 4 Week condition were the only test animals to spend significantly more time huddling ($p = 0.000$) with Partner 2 than Partner 1 (48 Hour, $p = 0.981$; 2 Week, $p = 0.406$). **B)** Test animals in the 4 Week condition stayed significantly closer ($p = 0.000$) to Partner 2 than Partner 1.While test animals in the 48 Hour ($p = 0.573$) and 2 Week ($p = 0.315$) conditions did not show a difference in proximity to Partner 1 versus Partner 2.

4. Discussion

Voles are a valuable model for studying pair-bonding and monogamy. While recent studies have shown that voles can form sequential bonds following partner loss, relatively little work has focused on how previous bond formation affects subsequent bonding in this species.

The goal of this study was to examine how a previous pair bond altered subsequent bonding behavior with a new partner. In line with previous research, we found that male prairie voles formed a sequential partner preference following the loss of their first partner. However, the formation and stability of that preference as well as whether the new preference supplants the old one are dependent on mating latency and separation time. Only voles separated from the first partner for 4 weeks formed a consistent second bond that supplanted the first. Overall this suggests that total dissolution of a partner bond is dependent on time, which could be due to a loss of motivational salience or diminished memory for the first partner.

In accordance with a prior study, male prairie voles reliably demonstrated a sequential partner preference regardless of previous bonding experience (Kenkel et al., 2019). This would suggest that prior bonding experience does not nullify the ability of male prairie voles to develop a subsequent preference for a new individual. This study builds on prior studies in three key ways. First, we monitored mating latency and found that longer latency to mate predicts weaker partner preference with the second partner. This could be an effect of experiential familiarity, meaning, once animals are no longer sexually naive, the propensity to mate soon after divider removal may play a larger role in their assessment of bond quality. Second, we assessed partner preference stability by performing partner preference tests at short-term and long-term timepoints following partner introduction. Third, we included a head-to-head test designed to ask when the second bond supplants the first. Only voles separated from their first partner for at least 4 weeks demonstrated a consistent, strong preference for the second partner across both tests and chose the second partner in a head-to-head test. This corresponds well with prior work indicating that prairie voles no longer show a partner preference after four weeks of separation from their pair-bonded partner. (Sun et al., 2014).

Testing partner preference at two timepoints provided an opportunity to examine behavioral stability across tests. A recent report (Ahern et al., 2019) suggests that huddling across different partner preference tests is not highly correlated. Here we show that huddling times with the partner and novel animals are highly correlated across tests. In the Ahern study, huddling was tested across different types of behavioral tests or contexts. While in our study huddling was tested within the same context of the partner preference test. Our findings indicate that inter-individual huddling is consistent when it is within-context.

5. Limitations and Future Directions

There are some aspects of this study that make the findings less representative of vole bonding in the wild. The head-to-head test places voles in a situation they are unlikely to encounter in the wild where re-pairing is a relatively scarce occurrence (Carter and Getz, 1993). There are several contributing factors to the lower rate of re-pairing in the wild. Life expectancy is shorter in the wild, thus limiting the pool of available partners (Getz et al., 1981). Even when a potential mate is available, pair-bonded males show significant aggression to novel females and vice versa up to 4 weeks post-separation from their previous partner (Ophir et al., 2008; Sun et al., 2014). In the lab setting, we use transparent dividers to minimize aggression and facilitate mating, which may contribute to the much higher rate of sequential preference formation than seen in the wild.

Our novel approach of measuring partner preference stability and administering a head-to-head test introduces a new path of inquiry in vole research. We do not have data on subsequent bond formation in females, whose mating behavior differs from that of males. If we are to thoroughly understand sequential partner preference formation in voles, replicating this study in females is a necessary next step. The effect of separation time on partner preference could be due to an alteration of the memory for the first partner or it could be that the voles still remember the first partner but no longer have the same motivational salience. Future studies should examine these possibilities.

6. Conclusions

Findings from this study provide a foundation upon which we can investigate the neurobiological mechanisms subserving adaptation to bond dissolution in species whose social biology resembles that of humans. In order to successfully recover from loss, humans must successfully adapt and form new bonds to reduce their risk of developing a myriad of mental and physiological disorders stemming from unresolved grief. By showing that voles can form more stable secondary bonds when given adequate time following separation from their first partner, we have provided the beginnings of a behavioral model for adaptation to loss that might one day be translated to clinical interventions for humans struggling to overcome grief.

