Quantifying Variability in the Social Behavior of Free-living Prairie Voles Using Advanced Bio-logging Approaches & Transgenic Manipulations

by

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Abstract

Understanding consistent individual variation in animal behavior is crucial for unraveling its ecological and evolutionary significance. Historically overlooked, such variation has profound implications for individual fitness, population dynamics, and species interactions. Genetic differences among individuals can significantly modulate behavior, yet studying this in natural environments remains challenging. Bio-logging technology offers a promising avenue for remotely quantifying animal behavior in the wild with real-time acquisition of abundant and precise data. Leveraging a genetic variant (*Shank3* mutation) in prairie voles (*Microtus ochrogaster*), known for their social monogamy, we developed a bio-logging system to investigate individual behavior variation in naturalistic environments. Our study aimed to evaluate the system's effectiveness and explore the impact of the *Shank3* gene on vole behavior. We found reduced sociality in *Shank3* mutant female voles, reflected in increased interaction distances and possibly decreased huddling. Additionally, no differences were found in trappability, home range sizes, body weight, and survival days between *Shank3* mutant and wild-type voles. Our results partially supported the laboratory findings of social deficits in *Shank3* mutant female voles, but the underlying mechanisms may differ in natural settings. Overall, our study demonstrates the potential of bio-logging technology for studying animal behavior in complex ecological contexts and highlights the need for further research to understand the interplay of genetic and environmental factors shaping individual behavior.

Keywords: Animal behavior, bio-logging, *Shank3*, sociality, prairie vole

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Introduction

Animal behavior is inherently complex, marked by significant variation among individuals. Historically, variation among individual animals within a species has been overlooked, perceived as inconsequential nuances unworthy of investigation. However, the growing recognition of this diversity in behavior reveals its profound implications for key ecological and evolutionary processes. It is now understood to significantly impact individual fitness, including growth, survival, and reproductive success, as well as population dynamics, encompassing collective behavior, social networks, dispersal, and speciation, along with species interactions such as predator-prey or host-parasitoid interactions (Laskowski et al., 2022). For example, in a meta-analysis, Smith & Blumstein (2008) found that bolder individuals had increased reproductive success, albeit at the expense of survival. More aggressive individuals tend to disperse more, facilitating colonization and range expansion (Cote et al., 2010). Active predators tend to consume inactive prey, while relatively inactive predators tend to consume active prey (Toscano et al., 2016). Behavioral differences in foraging frugivores and scatterhoarding animals can also impact seed dispersal as reviewed by Zwolak & Sih (2020). Additionally, understanding individual behavioral variation is essential for informing effective strategies for conservation, management of invasive species, and disease control, which are particularly important in the face of escalating anthropogenic environmental changes (Wolf & Weissing, 2012).

Many behaviors may have a substantial genetic component, with genetic variation therefore playing a significant role in modulating behaviors (Niepoth & Bendesky, 2020). For example, *Drd4* gene polymorphisms were found to be associated with variation in the level of novelty-seeking behavior in great tits (*Parus major*) (Fidler, 2007). Quantifying the impacts of genetic differences on behavior variation in the wild is challenging yet imperative because natural environments allow animals to express their full range of complex and ethologically significant behaviors, thereby enhancing the ecological authenticity of observed variation. Exciting advancements in bio-logging technology present a promising opportunity to remotely quantify and study the behaviors of free-living animals in their natural habitats. Bio-loggers, which are animal borne sensors placed on, near, or inside of the animal, enable real-time acquisition of abundant and precise information beyond human observation (Smith & Pinter-Wollman, 2020). Researchers have been using bio-loggers integrated with various sensors to study foraging behavior, migration and social interactions in a variety of populations (Wilmers et al., 2015).

In our study, we leveraged a genetic variant (*Shank3* mutation) in prairie voles (*Microtus ochrogaster*) to investigate individual behavior variation in their natural habitat using a self-developed bio-logging system. The *Shank3* gene has been identified as a

monogenetic factor contributing to a certain form of autism, leading to language and/or social communication disorders in humans (Durand et al., 2006). *Shank3* mutant mice have been shown to exhibit autistic-like behaviors and striatal dysfunction (Peça et al., 2011), but research on *Shank3* mutant mice has been limited to a single inbred mouse species in laboratory settings. Recent advances in *Shank3* mutant prairie voles offer new avenues for further research on this gene. Prairie voles, known for their socially monogamous behavior (Madrid et al., 2020), serve as an ideal model organism for studying social behavior due to their complex social behaviors, including bi-parental care (Thomas & Birney, 1979), social isolation-induced depression (Grippo et al., 2007), and empathy-based consoling (Burkett et al., 2016). There is also substantial within-species variability in the mating and pair-bonding (monogamous) behavior in this species (Madrid et al., 2020). Preliminary laboratory studies have revealed that female prairie voles (but not males) with the *Shank3* mutation exhibited social deficits (Larios, 2021). Specifically, they showed reduced social interaction with novel males and diminished preference for huddling with their partners. Prior studies have demonstrated that the natural behaviors of prairie voles can be fully observed and measured under semi-natural field settings (Sabol et al., 2018; Sabol et al., 2020), making it intriguing to investigate the effect of the *Shank3* gene on the prairie vole behavior in the field.

