Emergent intra-pair sex differences and organized behavior in pair bonded prairie voles (*Microtus ochrogaster*)

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Abstract

In pair bonding animals, coordinated behavior between partners is required for the pair to accomplish shared goals such as raising young. Despite this, experimental designs rarely assess the behavior of both partners within a bonded pair. Thus, we lack an understanding of the interdependent behavioral dynamics between partners that likely facilitate relationship success. To identify intra-pair behavioral correlates of pair bonding, we used socially monogamous prairie voles (*Microtus ochrogaster*) and tested both partners using social choice and non-choice tests at short- and long-term pairing timepoints. Females developed a preference for their partner more rapidly than males, with preference driven by different behaviors in each sex. Further, as bonds matured, intra-pair behavioral sex differences and organized behavior emerged—females consistently huddled more with their partner than males did regardless of overall intra-pair affiliation levels. When animals were allowed to freely interact with a partner or a novel vole in sequential free interaction tests, pairs spent more time interacting together than either animal did with a novel vole, consistent with partner preference in the more commonly employed choice test. Total pair interaction in freely moving voles was correlated with female, but not male, behavior. Via a social operant paradigm, we found that pair-bonded females, but not males, are more motivated to access and huddle with their partner than a novel vole. Together, our data indicate that as pair bonds mature, sex differences and organized behavior emerge within pairs, and that these intra-pair behavioral changes are likely organized and driven by the female animal.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

1 | INTRODUCTION

Interpersonal relationships require social cooperation to achieve shared goals, such as in socially monogamous pair bonds where two individuals share resources and offspring care. Because of these shared responsibilities and the lack of ongoing mate selection, monogamous species are often thought to exhibit fewer sex differences.[1,2] However, there are well documented examples of behavioral sex differences in monogamous species[3–6] which, unlike those observed in non-monogamous species, may emerge after a pair bond has formed to facilitate intra-pair cooperation and ensure reproductive success.

Among monogamous prairie voles (*Microtus ochrogaster*), there are sex differences in parental care.[7,8] Females and males exhibit similar parental behaviors, but they display these behaviors to different degrees across pup development and across subsequent litters.[8–10] By "trading off" duties, prairie vole parents can provide more active care for their pups, which promotes the pups' physiological and behavioral development.[11–13] However, whether reliable intra-pair sex differences and organized partner-directed behavior emerge as a function of relationship formation and maturation remains unexamined, especially as the vast majority of studies focus on only one member of a pair.

In addition to biparental care, prairie vole pair bonds are hallmarked by an affiliative partner preference that develops more rapidly in females than in males.[3,14] Here, we characterized the social behavior of both members of bonded pairs at short-term (2 days) and long-term (2 weeks) timepoints post-pairing. We employed complementary choice and non-choice social tests. The former test, which entails tethering of a partner and a novel vole at opposite ends of an arena while allowing the test animal to freely explore, has been used to infer partner preference as a proxy for pair bonding since its development and implementation nearly three decades ago,[15] while the latter test provides a more ethologically relevant assessment of pair behavior as neither vole's movement is hindered by tethering. We show that organization of intra-pair affiliative behavior emerges as a function of bond maturation, with distinct changes occurring in each sex, and that female, but not male behavior correlates with pair behavior across choice and non-choice assays.

While partner-directed affiliation is the gold standard for determining whether a pair bond has formed, these tests do not separate the appetitive and consummatory aspects of partner and novel interaction. To deepen our understanding of the underlying behavioral mechanisms that drive sex differences in pair bond behavior, we tested partner- and novel-directed social motivation in pair bonded voles using operant tasks in which voles press a lever to gain transient access to their partner. In accordance with prior reports, we found that females exhibited greater partner-directed motivation than males.[16,17] Together, this work has important implications for deepening our understanding of social behaviors

by uncovering behavioral mechanisms that reinforce pair bonds and delineating the interdependent dynamics between partners that facilitate relationship success.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult prairie voles were bred in-house in a colony descended from wild animals collected in Illinois. After weaning at 21 days, animals were housed in same-sex groups of 2−4 animals in standard static rodent cages (19 l × 10.5 w × 5 in. h) with ad-libitum access to water and rabbit chow (5326–3 by PMI Lab Diet). Diet was supplemented with sunflower seeds and alfalfa cubes, and cotton nestlets and plastic houses were given for enrichment. All voles were between 58 and 90 days of age at the start of the experiment. Beginning on day one, female/male pairs were co-housed in smaller static rodent cages (11.0 l × 8.0 w × 6.5 h. in.) with ad-libitum access to water and rodent chow, as well as cotton nestlets and houses for enrichment. Animals were kept at 23−26°C with a 14:10 light: dark cycle. All procedures were performed during the light phase and approved by the University of Colorado Institutional Animal Care and Use Committee.

2.2 | Timeline

Experimental timeline shown in Figure 1A was carried out for 16 female/male vole pairs. Briefly, baseline tests (day 0) consisted of two free interaction tests: one with the animal they would subsequently be paired with ("partner"), and one with an animal they would not be paired with ("novel"). After all 30-min free interaction tests were complete, animals were co-housed with their randomly pre-selected partner for the duration of the experiment. At the short-term timepoint 2 days post-pairing, we performed partner preference tests (PPTs)[15] sequentially for both animals within each pair followed by free interaction tests 1 day later (day 3). The pairs continued to cohabitate and were tested at a long-term timepoint via sequential PPTs on day 14 and free interaction tests on day 15. Each animal was tested with a different novel animal for each test, ensuring that the animals never saw the same novel animal twice. At 16 days post-pairing, animals were sacrificed to weigh the uterus and count embryos. Across all tests, test order for female and male was randomized, and for free interaction tests, the order of partner or novel presentation was randomized to account for potential order effects.

2.3 | Free interaction

Free interaction tests were performed in clear rectangular plexiglass arenas 50.7 cm long, 20.0 cm wide, and 30.0 cm tall. For each test, experimental animals were paired either with their partner or a novel opposite-sex animal, order randomized. All animals had an inter-trial interval of 30−90 min. Animals were individually placed on opposite sides of the chamber separated by an opaque divider. At the start of the test, the divider was removed and both animals were allowed to freely move about the chamber for 30 min. Overhead cameras (Logitech C925e webcam) were used to record four free interaction tests simultaneously.

Periods of social interaction between the two animals were scored post hoc using TopScan High-Throughput software v3.0 (Cleversys Inc). We adapted and optimized scoring methods

from Ahern et al.[18] and defined social contact by setting the "joint motion" parameter to <5. To confirm the accuracy of the TopScan software, two pairs were hand-scored using BORIS[19] for the following behaviors: interacting, affiliative behavior, neutral behavior, and aggressive behavior. Compared to the amount of interacting time scored by hand, the TopScan-scored interacting time differed by less than 6% in both videos.

2.4 | Partner preference test

Partner preference tests were performed as described in Scribner et al. 2020.[20] Briefly, both partner and novel animals were tethered to the end walls of three-chamber plexiglass arenas (76.0 cm long, 20.0 cm wide, and 30.0 cm tall). Tethers consisted of an eye bolt attached to a chain of fishing swivels that slid into the arena wall. Animals were briefly anesthetized with isoflurane and attached to the tether using a zip tie around the animal's neck. Two pellets of rabbit chow were given to each tethered animal and water bottles were secured to the wall within their access while tethered. After tethering the partner and novel animals, experimental animals were placed in the center chamber of the arena. At the start of the test, the opaque dividers between the chambers were removed, allowing the experimental animal to move freely about the arena for 3 h. Overhead cameras (Panasonic WVCP304) were used to video record eight tests simultaneously.

The movement of all three animals in each test was scored using TopScan software using the parameters from Ahern et al.[18] Behavior was analyzed using a Python script developed in-house (https://github.com/donaldsonlab/Cleversys_scripts) to calculate the following metrics: time spent in partner/novel chamber, time spent huddling with partner/ novel, average distance to partner/novel while in the respective chamber, latency to huddle with partner/novel, and total locomotion. The partner preference score was calculated as (partner huddle time/[partner huddle time + novel huddle time]) × 100%.

2.5 | Assessment of pregnancy status

Following the final free interaction test, female animals were sacrificed to weigh the uterus and to measure embryo head-to-rump length. Animals were euthanized using CO_2 and decapitation. Uteri were then dissected and weighed. From each uterus, embryos were counted and one embryo was removed to measure head-to-rump length.