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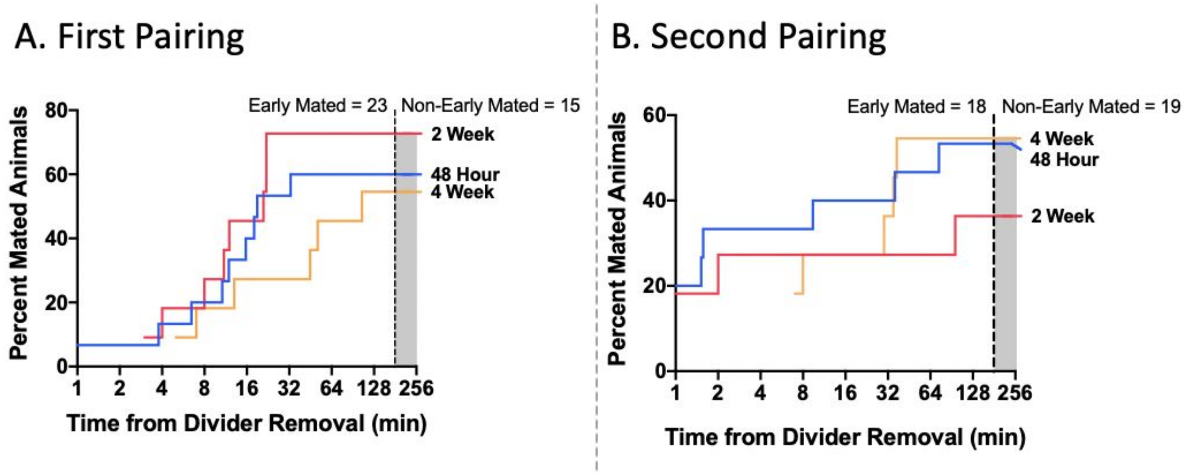
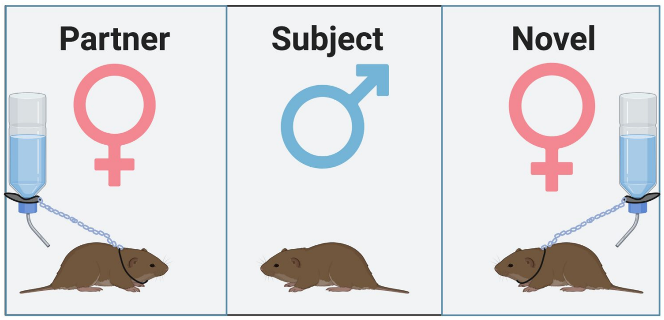
Supplemental Materials

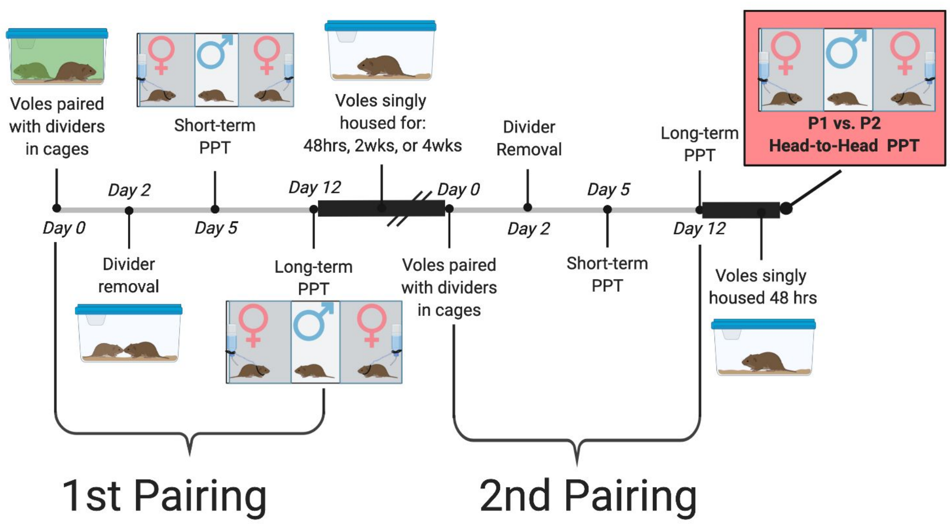


Supplemental Figure 1. Mating latency comparison across 3 conditions. A) There were no significant differences between conditions in mating latency with the first partner ($p = 0.505$). **B)** There were no significant differences between conditions in mating latency with the second partner ($p = 0.653$).

A.



B.