Our research aims to achieve two primary objectives. Firstly, we seek to evaluate the effectiveness of a self-developed Bluetooth Low Energy enabled bio-logging system by deploying it on wild-type and *Shank3* mutant (transgenic) prairie voles in field enclosures to track vole social interactions and physiological performances. This evaluation involves assessing device failure rates, examining the relationship between the received signal strength indicator (RSSI) and distance, monitoring battery life, and evaluating quantity and quality of collected data. Secondly, we aim to investigate the impact of the *Shank3* gene on vole behavior using the bio-logging system we developed. This involves studying their social behavior by constructing social networks and assessing interaction duration and distance, as well as investigating various behavioral aspects, as sociality can be correlated with other behavioral traits (Gartland et al., 2021). These include trappability, home range size, activity level and local temperature. Additionally, we intend to examine the consequences of the mutation on their body weight and survival.

We anticipated that transgenic female prairie voles would exhibit reduced levels of sociality, as suggested by the previous laboratory findings. Specifically, we expected a reduction in the interaction duration and an increase in the interaction distance of transgenic females. Furthermore, sociality was expected to be negatively correlated with boldness (Gartland et al., 2021). We predicted that transgenic females would have a higher likelihood of being trapped, as bold individuals are often trapped more easily and

more frequently than shy ones (Johnstone et al., 2021). We also predicted that transgenic females would have larger home range sizes because bold individuals were found to occupy larger areas and move longer distances compared to shy ones (Schirmer et al., 2019). Given the highly social nature of prairie voles, we anticipated that transgenic females would display reduced activity levels due to decreased sociality, leading to less frequent encounters with other individuals (Gartland et al., 2021). Moreover, we expected that transgenic females would exhibit lower mean body temperatures, as they lack social thermoregulation (Campbell et al., 2018). As sociality can have consequences for body condition and survival (Gartland et al., 2021), we expected transgenic females to exhibit differences in weight and survival days compared to their wild-type counterparts. However, it is also possible that transgenic females can still function typically in the wild. If this scenario holds true, then we would expect that their body weight and survival days would not differ from those of the wild-type females.

Methods

Study Site

Fieldwork was conducted at the Ecology Research Center in Miami University in Oxford, Ohio, U.S.A. (39° 53' N, 84° 73' W) from May to September 2023. Four 0.1 ha enclosures (32 × 32 m) were surrounded and separated by 20-gauge sheet metal walls, 75 cm above and 45 cm below the ground to prevent voles from escaping or moving between enclosures (Cochran and Solomon, 2000). We mowed the grass 1 m around the edge of the enclosures regularly to discourage voles from digging near the walls. We also checked the edge of the enclosures every day for holes in the ground or gaps between the walls. An electric wire over the enclosure walls was turned on when researchers left the enclosures to prevent mammals such as raccoons from entering the enclosures and disturbing traps. Two of the enclosures containing transgenic voles were covered by bird netting (#NKH2 100-150 from 3-T products) to avoid avian predation, and we checked the bird netting every day to make sure no bird got entangled. Four infrared cameras monitored the enclosures when researchers were not there. Despite all these efforts, we still had raccoons (*Procyon lotor*) enter the enclosures from approximately May 30 to June 20 when the electric wire on one of the enclosures was not working (this was fixed as soon as we realized it). We also observed 2 snakes (*Thamnophis* spp.) inside the enclosures.

Prior to releasing the voles into the enclosures, we live-trapped all enclosures for 3 consecutive days to get rid of any small mammals. Throughout the field season, we incidentally caught 110 mice (*Peromyscus* spp.), 4 rabbits (*Sylvilagus* spp.), 1 chipmunk (*Tamias* spp.) and 1 shrew (*Blarina* spp.) and released them outside the enclosures.

Study Animals

We used F3 generation wild-type prairie voles and Shank3 mutant (transgenic) prairie voles, generated through CRISPR-mediated mutagenesis in the laboratory of Dr. Devanand Manoli (University of California, San Francisco), and bred in the laboratory of Dr. James Burkett (University of Toledo). The genetic diversity of Shank3 mutant prairie voles was maintained since they were outbred from wild-caught stock in Illinois. They were descended from 26 unique parents (13 males, 13 females) representing 14 different lineages. Enclosures A and G were for the wild-type voles, while enclosures C and E were for the transgenic voles. The ages of the individuals released into enclosures A (mean 82.9, range 47 ~ 113) and G (mean 83.5, range 62 ~ 112) and enclosures C (mean 83.1, range 47 ~ 113) and E (mean 88.5, range 47 ~ 113) meant the ages of the wild-type (mean 83.2, range 47 ~ 113) and transgenic (mean 85.7, range 47 ~ 113) voles were roughly the same. We also tried to minimize the number of individuals from the same litter or different litters but born to the same parents. Specifically, enclosure A housed 12

males from 6 breeding pairs and 12 females from 7 breeding pairs, with at most 3 individuals from the same breeding pair. In enclosure G, there were 12 males from 7 breeding pairs and 6 females from 6 breeding pairs, with at most 4 individuals from the same breeding pair. Enclosure C housed 11 males from 6 breeding pairs and 12 females from 6 breeding pairs, with at most 6 individuals from the same breeding pair. In enclosure E, there were 11 males from 5 breeding pairs and 11 females from 4 breeding pairs, with at most 4 individuals from the same breeding pair.

Prior to their release, all founding voles were implanted (subcutaneously on their dorsal surface between the scapula) with passive integrated transponder (PIT) tags (Biomark: Boise, Idaho, 12 mm HPT tags) so that each vole could be uniquely identified. We encountered four cases where the trapped voles did not have functional PIT tags, and we implanted them with new ones. Two of them were identified by ear tags, while the other two remained unidentified.