2.6 | Statistical analyses

Data were analyzed using the SciPy Stats package[21] (version 1.7.0) and Pingouin package[22] (version 0.3.12) in Python (version 3.8.10) and the lme4 package[23] (version 1.1−23) and emmeans package (version 1.6.3) in R. Details of all statistical tests can be found in Table S1. To determine the statistical significance of the partner preference score (i.e., whether a partner preference was formed), we used a one-sample t-test comparing to a value of 50% (no preference for partner/ novel). To assess the intra-pair effects of sex and the within pair effects of time, we used linear mixed models with pair ID as a random term. Because the females and males are intrinsically paired, and within a pair, female and male behavior are not independent, we performed pairwise contrasts of estimated marginal means[24] with Bonferroni correction for our post hoc comparisons. For analysis of the PPT partner preference scores, we used repeated measures ANOVA (RM-ANOVA) with Wilcoxon rank

sum tests for our post hoc comparisons because the scores are not normally distributed. For our correlation analyses, we calculated all of our correlations using Spearman's Rho to avoid assumptions of linearity and account for order effects, neither of which are possible using the more traditional Pearson's R. Throughout the paper, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$. Asterisks in figures provided for significant post hoc tests only with main and interacting effects provided in figure legends and Table S1. In the correlation matrices, Rho values with associated p values <0.05 are colored.

2.7 | Open source custom operant chamber

Operant chambers contained 3 chambers separated by 2 motorized doors, 3 separate retractable levers (one for each type of reward), and one motorized pellet dispenser and trough (Figure 4A). Chambers were constructed from a mix of laser cut acrylic and 3D printed ABS plastic. A bill of materials and chamber designs can be accessed at https://github.com/donaldsonlab/Operant-Cage/tree/main/V2.

The box was controlled via custom scripts and code (https://github.com/dprotter/RPi_Operant) run on Raspberry Pi computers (Raspberry Pi Foundation). Servos were controlled via an Adafruit HAT (Adafruit 2327). Each chamber was controlled by a corresponding Raspberry Pi. Food rewards were 20 mg pellets (Dustless Precision Pellets Rodent Grain-Based Diet; VWR 89067−546) delivered to a trough. Pellet dispensal and retrieval was detected by an IR beam break in the trough. Tones were generated via PWM on the Raspberry Pi (pigpio), and played through an amplified speaker (Adafruit 3885).

2.8 | Operant timeline

Animals ($n = 12$, 6 M, 6F) were trained using in-house constructed operant chambers to perform a social choice operant task. Partners for test animals were sterilized either by tubal ligation or vasectomy (as described in Donaldson et al.[25] and Harbert et al.[26]) at least 2 weeks prior to pairing. Test animals were paired and cohabitated for 18 days before the start of operant training. Animals underwent 3 days of magazine training, 1 day of partner preference test, 4 days of food training, 5 days of social training (i.e. social non-choice), and 5 days of social choice testing. Animals were not trained or tested on weekends. Novel stimulus animals were rotated to minimize potential familiarity. All sterilized partners were used as novel stimuli, along with 5 additional unpaired, intact males and 3 unpaired, intact females.

2.9 | Magazine training

Animals underwent 15 trials per day, the goal of which was to learn associations between the lever, tone, and food reward. For each trial, a tone was played to indicate the start of the trial (5000 Hz, 1 s). The food lever was then extended for 2 s, a pellet cue (2500 Hz, 1 s) was played, and a pellet was delivered to the trough. The lever was retracted 2 s later. If an animal pressed within the first 2 s of lever access, it immediately triggered pellet delivery. No more than 1 pellet was delivered per trial. Total trial time was 90 s.

2.10 | Food training

Animals underwent 15 trials per day in which pellets were dispensed on every trial after 30 s of lever presentation, but lever pressing elicited an immediate reward. During each trial, a tone was played to indicate the start of the trial (5000 Hz, 1 s). The food lever was then extended for 30 s. After 30 s, the lever was retracted, a pellet cue (2500 Hz, 1 s) was played, and a pellet delivered to a trough. If the animal pressed the lever prior to the end of the 30 s extension period, the lever was immediately retracted, the pellet cue was played, and a pellet was immediately dispensed. In order to provide a window to observe anticipatory behavior, animals experienced a delay between lever pressing and reward as follows: (day 1: no delay, day 2: no delay, day 3: 1 s, day 4: 1 s). Total trial time was 90 s.

2.11 | Social training/social non-choice

Animals underwent 20 trials of social training per day, the design of which mirrored food training but where the reward was social access. They were administered alternating sets of five trials for each door, starting with the partner door (5 partner, 5 novel, 5 partner, 5 novel). The partner and novel stimulus animals were tethered at opposite ends of the apparatus and farthest from the doors in a similar fashion to the PPT. The tethering location of partner and novel stimulus remained consistent across days. On each trial, a tone was played to indicate the start of the trial (5000 Hz, 1 s). The corresponding social lever (the closest lever to the corresponding animal) was extended for 30 s. After 30 s, the lever was retracted, a door-opening cue was played (10,000 Hz, 1 s), and the corresponding door opened. If the lever was pressed prior to the end of the 30 s extension, the lever was retracted, the door-opening cue was played and the door opened immediately. At the end of the trial, a door close tone was played (7000 Hz, 1 s) and the door was closed. Total trial time was 110 s, with 20 s allocated for researchers to return the test animal to the central chamber in between trials, if necessary. Therefore, all animals always received a minimum of 60 s of partner or novel access on a trial, but animals that pressed more quickly received longer access. Delays between pressing and door opening were as follows: (day 1: no delay, day 2: no delay, day 3: 1 s, day 4: 1 s, day 5: 2 s).

2.12 | Social choice

Animals underwent 30 trials of social testing per day. The location of the partner and novel animals was kept the same as in social non-choice. On each trial, a tone was played to indicate the start of the trial (5000 Hz, 1 s). Both social levers were extended for 30 s. One lever press per trial was allowed, making social access mutually exclusive on each trial. If a lever was pressed, both levers were retracted, the door-opening cue was played (10,000 Hz, 1 s) and the corresponding door opened after a 1 s delay. If no lever was pressed within 30s, both levers were retracted and no door was opened. At the end of successful lever pressing trials, a door close tone was played (7000 Hz, 1 s) and the door was closed. Total trial time was 110 s, with 20 s allocated for researchers to return the test animal to the central chamber in between trials, if necessary. Therefore, during successful trials, animals that pressed more quickly received more social access, with a minimum of 60 s social access.

3 | RESULTS

We tested both members of bonded pairs in the partner preference test (PPT) and the free interaction test, at short- and long-term pairing timepoints (Figure 1A), enabling us to identify consistent intra-pair sex differences that emerge as a function of bond maturation and examine how pair bonds develop over time. With the exception of partner preference score, all PPT and free interaction metrics were analyzed in a pairwise fashion using linear mixed models with pair ID as a random term. p-values reported below represent post hoc pairwise contrasts of estimated marginal means[24] with Bonferroni correction with all additional statistics available in Table S1. To assess the presence of a partner preference for each sex at each timepoint, preference scores were compared to an expected null value of 50% (no preference) using one-sample t-test. Partner preference sex and/or timepoint differences were assessed via repeated measures ANOVA (RM-ANOVA) with Wilcoxon rank sum post hoc tests, as the scores are not linearly distributed. All operant metrics were analyzed using RM-ANOVA with post hoc paired t-tests with Bonferroni correction.

3.1 | Consistent intra-pair sex differences in PPT behavior emerge as a function of bond maturation

We first examined social behavior metrics in the classic partner preference test in both members of pairs following short-term (2 days) and long-term (2 weeks) cohabitation (Figure 1A). We calculated a partner preference score (partner huddle/[partner + novel huddle]) to determine whether pair bonds had formed. Compared to a null value of 50%, we found that at the short-term timepoint, only females display a partner preference (females: $p = 0.0049$, males: $p = 0.28$), but both females and males have a partner preference at the long-term timepoint (Figures 1C and S1A, females: $p = 3.3 \times 10^{-11}$, males: $p = 2.1 \times 10^{-5}$). Further, there is an increase in partner preference score between the short- and long-term timepoints for males ($p = 0.044$), but not females ($p = 0.42$). This is consistent with prior data indicating that males take longer than females to establish a partner preference.[3,14]

We next asked what specific behaviors within the PPT contribute to our observed sex difference in the emergence of partner preference. When looking at raw huddle time, we saw that females, but not males, increase their partner huddle time as they transition from short- to long-term timepoints (Figures 1D and S1B, females: $p = 4.0 \times 10^{-4}$, males: $p = 0.43$). Conversely, there was no change in novel-directed huddle between timepoints for females or males (females: $p = 0.29$, males: $p = 0.17$, Figure 1G). Accordingly, we can conclude that the formation and strengthening of partner preference over time occurs via different behavioral processes in females and males. Females increase partner-directed huddle even after a partner preference has already developed with no associated decrease in novel-directed huddle. Conversely, emergence of partner preference in males at the long-term timepoint is via more complex behavioral changes which reflect reorganization of partner and novel huddle at the level of individual behavior rather than an exclusive change in partner- or novel-directed huddle evident at the group level.