Supplemental Figure 2 and Partner Preference Test and Experiment Design. A) At the start of each partner preference test, the male vole is placed in the center chamber with dividers on either side blocking his way to each side chamber. One of which contains his partner and another contains a novel animal. In the final PPT these chambers contain Partner 1 and Partner 2. When the test begins, the dividers are removed and the male is free to roam among the three chambers while an overhead camera records his movements to later determine which female he prefers. **B)** Male voles were paired with naïve females and placed in small cages with a lengthwise divider for 2 days. Once dividers were removed the voles interacted and mated for 3 days at which point they underwent a short-term PPT. The pairs then cohabitated for a period of 7 days before undergoing a long-term PPT. Immediately following the long-term PPT all voles were singly housed according to their assigned separation condition of 48 hours, 2 weeks, or 4 weeks. After their assigned duration, the voles were paired with a new naïve female and put through the same stages as the first pairing. Divider placement, divider removal, short-term PPT followed by 7 days of cohabitation and a long-term PPT. Immediately after the long-term PPT all voles were singly housed for 2 days at which point the final, head-to-head PPT was performed. In this PPT the males were placed with their first partner and their most recent partner (Partner 2) to test if the new bond formed with Partner 2 supplanted the bond formed with Partner 1.

A.

Animal	Condition	PPT excluded from	Why
1872	2 Week	Test 4	Partner died
1850	4 Week	Test 5	Partner died
1867	4 Week	Test 5	Partner died
1924	2 Week	Test 5	Partner died
2242	48 Hour	Test 2-5	Partner died
2245	48 Hour	Test 5	Escaped PPT box
2262	48 Hour	Test 4	Camera shift so box was out of frame
2267	48 Hour	Test 4	Camera shift so box was out of frame

Supplemental Table 1 Excluded Animals Table.

Key:
PPT1 = Partner 1 Short-term
PPT2 = Partner 1 Long-term
PPT3 = Partner 2 Short-term
PPT4 = Partner 2 Long-term
PPT5 = Partner 1 vs. Partner 2

(A) All partner losses were due to injuries incurred from aggressive males whilst the females played the novel in other partner preference tests.

Experiment	Part-ner	Measurement	Statistical Test	Comparison	° of freedom, error	F or T	p	*	Group Size	Fig.	Notes
Partner Preference	1	Prop. Partner huddle	RM-ANOVA	Time (RM)	1, 36	1.726	0.197		n = 37	1A	
		Short Term	one way T-test	relative to 50%	37	2.495	0.017	*	n = 38		
		Long Term	one way T-test	relative to 50%	36	4.667	0.000	***	n = 37		
		Group differences prop. Partner huddle	RM-ANOVA	Time (RM)	1, 34	1.444	0.238		48 hr = 15, 2 wk = 11, 4 wk = 11	not shown	
				Time x group	2, 34	2.19	0.127				
				Group	2, 34	0.091	0.913				
		48hr, short term	one way T-test	relative to 50%	16	1.428	0.174				
		48hr, long term	one way T-test	relative to 50%	15	4.24	0.001	**			
		2 wk, short term	one way T-test	relative to 50%	10	0.77	0.459				
		2 wk, long term	one way T-test	relative to 50%	10	3.261	0.009	**			
		4 wk, short term	one way T-test	relative to 50%	10	2.163	0.056				
		4 wk, long term	one way T-test	relative to 50%	10	1.009	0.337				
		Short vs long term, partner 1	partner time	pearson, spearman		0.335, 0.341	0.043, 0.039	*	n = 37		
			partner huddle	pearson, spearman		0.347, 0.417	0.036, 0.010	*	n = 37		
			percent phuddle	pearson, spearman		0.290, 0.387	0.081, 0.018	*	n = 37		
			novel time	pearson, spearman		0.413, 0.423	0.011, 0.009	*	n = 37		
			novel huddle	pearson, spearman		0.294, 0.353	0.077, 0.032	*	n = 37		
		Ndist/Pdist corr with PctPhuddle	Short term	pearson, spearman		0.782, 0.827	6.7e-9, 1.55e-10	***	n = 38	1D	
		Ndist/Pdist corr with PctPhuddle	Long term	pearson, spearman		0.760, 0.790	1.55e-10, 4.73e-8	***	n = 37		
Huddle time	1	Partner huddle time	paired t-test	Time (RM)	36.000	0.192	0.849		n = 37	1B	
Huddle time	1	Novel huddle time	paired t-test	Time (RM)	36.000	2.347	0.025	*	n = 37	1B	
			RM-ANOVA	Time (RM)	1, 36	4.090	0.051		n = 37		
				Tethered animal (RM)		24.252	0.000	***			
				Time x tethered		1.534	0.224				