All voles were released into the four enclosures on May 5, except for the three transgenic voles that died during the process of handling and release into the enclosures. These handling deaths appeared to be due to the animals having seizures, which are also known to occur in the laboratory (James Burkett, Univ Toledo, personal communication). Initially, enclosures A and G each contained 24 wild-type voles (12 males, 12 females), enclosure C contained 23 transgenic voles (11 males, 12 females), and enclosure E contained 22 transgenic voles (11 males, 11 females). One wild-type vole, intended for enclosure A, was mistakenly placed in enclosure E and subsequently released back into enclosure A on June 3. All behavioral data associated with this vole were excluded from our analyses described below. Due to the high mortality rate of voles (see Fig. 9), as inferred from the low trapping rate, starting on June 27, all voles caught in enclosures E and G were combined into enclosures A and C of the same genetic type. Specifically, 2 wild-type males and 5 wild-type females were caught in enclosure G and released into enclosure A (which only contained wild-type voles), while 7 transgenic males and 3 transgenic females were caught in enclosure E and released into enclosure C (which only contained transgenic voles) after two sets of consecutive nights of trapping from June 26 to 29 and from July 5 to 9 (i.e., 7 nights of overnight trapping total). Enclosures E and G were continually trapped throughout the remaining field season whenever traps could be set to ensure the removal of all voles. No adult voles were caught in enclosures E and G after July 8.

Offspring were left inside the enclosures until weaning, which is estimated to be 21 days (Richmond & Conaway, 1969), as we initially aimed to record parental behavior for a separate experiment not described here. Once we caught juveniles that were estimated to be over 21 days old (described below in the “Live Trapping” section), we euthanized them using CO2 inhalation to maintain a constant population density and also

to minimize the number of transgenic animals we generated. All offspring captured were not uniquely identified or included in our analyses described below. The voles relied on the vegetation within the enclosures for food and cover. No additional food was supplied besides the cracked corn, which is a low-quality food used to bait traps (Sabol et al., 2020).

Bio-logging: Juxta

We used a newly-developed bio-logging system (Juxta) to automatically record contacts between the free-ranging voles in the enclosures. The Juxta device is centered on a Bluetooth Low Energy (BLE) Microcontroller Unit connected to a battery voltage sensor, an accelerometer, a temperature sensor and 2Gb non-volatile NAND memory. It is powered by a rechargeable 40mAh 3.7V lithium polymer (LiPo) battery regulated to 1.8V. The size of an entire functional device is 11×15×8 mm. It can be connected and configured using a corresponding smartphone application (app) on iOS devices. Prototypes of these devices have been described previously (Gaidica et al., 2024), but we provided the first comprehensive test of their performance under field conditions.

The Juxta device has two operation modes: shelf and interval. In shelf mode, the device advertises at a user-selected rate but does not scan. It simply waits for connection without any data logging and consumes ultra-low power. In interval mode, the device both advertises and scans at user-selected rates. When devices get close, their unique IDs (MAC addresses) will be scanned and logged periodically together with Received Signal Strength Indicator (RSSI) and time as "log" data. Battery voltage, movement and temperature are also logged as "meta" data. All data can be directly dumped out as comma-delimited text from the smartphone app.

Movement is detected by an inertial sensor. It records a movement event as "xl" if the acceleration exceeds the threshold within a one-minute interval, resulting in a maximum of 60 "xl" records per hour. This serves as an indicator of vole activity levels. Device temperature is monitored by a temperature sensor and logged every 5 minutes. While it reflects a combination of the ambient local temperature and vole body temperature, we can infer vole nest use behavior through device temperature variation because voles sleep in insulated nests either underground in a burrow or above-ground. For instance, an increase in device temperature likely reflects entry into a nest as it is heated by the body heat of an animal inside of the insulated nest (Studd et al., 2018). Additionally, we investigated the relationship between the 24-hour rhythms of device temperature and activity level to gain a deeper understanding of how the voles utilized their environment and responded to external stimuli or changes.

When deployed on the voles, each device was taped with Kapton tape to prevent the battery from detaching and enclosed in a two-piece 3D-printed (Anycubic Photon

Mono 4K) case made from ABS-like resin. The case was sealed by all-purpose RTV silicone (JB Weld) to make it waterproof. We then secured the packaged device on the neck of the vole as a collar using a zip tie enveloped by shrink wrap. The total weight of the Juxta collar was about 3 grams, which corresponded to 6.79% of the average vole body weight (44.2 g). This slightly exceeded the generally accepted 5% rule for terrestrial animals (Williams et al., 2019), but the fossorial nature of voles meant that they were not restricted in terms of flight patterns. The Juxta device can also be used as a static base station when the device is connected to a base station board and a larger battery (1800 mAh) for longer runtime. We enclosed it in a waterproof Zulkit junction box and put it near the burrow entrance once the location of a nest was identified using either VHF telemetry or by tracking the fluorescent trails of voles (described below in the "Locating Nests" section).

All voles were equipped with Juxta collars upon their release on May 5 2023. The initial configuration was set to advertise every 2 seconds and scan every 30 seconds. Because this resulted in a short battery life of 2 to 3 days, starting from May 11 2023, we adjusted all retrieved devices to advertise every 2 seconds and scan every 60 seconds in an attempt to increase battery life. From May 15 2023, the configuration of all retrieved devices were further modified to advertise every 5 seconds and scan every 60 seconds. As of May 26 2023, all retrieved devices were configured to advertise every 10 seconds and scan every 60 seconds, finally achieving a maximum battery life of around 6 to 7 days (see Table 2). We made these adjustments in an effort to increase battery life of the Juxta devices and to try to maximize the number of devices on voles at the same time.