We followed our longitudinal analysis of male and female behavior by asking whether there were intra-pair sex differences within either timepoint. There was no difference in partner preference score between females and males at the short-term timepoint (Figure 1C, $p =$

0.30), but by the long-term timepoint, females had higher preference scores than males (Figure 1C, $p = 0.037$). When we examined individual partner preference behaviors, we found that, at the group level, females and males huddled with their partner comparable amounts at the short-term timepoint ($p = 0.41$). Further, there was no consistent trend regarding which member of a pair huddled more with their partner; in 10 pairs, the female huddled more and in six pairs, the male huddled more (Figure 1D). However, at the long-term timepoint, females consistently huddled more than their male partner (F > M in 15 of 16 pairs; $p = 3.8 \times 10^{-4}$). In the one pair that the male huddled more than the female, the difference in huddle times was negligible (less than 1% of the average huddle time for that pair; Figure 1D). In contrast, there was no difference between females and males in novel-directed huddle (Figure 1G). Together, these data demonstrate that intra-pair sex differences in partner-directed, but not novel-directed, huddling emerge as bonds mature.

We next examined the time spent in partner and novel chambers when the test animal was not huddling (chamber time - huddle time). There was no main effect of sex in the amount of non-huddle time in the partner chamber (Figure 1I). However, at both timepoints, males spent more non-huddle time in the novel chamber than did females (Figure 1J, short-term: $p = 4.6 \times 10^{-4}$, long-term: $p = 1.3 \times 10^{-4}$). While these behaviors differed by sex within pairs, there was no main effect of pairing time on within-sex behavior (Figure S1E). Thus, these sex differences likely either reflect innate female/male differences or emerge extremely rapidly after pairing. Finally, there were no effects of sex or timepoint in the amount of time spent in the center chamber (Figure 1K) or in locomotion (Table S1).

3.2 | Emergent organization of intra-pair behavior

We next examined the relationship between female and male behavior within pairs. We found that at the long-term, but not short-term timepoint, female partner huddle and male partner huddle were correlated (Figure 1D, E, short-term: Rho = 0.17, $p = 0.53$, long-term: Rho = 0.53, $p = 0.035$). To further explore how this correlation emerges over time, we calculated the change in partner huddle between the short- and long-term timepoints for each animal. This enabled us to ask whether partners are changing their behavior in the same way or to the same degree between short and long-term timepoints. The change in partner huddle was not correlated between males and females (Figures 1F, H, S1B, Rho = 0.097, $p = 0.72$), indicating that the emergence of correlated female:male partner huddle at the long-term timepoint is not due to the same changes occurring in the male and the female of a given pair. Instead, by the long-term timepoint, pairs in which the female spends more time huddling with her partner are the same pairs in which the male spends more time huddling with his partner, suggesting a simpler organizational mechanism in which males may be matching their behavior to that of their female partner, as female partner huddle consistently increases between timepoints while male partner huddle does not.

We next calculated a pairwise measure of affiliation via "total pair huddle" (female partner huddle + male partner huddle) at each time-point. We found that how much a female changes her partner huddle between short- and long-term timepoints huddle was inversely correlated with the total pair huddle at the short-term timepoint (Rho = −0.73, $p = 0.0013$) but not the long-term timepoint (Rho = 0.19, $p = 0.48$, Figure 1G, H). Conversely, male

changes in partner-directed huddle over time were positively correlated with pair huddle at the long-term timepoint (Rho = 0.78, $p = 4.0 \times 10^{-4}$) but not the short-term timepoint (Rho = −0.17, $p = 0.53$, Figure 1F, H).

One interpretation of this data is that pairs in which high total huddle times are already established by the short-term timepoint, females change their behavior least and that total pair huddle at the long-term timepoint is predominately driven by the amount the male increases his partner-directed huddling. This mirrors our partner preference data in which female partner-directed huddling levels are already established by the short-term timepoint, while males increase partner-directed huddling as the bond matures.

Given the striking and consistent pattern of a F > M huddle pattern at the long-term timepoint, we asked whether the ratio of F:M huddle was consistent across pairs and/or was predicted by overall affiliation levels (i.e., total pair huddle). At the short-term timepoint, reflecting a lack of consistent sex difference in partner huddle, we did not observe any consistent F:M ratio or any relationship with total pair huddle. However, at the long-term timepoint, a striking pattern emerged in which pairs with the highest total pair huddle also had a female:male partner huddle ratio between 1.25 and 1.75 (Figure S1G, $\log_2$[female/male] between 0.32 and 0.81), suggesting that the most affiliative pairs are also those in which partners reliably and precisely match their behavior to that of their partner. Together, these analyses demonstrate that intra-pair affiliative behavior becomes organized as bonds mature.

3.3 | Affiliative behavior as a function of pregnancy status

Previous work suggests that pregnancy status can influence bond-related behaviors.[27] Thus, we aimed to uncover any correlations between pregnancy status and behavior. Nearly all pairs (15 of 16) became pregnant during the 2 weeks of pairing. At 16 days post-pairing, females were sacrificed and uteri were weighed. We found that uterine weight was positively correlated with female partner huddle time (Rho = 0.54, $p = 0.031$), but not male partner huddle time (Rho = 0.14, $p = 0.61$), at the long-term PPT (Figure 1E). While raw male partner huddle time was not correlated with uterine weight, male partner huddle time was correlated with female partner huddle time at the long-term timepoint (Rho = 0.53, $p = 0.035$). Interestingly, the only pair that did not become pregnant over the course of our experiment is the same pair that showed no sex difference in partner huddle time in the long-term PPT. Together, this suggests that pregnancy status may alter female behavior which, in turn, may drive changes in male behavior.

3.4 | Non-choice free interaction tests reflect partner preference and dyadic behavior

While PPT provides a valuable means to assess an individual's behavior in the context of social choice, social interactions in the wild are not independently constrained to the actions of one individual. Thus, we also performed non-choice sequential free interaction tests, where we placed each animal, untethered, in a chamber with their partner or a novel (randomly ordered), allowing them to freely interact for 30 min. Free interaction tests were performed upon the animals' initial introduction (baseline), and then again the day after short- and long-term PPTs (Figure 2A, B). In this free interaction test, huddling was

qualitatively much less common than in the PPT, which may reflect the shorter duration of the test, limitations placed on social behavior due to tethering, and/or huddling as a form of consolation in prairie voles.[28,29] In addition, as there was no consistent way to parse the direction of interaction (e.g., male to female directed or vice versa), we scored total interaction time for each dyad (pair or each partner + novel).

We found that at the group level, there are no differences in pair interaction time across timepoints (Figure 2C). However, while the majority of pairs (10 of 16) show modest increases in interaction between the baseline and long-term timepoints, six pairs decrease their interaction between these timepoints. Strikingly, four out of the six pairs that decrease are the same four pairs that exhibit notably higher levels of interaction than other pairs at the baseline timepoint. Despite this within-pair decrease, three of these pairs remain those with the highest interaction times at the long-term timepoint. Further, the percent change between the baseline and long-term timepoints is strongly correlated with the amount of time spent interacting in the baseline test (Spearman's Rho = -0.90, $p = 1.7 \times 10^{-6}$). Thus, although there is notable behavioral diversity between pairs, this demonstrates that within-pair behavior may change, but the pair's behavior relative to other pairs remains consistent over time. We next asked whether partner preference was evident in our free interaction paradigm by comparing the amount of interaction time with the partner and with the novel. At the short-term timepoint, males, but not females, spent more time interacting with their partner than the novel animal (females: $p = 0.12$, males: $p = 0.029$, Figure 2E). By the long-term timepoint, both females and males spent more time with their partner than a novel (females: $p = 0.0042$, males: $p = 0.015$, Figure 2E). There were no differences between female and male novel-directed huddle ($p > 0.99$). We further calculated a free interaction partner preference score (pair interaction/[pair interaction + novel interaction]) for each animal (Figure 2D). In this paradigm, both females and males show a partner preference by the short-term timepoint (females: $p = 1.9 \times 10^{-5}$, males: $p = 1.0 \times 10^{-3}$), which is maintained at the long-term timepoint (females: $p = 1.3 \times 10^{-4}$, males: $p = 8.0 \times 10^{-5}$). Compared to the PPT, this test did not reveal the same sex differences related to the strengthening of bonds over time, which may be partly due to the inability of this test to isolate behavior of one member of an interaction dyad.

We then asked whether there were any correlations between pair and novel free interactions to delineate which behavioral features correspond with bonding and which may reflect individual or sex-based differences in non-discriminate sociality. We used Spearman's Rho to calculate correlation coefficients to avoid assumptions of linearity and account for order effects within the data, which is important for addressing behavioral consistency (e.g., do the pairs that spend the most time interacting at short term also do so at long term?). Only 3 of 36 potential correlations met an unadjusted significance threshold of $p < 0.05$ as indicated by the colored boxes in Figure 2F. Specifically, we found that, at baseline, female interaction with their future partner or with the "novel" male was positively correlated (Rho = 0.55, $p = 0.027$), suggesting that some females may simply be more social than others, regardless of male interaction partner (Figure 2F). Notably, this was not true for males. Similarly, we found that female novel social interaction is positively correlated between baseline and short-term timepoints (Rho = 0.65, $p = 0.0061$), but neither baseline nor short-term is correlated with the long-term time-point, indicating that this general sociality erodes as

pair bonds mature (Figure 2F). We also found that partner social interaction is correlated only between short-term and long-term timepoints (Rho = 0.73, p = 0.0012, Figure 2F). This demonstrates enhanced intra-pair consistency over time with some pairs showing more interaction than others.