Avg. distance from tethered partner	1	Distance when in chamber	48 hr; Paired t-test short term		14	-3.899	0.002	**	n = 15	1C	
			48 hr; Paired t-test long term		14	-3.727	0.002	**			
			2 wk; Paired t-test short term		10	-2.765	0.020	*	n = 11		
			2 wk; Paired t-test long term		10	-3.615	0.005	***			
			4 wk; Paired t-test short term		10	-3.367	0.007	***	n = 11		
			4 wk; Paired t-test long term		10	-2.334	0.042	*			
Distance	1	Pdistance minus Ndistance	RM-ANOVA	Time (RM)	1, 36	1.534	0.224		n = 37	not shown	
			RM-ANOVA	Time (RM)	1, 34	1.224	0.276				
				Group	2, 34	0.545	0.585				
				Time x group	2, 34	1.276	0.292				
Huddle time	2	48hr, 2nd partner; Partner huddle	RM-ANOVA	Time (RM)	1, 12	3.392	0.090		n = 13	2C	
Huddle time	2	48hr, 2nd partner; Novel huddle time	RM-ANOVA	Time (RM)	1, 12	2.106	0.172		n = 13		
Huddle time	2	2wk, 2nd partner; Partner huddle time	RM-ANOVA	Time (RM)	1, 9	2.754	0.131		n = 10	2F	
Huddle time	2	2wk, 2nd partner; Novel huddle time	RM-ANOVA	Time (RM)	1, 9	6.424	0.032	*	n = 10		
Huddle time	2	4wk, 2nd partner; Partner huddle time	RM-ANOVA	Time (RM)	1, 10	0.322	0.577		n = 11	2I	
Huddle time	2	4wk, 2nd partner; Novel huddle time	RM-ANOVA	Time (RM)	1, 10	0.343	0.571		n = 11		
Avg. distance from tethered partner	2	48hr: Distance when in chamber	RM-ANOVA	Time (RM)	1, 12	6.405	0.026	*	n = 13 - 15	2B	
				Tethered animal (RM)		5.007	0.045	*			
				Time x tethered		1.669	0.221				
			Paired t-test short term		14	-3.727	0.002	**			
			Paired t-test long term		12	-1.371	0.196				
Avg. distance from tethered partner	2	2wk: Distance when in chamber	RM-ANOVA	Time (RM)	1, 9	2.636	0.139		n = 10 - 11	2E	
				Tethered animal (RM)		8.734	0.016	*			
				Time x tethered		3.290	0.103				
			Paired t-test short term		10	-3.615	0.005	**			
			Paired t-test long term		9	-0.623	0.549				

Avg. distance from tethered partner	2	4 wk: Distance when in chamber	RM-ANOVA	Time (RM)	1, 10	1.655	0.227		n = 11	2H	
				Tethered animal (RM)		19.06	0.001				
				Time x tethered		1.872	0.201				
			Paired t-test short term		10	-2.334	0.042	*			
			Paired t-test long term		10	-4.1	0.002	**			
Partner preference	2	Proportion partner huddle time	RM-ANOVA	Time (RM)	1, 31	4.991	0.033	*		2A	
				Time x group	2, 31	3.263	0.052				
				Group	2, 31	0.342	0.712				
		48hr, short term	one way T-test	relative to 50%	14	2.584	0.022	*	n = 15		
		48hr, long term	one way T-test	relative to 50%	12	1.119	0.285		n = 13		
		48hr	Paired t-test short vs long term	Percent huddle	12	2.031	0.065				
		2 wk, short term	one way T-test	relative to 50%	10	3.833	0.003	**	n = 11	2B	
		2wk, long term	one way T-test	relative to 50%	9	0.194	0.850		n = 10		
		2 wk	Paired t-test short vs long term	Percent huddle	9	3.297	0.009	**			
		4wk, short term	one way T-test	relative to 50%	10	1.985	0.075		n = 11	2B	
		4 wk, long term	one way T-test	relative to 50%	10	2.901	0.016	*			
		4 wk	Paired t-test short vs long term	Percent huddle	10	-0.553	0.592				
		Short vs long term, partner 2	partner time	pearson, spearman		0.557, 0.593	0.001, 0.0003	**	n = 33	not shown	excludes 1872, 2262, 2267, 2245, no data for 1850, 1867, 1924
			partner huddle	pearson, spearman		0.660, 0.732	0.00003, 0.000001	**			
			percent phuddle	pearson, spearman		0.535, 0.560	0.001, 0.001	**			