As the field season unfolded gradually, many Juxta devices were lost in the enclosures and could not be retrieved, with a total of 29 out of the 98 devices placed on the voles confirmed lost by July 20 2023. This was most likely because the collars were not tight enough and slipped off the voles' necks. Because not every trapped vole was able to be equipped with a Juxta collar, Juxta devices were prioritized for females and for base stations placed at nest entrances that we located. Starting on July 20 2023, we manufactured an additional number of Juxta devices, enabling us to put a Juxta collar on each trapped vole.

- *Grid Validation: RSSI vs. Distance*

The received signal strength indicator (RSSI) is measured in decibels with the unit dBm. It operates on a logarithmic scale, thereby yielding negative values. A lower (more negative) number indicates a weaker signal and thus a farther distance. To validate the relationship between RSSI and distance, we utilized the grid validation routine developed by Kirkpatrick et al. (2021). We laid out two measuring tapes in a cross on a flat area with short grass. One logger was placed at the center of the cross, and four loggers were

positioned at equidistant points along each arm. All loggers were packed up in 3D-printed cases, as described above. The four loggers were initially 2.5 cm from the center one, and were subsequently moved along the arms in 2.5 cm increments until reaching a distance of 30 cm (Fig. 1). All loggers were configured to advertise every 1 second and scan every 20 seconds. The loggers remained stationary for 1 minute between each move to ensure stable log records. The timestamp and actual distances associated with each move were recorded, and the log data were downloaded from the smartphone app after the experiment concluded.

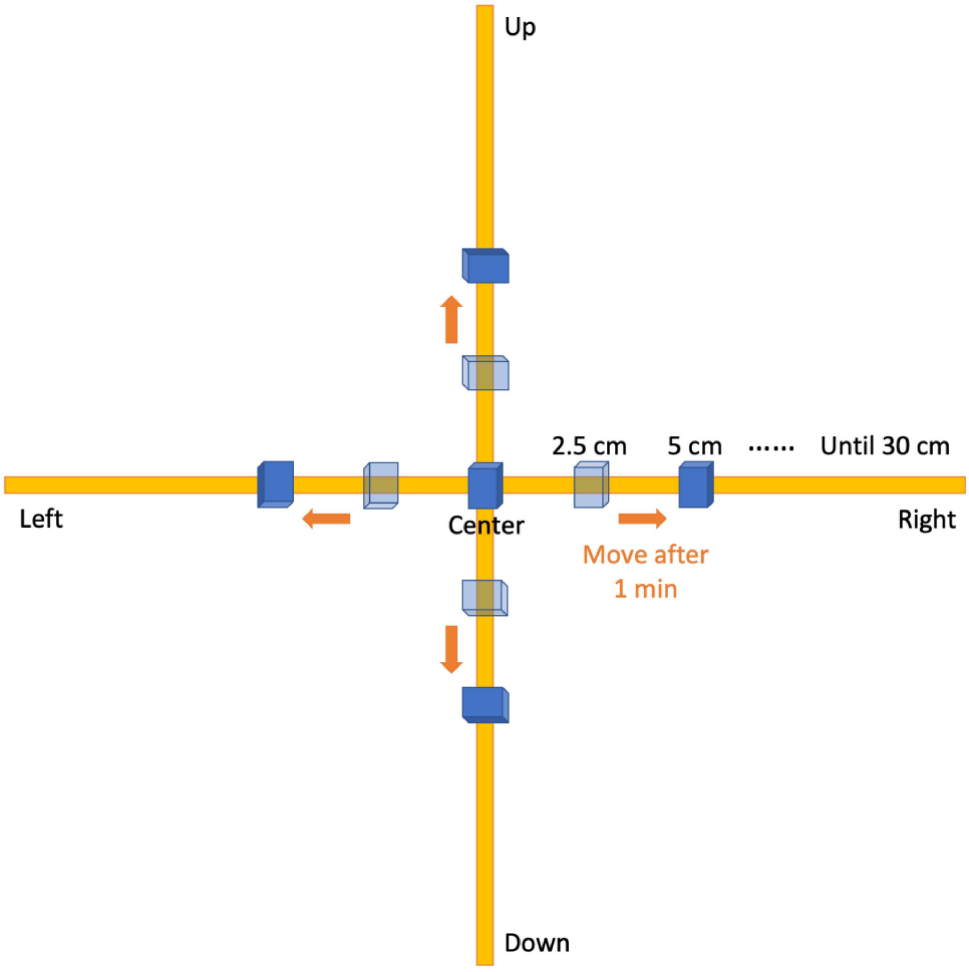

Figure 1. Grid validation process for the relationship between RSSI and distance of the Juxta devices. Four loggers (represented by the blue cubes) were placed on the four arms of the two crossing measuring tapes (represented by the yellow elongated rectangles), and were gradually moved away in 2.5 cm increments from the center logger (loggers prior to movement are depicted as transparent blue cubes). A one-minute interval was allowed for the loggers to record each other between each move.

Live Trapping

We live-trapped all four enclosures frequently throughout the field season. In each enclosure, 25 stakes (rebars) were arranged in a 5×5 grid. Initially, we placed 1 Sherman trap and 2 Ugglan traps (Granhab, Hillerstorp, Sweden) at each rebar (corresponding to 3 traps per grid rebar or 75 total traps per enclosure). To increase the trapping rate, we added one more Sherman trap to each rebar on June 2, resulting in 4 traps per grid rebar or 100 total traps per enclosure. The Sherman traps were entirely enclosed with sheet metal, typically allowing the capture of one animal at a time. However, there was one occasion when we captured three adult voles in the same Sherman trap. The Ugglan traps were meshed and covered by sheet metal from the top, enabling the capture of multiple animals per trap. The same number and types of rebar traps were set in each enclosure during the same trapping session. Once a nest was located with VHF radio telemetry and/or UV powder tracking (described below in the "Locating Nests" section), two additional traps were placed near the nest to increase the chance of capturing the adult voles residing there and/or their offspring. The type of nest traps (Sherman or Ugglan) depended on availability.