3.5 | Female behavior converges as bonds mature and correlates with pair behavior

To reduce dimensionality and further explore sex-associated patterns within our PPT data, we performed a principal component analysis (PCA) on partner huddle time, novel huddle time, partner non-huddle, novel non-huddle, center chamber time, average distance to partner while in partner chamber, average distance to novel while in novel chamber, and total distance traveled. Upon plotting the first three components, we found that at the short-term timepoint, female and male points largely overlap, and neither sex clustered together nor apart from the other sex (Figure 3A). However, by the long-term time-point, females clustered together and apart from males, while males remain relatively dispersed (Figure 3B). This suggests that female behavior converges as a function of pair bond maturation while males retain larger individual differences.

To determine which PPT metrics were driving each principal component, we performed a factor extraction, focusing on factors with a loading value >0.3, indicating that 30% of the variance in that variable is explained by the principal component. At both timepoints, there is notable consistency in the specific behavioral factors that drive each principal component (Figure 3A, B). Specifically, PC1 is driven by partner and novel huddle time, novel non-huddle, and average within-chamber distance to the novel, with addition of partner non-huddle at the long-term timepoint. At both timepoints, PC2 is driven by partner huddle time, partner non-huddle, average within-chamber distance to partner, total distance traveled, and center chamber time. Finally, PC3 is driven by partner non-huddle, novel non-huddle, average within-chamber distance to novel, and center chamber time. At the long-term timepoint, PC3 is driven by partner non-huddle, total distance traveled, and center chamber time. Altogether, the first and second principal components broadly represent novel versus partner-directed behaviors, respectively, and this remains consistent over time.

We next compared behavior across choice and non-choice interaction tests. We calculated and included metrics that are likely to represent similar behavioral components across tests. Specifically, we reasoned that interaction in the free interaction test was conceptually similar to the time an animal chose to interact with a tethered vole when it was near that animal. Thus, we calculated the huddle ratio–the percent time in the partner or novel chamber spent huddling (i.e., huddle time/chamber time). In addition, we calculated the within-pair Euclidean distance from the PCA of PPTs at each time point as a comprehensive indicator of within-pair similarity across multiple PPT metrics, with a greater distance between partners representing more disparate behavior (Figure 3A–C).

We found that all PPT and free interaction metrics were uncorrelated at the short-term timepoint. However, correlations between these test metrics emerged by the long-term timepoint, suggesting a stabilization of a pair's behavioral structure that emerges as a function of bond maturation. We found that at the long-term timepoint, pair interaction in the free interaction test is correlated with female partner huddle ratio, indicating that

females that interacted more with their partner in the free interaction test also preferred to huddle when in proximity to their partner in the PPT (Rho = 0.60, p = 0.013, Figure 3D). Additionally, female novel interaction in the free interaction test is positively correlated with the Euclidean distance between partners (Rho = 0.56, p = 0.023, Figure 3D). In other words, among pairs with lower intra-pair behavioral similarity in the PPT (a larger Euclidean distance), the female spends more time interacting with the novel in the free interaction test.

In addition to within-timepoint correlations, we also found that aspects of PPT behavior at the long-term timepoint correlated with a subset of metrics in the free interaction test at the short-term time-point. This may suggest that even at the earlier bonding timepoints some behaviors are beginning to stabilize and are predictive of future behavior. The partner interaction time at the short-term timepoint was negatively correlated with the Euclidean distance between partners at the long-term timepoint (Rho = −0.59, p = 0.015, Figure 3D); the more time the pair spent together in the short-term free interaction test, the smaller the Euclidean distance between partners at the long-term timepoint. Partner interaction at the short-term timepoint was also correlated with female partner huddle ratio in the long-term PPT (Rho = 0.70, p = 0.0027, Figure 3D). Unlike females, male PPT partner behavior does not correlate with pair free interaction behavior. However, male free interaction with the novel at the short-term timepoint weakly correlates with male novel huddle time in the long-term PPT (Rho = 0.49, p = 0.055, Figure 3D). Taken together, our data suggest that female behavior can predict pair behavior specifically, while male behavior does not.

3.6 | Females display greater partner-directed motivation than males

To determine if sex differences observed in PPT and free interaction behavior may be partially explained by differences in selective social motivation, we trained 6 female and 6 male prairie voles to press for social access. To confirm that voles had bonded with their sterilized mates, we performed a three-hour PPT. As previously observed (Figure 1C), male voles displayed more variability in their partner preference scores, with 2/6 males displaying a novel preference, and 3/6 displaying scores greater than 80%. In contrast, all females displayed preference scores greater than 80% (Figure 4C). While not statistically significant (p = 0.069), females displayed greater partner huddle times than males, consistent with earlier observations (e.g., Figure 1D, long-term).

Prairie voles rapidly learned to press for food pellets in our training paradigm, with most animals pressing on more than 50% of trials after 3 days of magazine training and 4 days of food training (males: 4/6, females: 5/6). Additionally, across training days both male and female latency to lever press decreased (Figure S2A, main effect of day, p = 0.0021) and the percentage of trials in which they successfully lever-pressed increased (Figure S2B, main effect of day, p = 0.017), two indicators that the animals learned the task.

Following food training, voles were trained to press for social access via a similar paradigm. This was a non-choice social task, in which the animal was presented with either the partner lever or the novel lever on any given trial. Of note, in this task, animals were always given the access to the corresponding stimulus animal, but could gain access more quickly and for a longer total duration by pressing the lever. Similar to food training, animals pressed the lever in a greater percentage of trials after the first day (Figure S2C, main effect of day, p =

0.0091), indicating they learned the social non-choice task consistent with prior reports.[30,31] Interestingly, even though most animals display a preference in the PPT, we did not observe a preference in pressing when presented with only one lever at a time (Figure 4F).

To more directly ask if prairie voles displayed differential motivation to access their partner versus a novel vole, we then performed a social choice test. We extended both social levers simultaneously such that the test animal was faced with a mutually exclusive choice to gain access to the partner or the novel vole. Females learned this task by the 5th day of testing, which was demonstrated by correctly orienting towards the selected door prior to the door opening. (Figure S2E,F, one sample t-test vs. 50%, $p = 3.1 \times 10^{-4}$), Male orienting behavior, as a group, was not better than chance ($p = 0.074$), although this was primarily driven by one individual (Figure S2F). When removed, the remaining five males correctly oriented more frequently than chance ($p = 0.0065$ excluding one outlier vole).

We found that in the social choice test, female voles developed a significant preference for the partner lever (one sample t-test vs. 50%, day 4: $p = 0.014$, day 5: $p = 0.047$). Conversely, male vole lever-pressing did not indicate a partner or a novel preference, and on day 5 only 1/6 males pressed more for their partner (Figure 4G, day 5: $p = 0.45$). This observation in female voles contrasts with the lack of preference observed in the non-choice training phase (Figure 4F). As selective social motivation likely impacts preference in the PPT, we compared PPT preference scores to operant choice pressing preference (Figure S2D) and found that males tended to show a weaker preference than females for both metrics.

Finally, we asked if males and females showed different behaviors after pressing to gain social access. Although we did not observe any statistically significant differences between males and females, their post-pressing behavior trends match those observed in pairs in the PPT (Figure 1), with a trend for females to huddle more with their partners ($p = 0.10$) and a trend for males to spend more non-huddling time in the novel chamber (Figure S2G, $p = 0.088$). Males and females displayed similar levels of aggression, with greater aggression directed towards the novel than the partner, although this did not reach threshold for significance (Figure S2H, main effect of interaction partner, $p = 0.13$).

4 | DISCUSSION

The vast majority of studies examining sex differences in prairie voles do so in the context of parenting behavior. While important, this leaves us with a relative lack of understanding of behavioral sex differences that may be critical to forming and maintaining a bond pre-parenting. Critical work performed nearly three decades ago demonstrated that partner preferences develop more rapidly post-pairing in females than males, which has since been replicated.[3,14] However, whether sex differences are seen within individual pairs, and whether these contribute to the organization of intra-pair behavior as bonds mature, has remained unexplored.