			novel time	pearson, spearman		0.594, 0.520	0.000268, 0.002	**			
			novel huddle	pearson, spearman		0.614, 0.374	0.000147, 0.032	**			
Huddle time	1 vs 2	Partner 2 huddle time	ANOVA	Group	1, 30	1.06	0.359		48 hr = 14, 2 wk = 10, 4 wk = 9	4A	
		Parnter 1 huddle time	ANOVA	Group	1, 30	2.455	0.103				
Partner preference	1 vs 2	48 hr group	one way T-test	relative to 50%	13	-0.03	0.981		n = 14	not shown	
		2wk group	one way T-test	relative to 50%	9	0.871	0.406		n = 10		
		4wk group	one way T-test	relative to 50%	8	10.66	0.000	***	n = 9		
Avg. distance from tethered partner	1 vs 2	Distance when in chamber	RM-ANOVA	Group	2, 30	2.281	0.120		48 hr = 14, 2 wk = 10, 4 wk = 9	4B	
				Tethered animal (RM)	1, 30	17.062	0.000	***			
				Group x tethered	2, 30	6.480	0.005	**			
			48 hr: Paired t-test		13	-0.578	0.573				
			2 wk: Paired t-test		9	-1.064	0.315				
			4 wk: Paired t-test		8	-7.953	0.000	***			
Mating latency 1st partner - survival analysis	48 hr vs 2 wk vs 4wk	Latency to mate	Kaplan Meyer with Log Rank for overall comparison		Chi sq = 1.367	df = 2	0.505			S1A	
Mating latency 2nd partner - survival analysis	48 hr vs 2 wk vs 4wk	Latency to mate	Kaplan Meyer with Log Rank for overall comparison		Chi sq = 0.852	df = 2	0.653			S1:B	
Mating latency 1st vs 2nd partner	1st vs 2nd	Latency to mate	Kaplan Meyer with Log Rank for overall comparison		Chi sq = 0.565	df = 1	0.452			3C	
Proportion mated within 3 hours	1st vs 2nd		Fisher exact		F = 0.313		0.187			3A:A, B	
Mating latency 1st vs 2nd partner	Correlation	Latency to mate		pearson, spearman		0.193, 0.176	0.253, 0.296				

Avg. distance from tethered partner for early and late mating	1	Distance when in chamber	RM-ANOVA	Time (RM)	1, 35	0.220	0.642		Early mating = 23; Late mating = 14	not shown	
				Time x condition	1, 35	0.683	0.414				
				Condition (early vs late)	1, 35	0.029	0.866				
Avg. distance from tethered novel for early and late mating	1	Distance when in chamber	RM-ANOVA	Time (RM)	1, 35	3.818	0.059		Early mating = 23; Late mating = 14	not shown	
				Time x condition	1, 35	0.007	0.933				
				Condition (early vs late)	1, 35	0.507	0.481				
Avg. distance from tethered partner minus novel for	1	Distance when in chamber	RM-ANOVA	Time (RM)	1, 35	1.122	0.297		Early mating = 23; Late mating = 14	not shown	
				Time x condition	1, 35	0.287	0.595				
				Condition (early vs late)	1, 35	0.097	0.757				
Partner preference	1	Proportion partner huddle time	RM-ANOVA	Time (RM)	1, 35	1.21	0.279		Early mating = 23; Late mating = 14	3B:A, B	
				Time x condition	1, 35	0.458	0.503				
				Condition (early vs	1, 35	0.002	0.969				
		Early maters, short term	one way T-test	relative to 50%	22	1.752	0.094				
		Late maters, short term	one way T-test	relative to 50%	14	1.766	0.099				
		Early maters, long term	one way T-test	relative to 50%	22	4.13	0.000	**			
		Late maters, long term	one way T-test	relative to 50%	13	2.291	0.039	*			
Partner preference	2	Proportion partner huddle time	RM-ANOVA	Time (RM)	1, 32	4.533	0.041	*	Early = 16, Late = 18	3B:C, D	
				Time x condition		1.296	0.263				
				Condition (early vs		9.077	0.005	**			
		Early maters, short term	one way T-test	relative to 50%	17	10.3	..9*10^-9	****			
		Late maters, short term	one way T-test	relative to 50%	18	1.048	0.309				
		Early maters, long term	one way T-test	relative to 50%	15	3.832	0.002	**			
		Late maters, long term	one way T-test	relative to 50%	17	0.501	0.623				