Enclosures were alternately trapped. For each trapping session, we typically set all traps in the enclosures, including 75 or 100 rebar traps per enclosure before or after June 2 2023, along with all nest traps if nests were identified. From June 6 to June 20, 2023, only Sherman traps were set at the rebars, along with nest traps, due to limitations in personnel and time availability. Most of the time, we set traps between 1900 and 2100 h. Traps were left open overnight, and checked and closed in the morning, typically starting at 0700 h. Occasionally, setting traps started in the early morning (around 0630 h), and traps were checked and closed 2 hours later. We typically trapped each enclosure 2 to 3 times a week, with increased trapping sessions in enclosures E and G when attempting to combine the voles into enclosures A and C. We never set traps when thunderstorms were forecasted to allow the voles to seek shelter from the rain. During the first month, two enclosures were trapped per trapping session and we alternated between enclosures A & C and enclosures E & G every 4 days. Before mid-June, we trapped enclosures A & C and enclosures E & G, respectively, for 3 consecutive nights to deploy as many voles with functional Juxta collars as possible simultaneously. At the end of June, we trapped enclosures E & G for three consecutive nights to eliminate all voles in enclosures E & G and combine them into enclosures A & C. Then enclosures A & C were alternately trapped every 3 days. Whenever possible, we also set traps in enclosures E & G when we trapped enclosures A or C. One week before the end of the experiment, as we acquired more Juxta devices, enclosures A & C were trapped for 3 consecutive nights to deploy as many voles as possible with Juxta collars. From July 26 to 28 2023, we trapped enclosures A & C for 2 consecutive nights to retrieve all Juxta devices. Starting from

August 2 2023, we trapped all 4 enclosures for three consecutive nights to euthanize voles and recover any remaining Juxta devices and then another four consecutive nights from August 6-10 2023 to ensure no voles remained in the enclosures (this represented 448 total traps being set per night) From September 8 to 11 2023, a final 3 consecutive trappings were conducted to ensure the removal of all voles from the enclosures. As evidenced by our trapping data from August 6 to September 11 2023 (representing 9 trap nights, over 400 total traps set per night) where we caught 0 adult voles and 3 pups, we believe that all voles were removed from the enclosures. Full details on trapping schedule can be found in Table S1.

For each trapped vole, we recorded its vole ID (PIT tag ID) by reading with an external transponder, noted the trap location, checked whether there were other individuals in the same trap or at the same stake, weighed it with a Pesola spring scale, collected a fecal sample, and assessed its reproductive condition when possible. For females, we determined whether they were pregnant or lactating; for males, we checked for the presence of scrotal testes. If it had a Juxta collar, we cut the collar off, swapped the battery, downloaded the data, reset and repacked the device, and put it back onto the vole. The time when the vole was released back into the enclosure was also recorded. If a vole was trapped consecutively, we recorded its vole ID and trap location and released it immediately without further processing. If we caught an offspring, we estimated its age based on appearance (teeth, tail length and fur growth) and weight (Swanson et al., n.d.). Offspring under three weeks old were released into the enclosure, while those three weeks or older were euthanized as described above.

Locating Nests

To locate nests, we used both VHF radio telemetry and UV powder tracking. Starting from June 3 2023, for the adult female voles we captured, we replaced the Juxta collars with VHF collars (Holohil model BD-2C, approximately 1.5g), and attempted to locate them between 1:00 PM and 5:00 PM when we expected them to be least active (Sabol et al., 2018). Once the females were located, we searched the surrounding area to identify burrows or surface nests that were actively in use and flagged them. We repeated telemetry several times to confirm the location of their nests before taking off the VHF collars. Additionally, we dipped 20 female voles in UV fluorescent powder (Lemen and Freeman, 1985), released them, and followed their traces with a UV flashlight in the dark to locate their nests. In total, we recorded 31 nests: 18 in enclosure A, 6 in enclosure C, 3 in enclosure E, and 4 in enclosure G. Out of these, 17 were identified using UV powder, while 14 were located through VHF radio telemetry. The nest coordinates were measured in meters, as in distance from the edges of enclosures, using a measuring tape. Some nests

in A were flagged for the same vole because they were located at different locations, either at different times or by different methods.

Construction of Social Networks

Social networks were constructed using the R (version 4.3.1, R Core Team, 2023) package igraph (version 1.6.0). To construct social networks from the Juxta data, we first trimmed all log data files to start from the time when each vole was released into the enclosure and to end at the time when the first trap was set for the next trapping session when the vole was recaptured. For example, if a vole was caught and released into the enclosure on May 11 2023 at 1030 h, and was caught again on the morning of May 15 2023 as we set traps at the night of May 14 2023 starting from 1905 h, then the trimmed data file will consist of data from May 11 2023, 1030 h, to May 14 2023, 1905 h. This ensured the use of data only when the voles were moving freely in the enclosures. We also excluded all log data on the first day of release (May 5 2023) to allow a habituation period for the voles to spread out in the enclosures. Moreover, as Juxta loggers could detect each other at relatively far distances, which did not necessarily indicate individual interactions, we considered only the log data recorded within 20 cm as interactions. This was achieved by thresholding the RSSIs using the predicted RSSI value at 20 cm from the general linear model fitted to all data in the grid validation process. Since the number of logs is not a true reflection of the strength of association due to unsynchronized advertising and scanning schedule, inter-logger variation in performance, device loss and modified advertising and scanning intervals, we used the duration from the time of contact initiation by the first logger until the termination of contact by both loggers (Boyland et al., 2013). Interactions were considered continuous if the time intervals between consecutive logs were smaller or equal to 61 seconds. Given that higher associations may arise from voles wearing functional devices for longer durations, the duration between each dyad was then weighted by the simple ratio index (Cairns and Schwager, 1987), calculated as the duration the dyad was recorded together divided by the sum of the duration they were recorded together or separately, thereby accounting for variations in sampling effort. Social networks constructed from live-trapping data used both the records of the voles captured in the same traps and at the same rebars. To address the potential bias introduced by varying capture probabilities, the associations were also weighted by the simple ratio index, calculated as the number of times the dyad was trapped together divided by the sum of the number of times they were trapped together or separately.