4.1 | Choice and non-choice social tests reveal female-driven intra-pair organization

Here, we provide the first intra-pair comparisons of affiliative pair bonding behavior in prairie voles. We tested both members of female/male pairs in PPTs and in free interaction

tests at short- and long-term pairing timepoints. We first found that pair bonds mature over time via different mechanisms in females and males, with only females increasing their partner affiliative behavior over time. In males, partner preference arises only by the long-term timepoint, pre-sumably due to within-individual changes in partner and novel huddle. In addition, we documented an emerging organization of intra-pair behavior as bonds matured. Most prominently, males huddle less than their female partner, most commonly leading to a female:male huddle ratio between 1.25 and 1.75. How a given pair achieves this reliable affiliation ratio is not uniform; the direction of change in partner huddle for the male and female is not consistent across pairs.

We next tested prairie vole pairs in sequential free interaction tests, a non-choice paradigm in which the experimental animal has the option to either interact with their untethered partner or a novel in the absence of the other. Despite being a non-choice test, the free interaction test recapitulated results from the PPT, with animals choosing to spend more time interacting with their partner than a novel after pairing. Unlike the PPT, the free interaction test uniquely allowed us to test dyadic pair bonding behavior—behavior resulting from the actions of both partners at the same time—rather than isolating the partner-directed behavior of one animal as in the PPT.

When we compared individual partner-directed behavior in the PPT and dyadic pair behavior during free interaction, we found that female partner-directed behavior correlates with pair behavior, while male behavior does not. Once in proximity to their partner in the PPT (e.g., in the partner chamber), the experimental animal has the choice to either interact with their partner or not, which is analogous to the choice to interact with a non-tethered animal in the free interaction test. Thus, we calculated the partner huddle ratio (partner huddle time/partner chamber time) from the PPT and found that female partner huddle ratio at the long-term timepoint is correlated with pair free-interaction at both the short- and long-term timepoints. To further compare our two tests, we used PCA to reduce the dimensions of our PPT data and then calculated the Euclidean distance between partners within the same pair. Interestingly, we found that the Euclidean distance between partners at the long-term timepoint was inversely correlated with partner interaction at the short-term timepoint. As a larger Euclidean distance between partners represents more disparate behavior in the PPT, our data indicate that less pair interaction at the short-term timepoint predicts more dissimilar behavior at the long-term timepoint. In addition, Euclidean pair distance is positively correlated with female + novel free interaction at the long-term timepoint indicating that in the pairs with more dissimilar behavior in the PPT, the female spends more time interacting with the novel in the free interaction. Across the PPT to free interaction comparisons, female behavior correlated with pair behavior while male behavior did not. Together, this suggests that female behavior is a primary driver of pair behavior and therefore behavioral organization.

We employed both choice and non-choice tests, one benefit of which was to assess the reliability of behavior across tasks that differ in their ethological relevance. While this proved useful for identifying important sex differences, overall, few behavioral metrics were significantly correlated across choice and non-choice contexts. This indicates that conceptually related tasks may not be measuring the same social behavior. Recent work

in bats has also shown the ethological relevance of a given behavioral task (e.g., trained vs. natural bat calls) recruits different neuronal responses.[32] Together, these findings have important implications for how we study social behavior and the biological assumptions derived from more and less ethologically relevant paradigms.

4.2 | Emergent sex differences serve a function other than mate choice

Sex differences are thought to exist primarily for two intertwined purposes: mate choice and reproduction.[33–35] Compared to non-monogamous species, monogamous species typically exhibit fewer sex differences,[1] and in our experiment, pairs were randomly pre-assigned. Together, this strengthens the argument that the sex differences that emerge as pair bonds form and mature serve a different function than those that exist to drive mate choice and sexual selection.

Instead, emerging sex differences in pair bonding behaviors may help prime pairs to co-parent. Notably, our observed sex differences are organized within pairs, with intra-pair affiliation (partner huddle) consistently higher in females. Supporting a role for this behavioral organization in future parenting, female huddle is correlated with uterine weight, and as such, pregnancy status may be driving correlated female−male behavior by acting as a set-point for female partner huddle, which the male uses to calibrate his behavior. Interestingly, the one pair that did not become pregnant over the course of our experiment was also the one pair with inconsistent female−male behavior; the female did not show greater partner huddle time than her male partner in the long-term PPT. While it is unclear whether the lack of pregnancy drives the lack of coordinated behavior or vice versa, it does support a broad role for behavioral coordination in facilitating reproduction. Notably, similar mechanisms in which hormones drive female behavior which, in turn, changes male behavior have been observed in pair bonding bird species.[36]

4.3 | Sex differences in partner-directed motivation broadly reflect differences in partner affiliation

Using a separate cohort of animals, we dually employed social operant testing and PPTs. Replicating our previous experiment, there were notable sex differences in partner huddle time, with females huddling more with their partners than males. When provided with a fixed ratio of one lever press per social reward, in which voles pressed separately for either a partner or a novel in separate trials, females and males pressed equally for access to their partner and a novel vole in this testing paradigm. Although this differs from findings by Beery et al.[16] showing that female prairie voles work harder to access familiar males in a non-choice operant task, there are two important differences between our paradigms. Beery et al. employed a progressive ratio in which voles had to increase pressing across trials to get the same social access, and if they failed to press, they did not gain access. In contrast, our paradigm required substantially less effort and voles gained access to the social reward even if they did not press. Thus it is likely that sex differences are only evident under increasing task demands or in a choice context. Accordingly, when given a choice of pressing to gain access to their partner or a novel vole, females showed biased pressing to access their partner more frequently than accessing the novel. These sex differences in partner-directed motivation are broadly reflected in the sex differences in PPT partner-directed affiliation

(although these two metrics are not strongly correlated at the individual level). Given that females huddle with their partner more than males across both PPT experiments and in the operant paradigm, sex differences in partner-directed motivation may partially be responsible for increased affiliative behavior and, by extension, pair behavior. The present findings in the partner/novel operant choice test mirror those of similar tests conducted by Vahaba et al.[17] (also submitted to this issue). Of particular note, the results reported by Vahaba et al. and in this manuscript are consistent despite differences in testing apparatus, training paradigms, food restriction, colony origins, altitude, and other factors, indicating that sex differences in partner-directed effort are highly robust.

Notably, our operant experiment was designed to assess sex differences, but not within the context of a pair. This was largely due to constraints related to the daily training and testing required for this experiment. Further, the longitudinal nature of this experiment required sterilization of the untested partner. This experiment therefore indicates that pregnancy itself is not required for emergence of behavioral sex differences, although whether it is required for the intra-pair coordination of these behaviors remains unknown.

4.4 | Limitations and future directions

One limitation of the current work stems from the relatively sparse schedule of testing (3 timepoints), which occurred entirely before females were due to give birth. Future work is needed to resolve the time course of behavioral changes, pair-based variability, and the factors that may drive this variation. While we demonstrate that intra-pair affiliative behavior becomes more organized as bonds mature, it remains unknown whether or how this relates to previous reports of organized biparental behavior in this species. Manipulations of pregnancy status and/or pup presence may provide fruitful insight into this question. Finally, work is needed to determine whether our observed intra-pair behavioral organization reflects an active coordination of reciprocal behavior across pair members.

Together, our data demonstrate that prairie voles exhibit behavioral sex differences that contribute to reliable patterns of intra-pair behavior. These sex differences emerge as a bond matures and correlate with pregnancy. This emergent coordination may serve to facilitate future biparental care, or may simply be a result of cumulating social experience between two individuals. Together, this work and future work can uncover how coordinated behavior arises between bonded partners and its role in promoting success of a species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available via Figshare (https://doi.org/ 10.6084/m9.figshare.16619881, https://doi.org/10.6084/m9.figshare.16619896, and https:// doi.org/10.6084/m9.figshare.16619899) Complete statistical results, including effect sizes are reported in the supplementary Statistics Table. Code and operant apparatus design files are available via the Github links provided in the Methods section.

REFERENCES

1. Kleiman DG. Monogamy in mammals. Q Rev Biol 1977;52(1):39–69. [PubMed: 857268]

2. Sue Carter C, Courtney Devries A, Getz LL. Physiological substrates of mammalian monogamy: the prairie vole model. Neurosci Biobehav Rev 1995;19(2):303–314. [PubMed: 7630584]

3. DeVries AC, Carter CS. Sex differences in temporal parameters of partner preference in prairie voles (*Microtus Ochrogaster*). Can J Zooloogy 1999;77:5.

4. DeVries AC, DeVries MB, Taymans SE, Carter CS. The effects of stress on social preferences are sexually dimorphic in prairie voles. PNAS 1996;93(21):1980–1984.

5. Prounis GS, Thomas K, Ophir AG. Developmental trajectories and influences of environmental complexity on oxytocin receptor and vasopressin 1A receptor expression in male and female prairie voles. J Comp Neurol 2018;526(11):1820–1842. [PubMed: 29665010]

6. Bales KL, Carter CS. Sex differences and developmental effects of oxytocin on aggression and social behavior in prairie voles (Microtus ochrogaster). Horm Behav 2003;44(3):178–184. [PubMed: 14609540]

7. Gruder-Adams S, Getz LL. Comparison of the mating system and paternal behavior in *Microtus ochrogaster* and *M. pennsylvanicus*. J Mammal 1985;66(1):165–167.