Statistical Analyses

All statistical analyses were done in R. All models were fitted using the lme4 package (version 1.1-34). All variables, except categorical ones, were standardized by subtracting their sample mean and dividing by their standard deviation to mitigate issues related to multicollinearity in linear mixed-effects models and generalized linear mixed-effects models. For all models, we visually checked for normality of residuals and homoscedasticity of residuals and assessed multicollinearity among the predictors using the variance inflation factor (VIF) (Zuur et al., 2009). No significant violations of these assumptions were observed in any of our models. A VIF higher than 5 to 10 indicates multicollinearity among explanatory variables (Kim, 2019). For most of our models, we included an interaction between sex (female or male) and strain (transgenic or wild-type) to allow us to assess if the effects of the *Shank3* mutation on vole behavior or other characteristics differed between females and males. If this interaction term proved significant, we further conducted post-hoc analyses for pairwise comparisons between transgenic and wild-type voles within the same sex category using the emmeans package in R (version 1.8.9).

- *RSSI vs. Distance for Juxta Loggers*

The actual distances (in cm) were calculated for each log record corresponding to each pair of devices. To investigate the relationship between RSSI and distance for the Juxta loggers, we fitted a general linear model with RSSI as the response variable and included an interaction between distance and logger (represented as center, left, right, up and down) as the predictor. This interaction term aimed to quantify the variation among loggers in the relationship between RSSI and distance. VIFs were all below 3.11, suggesting no issues of multicollinearity. An ANOVA was performed to assess the significance of the predictors. Additionally, a general linear model was fitted to all the RSSI and distance data collected from the five loggers to determine a threshold for vole interactions.

- *Interaction Duration*

We examined whether different sex pairs of voles of different strains exhibited varying interaction durations by fitting a linear mixed-effects model to the interaction duration (n = 77 voles). The interaction duration was calculated as described above in the "Construction of social Networks" section, and was log transformed to improve normality of the residuals. The predictors included the interaction between strain and type of sex pair (male-male, male-female, or female-female), as well as the date of interaction. This interaction between strain and sex pair enabled us to assess whether transgenic and wild-type voles differed in their interaction duration and whether this difference depended on

the sexes of voles involved. Enclosure was included as a random intercept to account for any variation among enclosures. The highest VIF was 6.67, which was expected because it occurred in an interaction term between strain (transgenic) and type of sex pair (male-male).

- *Interaction Distance*

Since the RSSI values signify distances between loggers, we explored whether different sex pairs of voles of different strains interacted at different distances by fitting a linear mixed-effects model to the RSSIs (n = 84 voles). The predictors included the interaction between strain and type of sex pair (male-male, male-female, or female-female), along with date and hour. This interaction between strain and sex pair allowed us to examine whether transgenic and wild-type voles differed in their interaction distances and whether this difference depended on the sexes of voles involved. Enclosure served as a random intercept to account for any variation among enclosures. The highest VIF was 9.06, which was expected because it occurred in an interaction term between strain (transgenic) and type of sex pair (male-male).

- *Activity Level*

We examined how voles of different sexes and strains differed in their activity levels. To ensure that the only data we used in our analyses corresponded to periods when voles were freely moving within the enclosures rather than being in our traps, all meta data files were trimmed to start from the time when the vole was released into the enclosure and to end at the time when the first trap was set for the next trapping session when the vole was recaptured (following the same trimming procedure as described above for the log data files in the "Construction of Social Network" section). Subsequently, all remaining "xl" data for each vole were grouped by dates and hours and aggregated to calculate "xl percentage", which was the count of "xl" records per hour divided by 60, representing the percentage of movement per hour and serving as an indicator for vole activity levels. For statistical analysis, because three-way interaction terms complicate the interpretation of the model and we had relatively small sample sizes to interpret three-way interactions, we ran two separate models for the two sexes. We employed a binomial generalized linear mixed-effects model, with the count of "xl" per hour as the response variable (n = 58 voles). Fixed effects included an interaction between strain and date and an interaction between strain and hour (to capture any time-dependent differences in the effect of strain on vole activity levels), while vole ID was treated as a random intercept (because we had repeated observations of the same individual voles). Enclosure was not included as a random effect because it contributed little to the total variance and would result in overfitting. VIFs were all below 3.09 for females and 3.28

for males, except for the strain term, which exceeded 20. The high values were expected due to the interaction of strain with all other predictors. Subsequently, for voles of each sex, we conducted post-hoc comparisons of activity levels between transgenic and wild-type voles for each hour of the day.

- *Device Temperature*

We examined how device temperature differed in voles of different sexes and strains, which could imply differences in their nesting behavior as we expected steady and sustained elevations in device temperature reflected nest use. All metadata files were trimmed as described above in the "Activity Level" section to ensure that the only data we used in our analyses corresponded to periods when voles were freely moving within the enclosures. Data files with negative temperature values or fewer than 5 distinct temperature values were excluded, as they indicated malfunctioning temperature sensors. All remaining temperature data for each vole were grouped by dates and hours and aggregated as the mean temperature of each hour. We again split the data into two sexes to run separate models to simplify interpretation. We employed a linear mixed-effects model, with the mean device temperature as the response variable (n = 56 voles). Fixed effects included an interaction between strain and date and an interaction between strain and hour (to capture any time-dependent differences in the effect of strain on device temperature), while enclosure and vole ID were treated as random intercepts. VIFs were all below 3.15 for females and 2.98 for males, except for the strain term, which exceeded 20. The high values were expected due to the interaction of strain with all other predictors. Subsequently, for voles of each sex, we conducted post-hoc comparisons of device temperature between transgenic and wild-type voles for each hour of the day.

- *Trappability*

To investigate trappability between voles of different sexes and strains, we used a linear mixed-effects model for the number of times each individual was trapped in each enclosure where they lived (n = 75 voles), with an interaction between sex and strain, and the number of trapping sessions experienced by each individual in each enclosure within their survival days (see below for estimation of survival days) as fixed effects, and enclosure as a random intercept to account for any variation among the four enclosures and because we sampled voles within the same enclosures. The interaction between sex and strain allowed us to assess if wild-type and transgenic voles differed in terms of their willingness to enter traps and if this difference depended upon vole sex. We included the number of trapping sessions experienced by each individual in each enclosure within their survival days to control for the effects of trapping efforts. VIFs were all below 3.11, suggesting no issues of multicollinearity.

- *Home Range Size*

The home range sizes of individuals were calculated as the area of the minimum convex polygons generated from the spatial coordinates of their trapped locations, including both the rebar and the nest traps, using the chull function in the grDevice package (R base package). If an individual was captured only at one location but for three or more times, its home range size was estimated as 0. Individuals with two trapped locations were excluded since a minimum of three locations was required to form a polygon. To investigate whether voles of different sexes and strains differed in their home range sizes, we fitted a linear mixed-effects model to the home range size of each individual in each enclosure where they lived (n = 42 voles), with an interaction between sex and strain and the number of times each individual was trapped in each enclosure as fixed effects, and enclosure as a random intercept. We included the number of times each individual was captured in each enclosure to control for the effects of trapping frequency on home range size estimates. Home range sizes were log transformed to improve normality of the residuals. VIFs were all below 2.87, suggesting no issues of multicollinearity.

- *Vole Body Weight*

We investigated the effects of sex and strain and their interaction on both the mean body weight of each individual vole (n = 71 voles sampled over the entire experimental period from May to August 2023) and, for voles for which we had body weight measures both at the start (at release into the enclosures on May 5 2023) and end of the experimental period (defined as weight recorded on or after July 27 2023), the within-individual weight change across the experimental period (n = 22 voles). For mean vole weights, we ran separate models for the two sexes to simplify interpretation. We used a linear mixed-effects model with an interaction between strain and date, an interaction between strain and date^2 (to capture any non-linear trend in body weight, such as increases followed by decreases in vole body weight associated with pregnancy, and any time-dependent differences in the effect of strain on mean vole weights) and initial weight at release into the enclosures as fixed effects. Vole ID served as a random intercept term because we had repeated observations of the same individual voles, with date and date^2 as random slopes to assess if different voles varied in their weight across the experimental period. Mean weights were log transformed to improve normality of the residuals. We did not include a random effect of enclosure because it had 0 variance even if included in the model, meaning the variability among different enclosures is negligible. VIFs were all below 3.60 for females and 3.43 for males. For within-individual weight changes across the experimental period, we only considered voles with weights recorded on or after July 27 and used the change in weight (last recorded weight - initial weight) as the response

variable. We fitted a linear model with an interaction between sex and strain as the predictor. VIFs were all below 1.74.

- *Survival Days and Population Size*

The survival days of individuals in the enclosures was estimated based on the lifespan of PIT tags (vole IDs), calculated as the difference between the last recorded time and the first recorded time (on the day of release). It is of course possible that some voles that were put into the enclosures were just never caught again, but this seems unlikely given that we did 9 sessions of overnight trapping from August 6 to September 11 and caught 0 adult voles, indicating that all voles had either died naturally or been captured and euthanized. We assumed that a vole was alive until its last recorded PIT tag date and deceased thereafter. The vole population size for each date was then estimated by summing the number of live voles (i.e., the number of PIT tags recorded on or after that date). To investigate whether voles of different sexes and strains had different survival days, we fitted a linear mixed-effects model to the survival days of each individual (n = 93 voles), with an interaction between sex and strain as a fixed effect, and enclosure at release as a random intercept. The interaction term allowed us to examine differences in survival days between wild-type and transgenic voles and whether this difference was sex-dependent. Two later-inserted PIT tags, with unknown correspondence to the founding PIT tags, were excluded from this analysis. VIFs were all below 2.98, suggesting no issues of multicollinearity.

Results

Juxta Bio-logging System

- *Failure Rates*

During the field experiment, we used a total of 123 Juxta devices. Of these, 121 were deployed on the voles, while 16 were used as base stations. However, by the end of the experiment, 82 devices remained untrieved, indicating a high loss rate. In addition, 32 devices out of 123 total devices used encountered software or hardware issues, including incomplete or corrupted data, download failures, initialization errors, and app crashes due to excessive data volume. Two devices experienced overheating when connected to a battery and were consequently not used in the experiment. Several devices struggled or failed to establish connections towards the end of the experiment. Most of the issues, especially the software-related ones, were identified and resolved before June.