8. Lonstein JS, De Vries GJ. Comparison of the parental behavior of pair-bonded female and male prairie voles (*Microtus ochrogaster*). Physiol Behav 1999;66(1):33–40. [PubMed: 10222470]

9. Rogers FD, Rhemtulla M, Ferrer E, Bales KL. Longitudinal trajectories and inter-parental dynamics of prairie vole biparental care. Front Ecol Evol 2018;6:73. [PubMed: 31396513]

10. Solomon NG. Comparison of parental behavior in male and female prairie voles (*Microtus ochrogaster*). Can J Zoology 1993;71(2): 434–437.

11. Ahern TH, Young LJ. The impact of early life family structure on adult social attachment, alloparental behavior, and the neuropeptide systems regulating affiliative behaviors in the monogamous prairie vole (*Microtus ochrogaster*). Front Behav Neurosci 2009;3:17. [PubMed: 19753327]

12. Bales KL, Lewis-Reese AD, Pfeifer LA, Kramer KM, Carter CS. Early experience affects the traits of monogamy in a sexually dimorphic manner. Dev Psychobiol 2007;49(4):335–342. [PubMed: 17455224]

13. Rogers FD, Freeman SM, Anderson M, Palumbo MC, Bales KL. Compositional variation in early-life parenting structures alters oxytocin and vasopressin 1a receptor development in prairie voles (*Microtus ochrogaster*). J Neuroendocrinol 2021;33(8):13001.

14. Insel TR, Hulihan TJ. A gender-specific mechanism for pair bonding: oxytocin and partner preference formation in monogamous voles. Behav Neurosci 1995;109(4):782–789. [PubMed: 7576222]

15. Williams J Development of partner preferences in female prairie voles (*Microtus ochrogaster*): the role of social and sexual experience. Horm Behav 1992;26(3):339–349. [PubMed: 1398553]

16. Beery AK, Lopez SA, Blandino KL, Lee NS, Bourdon NS. Social selectivity and social motivation in voles. eLife 2021;10:2684.

17. Vahaba DM, Halstead ER, Donaldson ZR, Ahern TH, Beery AK. Sex differences in the reward value of familiar mates in prairie voles. Bio-Rxiv Animal Behav Cognition 2021.

18. Ahern TH, Modi ME, Burkett JP, Young LJ. Evaluation of two automated metrics for analyzing partner preference tests. J Neurosci Methods 2009;182(2):180–188. [PubMed: 19539647]

19. Friard O, Gamba M. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol Evol 2016;7(11):1325–1330.

20. Scribner JL, Vance EA, Protter DSW, et al. A neuronal signature for monogamous reunion. Proc Natl Acad Sci USA 2020;117(20):1076–1084.

21. Virtanen P, Gommers R, Oliphant TE, et al. SciPy 1.0: fundamental algorithms for scientific computing in python. Nat Methods 2020; 17(3):261–272. [PubMed: 32015543]

22. Vallat R Pingouin: statistics in python. J Open Source Software 2018; 3(31):1026.

23. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Softw 2015;67:1–48.

24. Searle SR, Speed FM, Milliken GA. Population marginal means in the linear model: an alternative to least squares means. Am Statist 1980; 34(4):216–221.

25. Donaldson ZR, Yang SH, Chan AWS, Young LJ. Production of germline transgenic prairie voles (*Microtus ochrogaster*) using lentiviral vectors1. Biol Reprod 2009;81(6):1189–1195. [PubMed: 19641177]

26. Harbert KJ, Pellegrini M, Gordon KM, Donaldson ZR. How prior pair-bonding experience affects future bonding behavior in monogamous prairie voles. Horm Behav 2020;126:104847. [PubMed: 32910950]

27. Curtis JT. Does fertility trump monogamy? Anim Behav 2010;80(2): 319–328. [PubMed: 20823948]

28. Burkett JP, Andari E, Johnson ZV, Curry DC, FBM de W, Young LJ. Oxytocin-dependent consolation behavior in rodents. Science 2016; 351(6271):375–378. [PubMed: 26798013]

29. Smith AS, Wang Z. Hypothalamic oxytocin mediates social buffering of the stress response. Biol Psychiatry 2014;76(4):281–288. [PubMed: 24183103]

30. Matthews TJ, Williams DA, Schweiger L. Social motivation and residential style in prairie and meadow voles. Open Behav Sci J 2013;7:16–23.

31. Beery AK, Lopez SA, Blandino KL, Lee NS, Bourdon NS, Ahern TH. Social selectivity and social motivation in voles. BioRxiv Neurosci 2021.

32. Rose MC, Styr B, Schmid TA, Elie JE, Yartsev MM. Cortical representation of group social communication in bats. Science 2021;22:9584.

33. Darwin C The Descent of Man; 1871.

34. Price T Sexual selection and natural selection in bird speciation. Philos Trans R Soc Lond B Biol Sci 1998;353(1366):251–260.

35. Andersson M, Iwasa Y. Sexual selection. Trends Ecol Evol 1996;11(2): 53–58. [PubMed: 21237761]

36. Martinez-Vargas MC, Erickson CJ. Some social and hormonal determinants of nest-building behaviour in the ring dove (*Streptopelia risoria*). Behaviour 1973;45(1/2):12–37. [PubMed: 4781222]
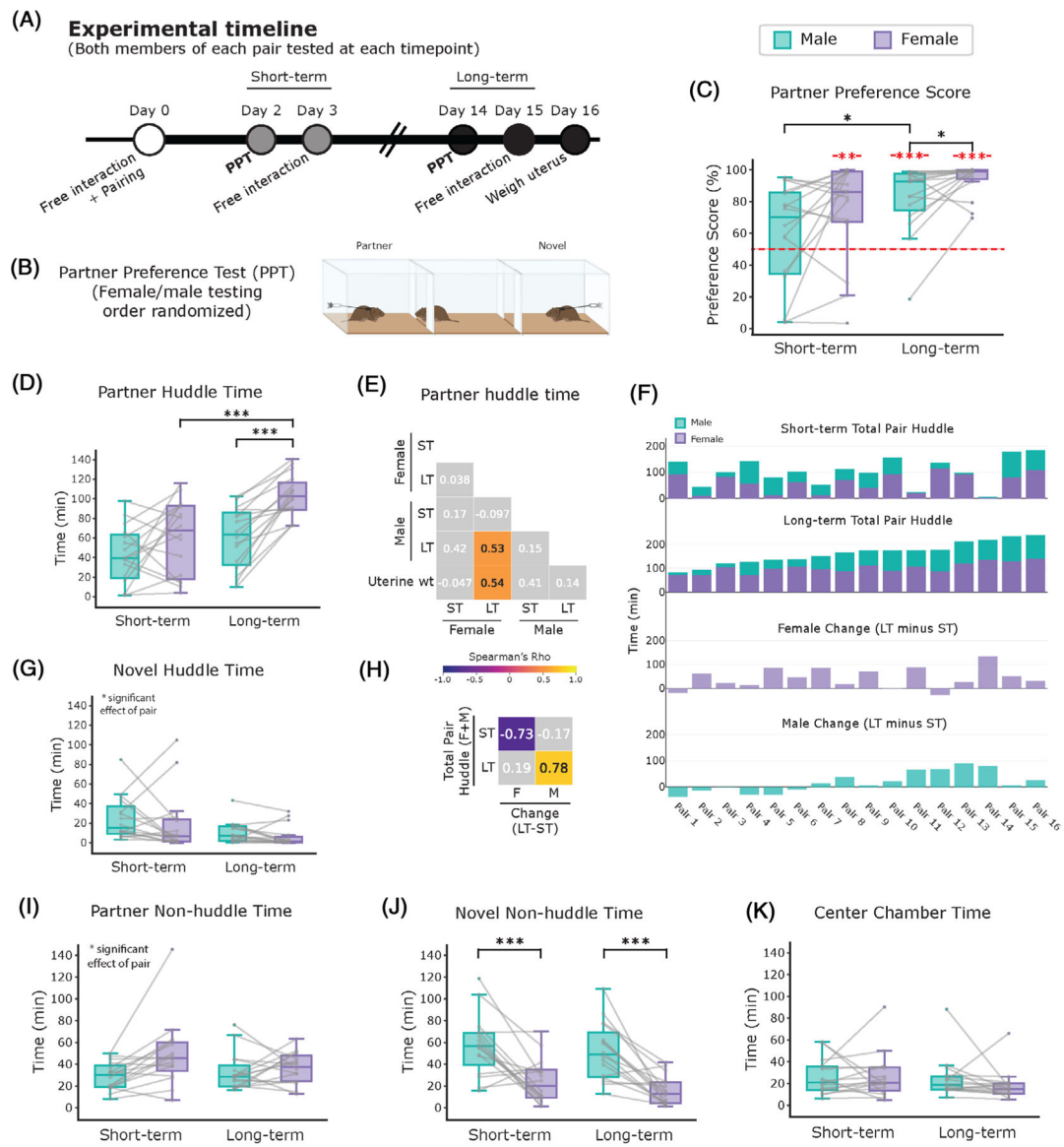
FIGURE 1.

Sex differences in partner preference metrics. (A) Schematic of experimental timeline. Animals (n = 16 F, 16 M) underwent a free interaction period with two novel animals: their eventual partner and a non-partner, before being paired for the remainder of the experiment. Partner preference tests (PPTs) were conducted 2 days (short-term) and 2 weeks (long-term) post-pairing. (B) Diagram of partner preference test. (C) Partner preference scores for females and males at short and long term timepoints, calculated for each animal as partner huddle time/(partner huddle time + novel huddle time) × 100%. Red asterisks denote significant difference from the null hypothesis of no preference (50%) using a one-sample t-test. Females form partner preferences by the short-term timepoint, while males do not. By the long-term timepoint, both females and males display a partner preference. Males show an increase in partner preference between short- and long-term. Sex differences in preference score are not apparent at the short-term timepoint but emerge by the long-term

timepoint. (D) Total partner huddle duration for females and males at short- and long-term timepoints. Females huddled more than their male partner at long-term but not short-term timepoints. Only females increase their partner huddle time between short- and long-term. (E) Correlation matrix of female and male partner huddle times at the short-term (ST) and long-term (LT) timepoints and uterine weight with colored squares indicating $p < 0.05$. Female and male huddle is correlated only at the long-term timepoint. Uterine weight is correlated with female partner huddle time at the long-term timepoint. (F) Top two plots are stacked bar graphs of female partner huddle + male partner huddle ("total pair huddle") at each timepoint. Third plot shows the change in female partner huddle between the short- and long-term timepoints. Fourth plot shows change in male partner huddle between short- and long-term timepoints. For all plots, pairs are ordered by total pair huddle at the long-term timepoint. (G) Total novel huddle duration decreased as a function of time, although post hoc tests did not reach significance for either sex over time. There was also a significant effect of pair. (H) Correlation matrix of total pair huddle versus female (F) and male (M) change in partner huddle between timepoints with colored squares indicating $p < 0.05$. Total pair huddle at the short-term timepoint is inversely correlated with female change. Total pair huddle at the long-term timepoint is positively correlated with male change. (I) Partner non-huddle time, calculated as partner chamber time minus partner huddle time. There was a main effect of timepoint, but no significant differences between timepoints for females or males in post hoc tests. There was a significant effect of pair. (J) Novel non-huddle time. Males spent more time investigating the novel than females did at both timepoints. (K) Time in the center chamber. No sex differences or time-dependent changes were observed
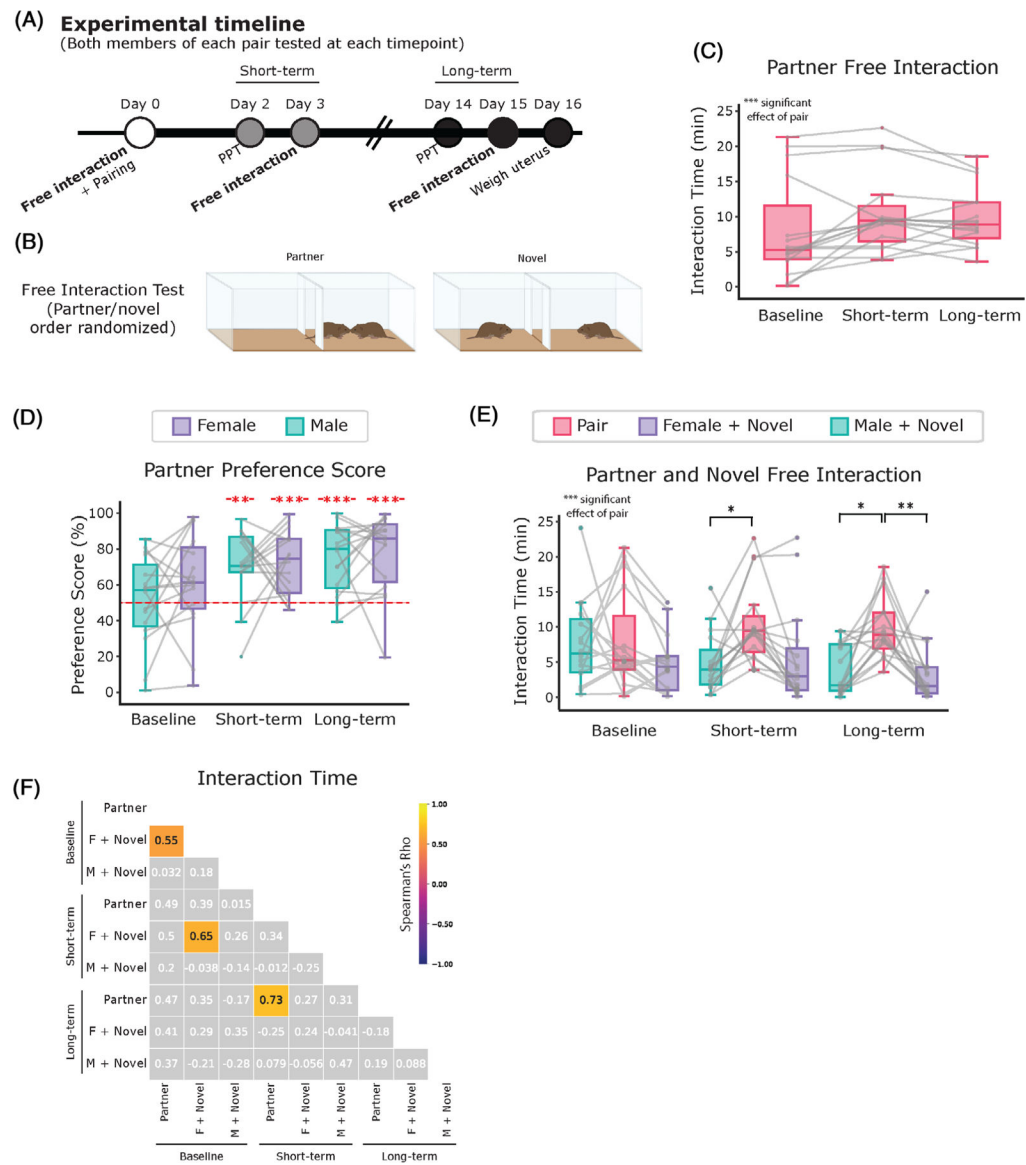
FIGURE 2.

Non-choice free interaction tests as a measure of partner preference. (A) Schematic of experimental timeline. Free interaction tests were conducted at baseline (day 0), short-term (3 days) and long-term (15 days) post-pairing. (B) Diagram of free interaction tests. Animals were placed in an open chamber and allowed to freely interact with a partner or novel animal for 30 min. After an inter-test interval of at least 30 min, the focal vole was tested with the other partner/novel (order randomized). (C) Interaction between partners at baseline (day 0), short-term (day 3), and long-term (day 15) timepoints. No significant differences in pair interaction across timepoints. There was a significant effect of pair. (D) Free interaction partner preference score calculated as pair interaction/(pair interaction + novel interaction) for each animal at each timepoint. Females and males show a significant partner preference at short- and long-term timepoints. (E) Partner and novel free interaction. Pair interaction was greater than male + novel interaction at the short-term and long-term timepoints. Pair

interaction was greater than female + novel interaction only at the long-term timepoint. There was a significant effect of pair. (F) Correlation matrix of free interaction metrics between timepoints calculated using Spearman's Rho. Significant correlations are colored according to Rho value
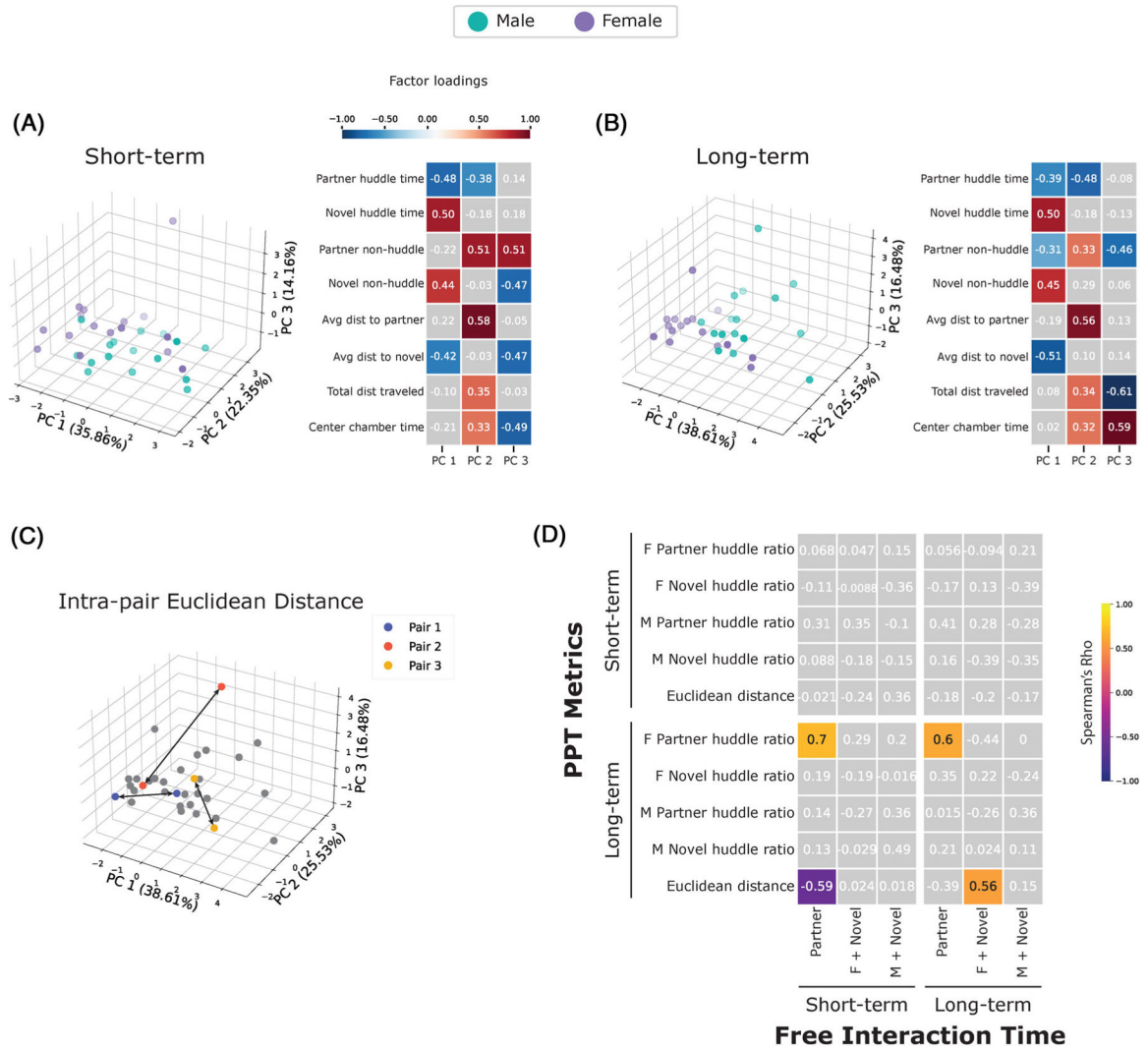
FIGURE 3.
Correlations between PPT and free interaction test. (A) Principal component analysis
(PCA) and factor extraction of mutually exclusive partner preference metrics at short-term
timepoint. Females and males are largely overlapping in the PCA. (B) PCA and factor
extraction of partner preference metrics at long-term timepoint. Females cluster separately
from males. (C) Diagram of how Euclidean distance was calculated between partners within
the same pairs from the PCAs in A and B. (D) Spearman's Rho correlations between
PPT and free interaction tests with colored squares indicating $p < 0.05$. Huddle ratio was
calculated as huddle time/chamber time. Short-term PPT and free interaction test metrics did
not correlate. The following metrics correlated significantly between the long-term PPT and
short-term free interaction tests: female partner huddle ratio versus partner free interaction,
PCA Euclidean distance versus partner free interaction. Significantly correlated metrics
between the long-term PPT and long-term free interaction tests include: female partner
huddle ratio versus partner free interaction, Euclidean distance versus female + novel free
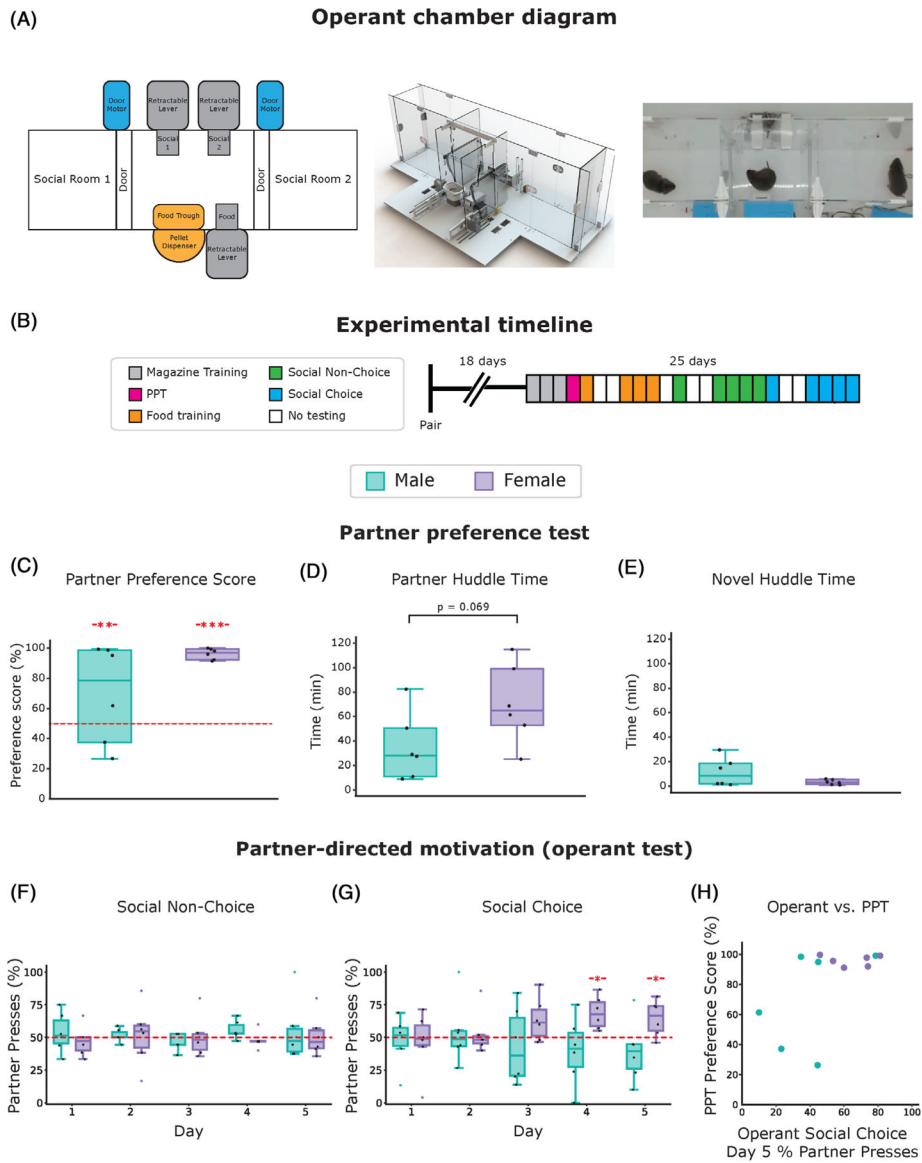interaction

FIGURE 4.

Operant paradigm for assessing partner-directed motivation. (A) Social choice operant apparatus. Left: schematic of relevant components for lever delivery and access to food or social reward. Middle: 3-dimensional diagram of apparatus designed in Solidworks and visualized in Photoview 360. Right: Top down screenshot of vole performing the social choice operant task. (B) Experimental timeline. Voles learned to associate relevant cues with food pellet delivery during magazine training (gray boxes), and then underwent training in which they received a food pellet faster if they pressed the lever (orange boxes). This was repeated for access to a non-choice social reward (partner or novel alternated in five trial bins; green boxes). Finally, social choice was assessed via an exclusive choice task in which both levers were presented and the test animal could receive access to either the partner or novel animal during each trial (blue boxes). (C) Partner preference scores (partner huddle time/(partner huddle time + novel huddle time) × 100%) for test conducted 3 weeks post-

pairing (pink box in B). Red asterisks denote significant difference from the null hypothesis of no preference (50%) using a one-sample *t*-test. Females show non-significantly stronger preference scores compared with males. (D) Females huddled more with their partner than males did, although this did not reach a $p < 0.05$ threshold. (E) No sex differences were observed in novel huddle duration. (F) When one lever is presented at a time in a non-choice paradigm, males and females will press equally for access to the partner or the novel. (G) In a social choice paradigm, a preference for partner access emerges by testing day 4 for females but not for males. Red asterisks denote significant difference from the null hypothesis of no preference (50%) using a one-sample *t*-test. (H) Scatterplot showing a general separation in female and male behavior based on partner preference score and percent partner lever presses in the operant choice paradigm