- *RSSI vs. Distance*

For all five loggers used in the grid validation, RSSI decreased in a linear fashion as the distance from one another increased (Fig. 2). Notably, the relationship varied significantly among the five loggers ($F = 27.19$, $df = 4$, $p < 0.0001$, Table 1). Large variation in RSSIs was observed for the same device at the same distances. The maximum detectable distances also varied from 30 to 60 cm among the five loggers. Pairs of loggers generally exhibited similar RSSIs when recording each other. However, there were occasions where the records were not symmetric, meaning one device recorded the other, but was not recorded by the other device. We noticed that this occurrence became more frequent as the distance increased. We concluded that the maximum distance at which loggers could reliably detect each other was about 20 cm. A general linear model fitted to the RSSI and distance data from all five loggers revealed an RSSI value of -80.87 dBm at the distance of 20 cm. A RSSI threshold of -80 dBm was used to indicate interactions between voles in the subsequent analyses.

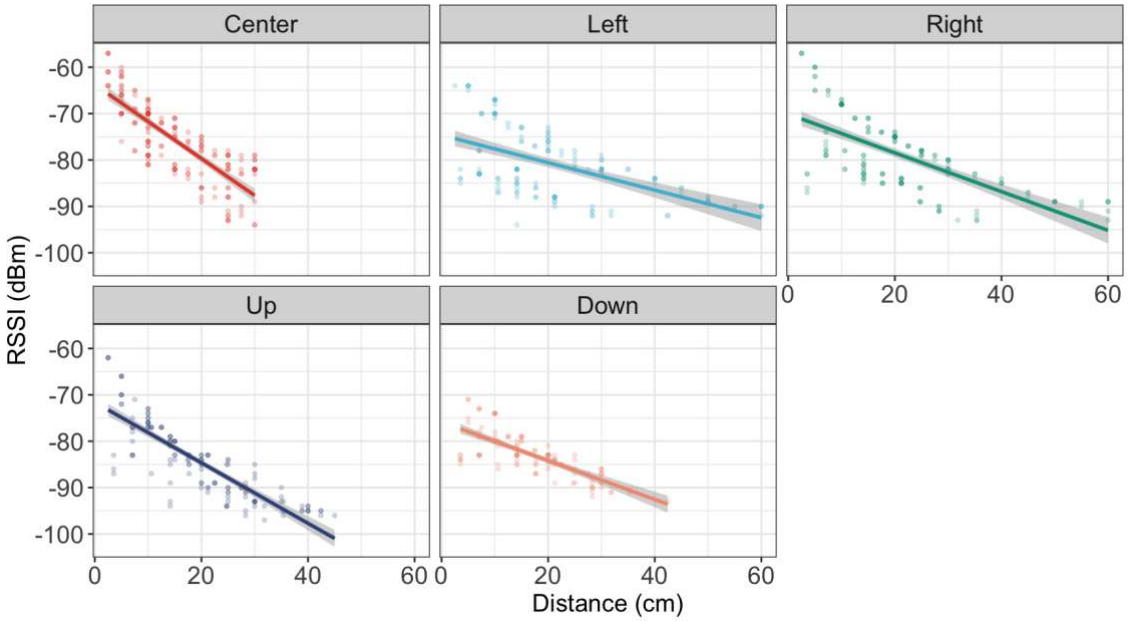

Figure 2. Correlation between the received signal strength indicator (RSSI) and the distance for the 5 loggers used in the grid validation. Negative linear relationships were observed (Table 1), with diverse detection ranges among devices and varying RSSIs at identical distances within devices.

Table 1. Results of the ANOVA for the factors influencing the received signal strength indicator (RSSI) in Juxta loggers.

	df	Sum Sq	Mean Sq	F	Pr (>F)
RSSI					
Distance	1	22524.5	22524.5	842.94	**< 0.0001**
Logger	4	5812.7	1453.2	54.38	**< 0.0001**
Distance × Logger	4	2906.4	726.6	27.19	**< 0.0001**

- *Battery Life*

The battery life was estimated based on the duration of the collected meta data, which was continuously recorded. The battery life of the 40mAh 3.7V lithium polymer (LiPo) batteries exhibited variation across different configurations of advertising and scanning intervals (Fig. 3). Moreover, significant variability was observed among devices sharing the same configuration (Fig. 3). It is important to note that the mean and median battery life for the initial configuration (advertising = 2s and scanning = 30s) were

underestimated, as the devices were turned on approximately one day before being reset and deployed on the voles on the first day of release (May 5 2023). The maximum lifespan of 3.5 days likely provides a more accurate estimation of the battery's performance under this configuration. Longer advertising and scanning intervals corresponded to extended battery life, with advertising intervals of 10 s and scanning intervals of 60 s resulting in the longest battery life (maximum = 7.1 days) and advertising intervals of 2 s and scanning interval of 30 s having the shortest battery life (maximum = 3.5 days, Table 2). Scanning intervals had a more pronounced impact on battery life, as scanning consumes more power than advertising. With a scanning interval of 60s, the ideal battery life ranged from 6 to 7 days (Table 2). Furthermore, we observed that devices with long advertising and scanning intervals but short battery life shut off with a high last recorded battery voltage, indicating that the batteries were not yet depleted. This phenomenon could be attributed to battery manufacturing issues or batteries becoming detached from the devices.

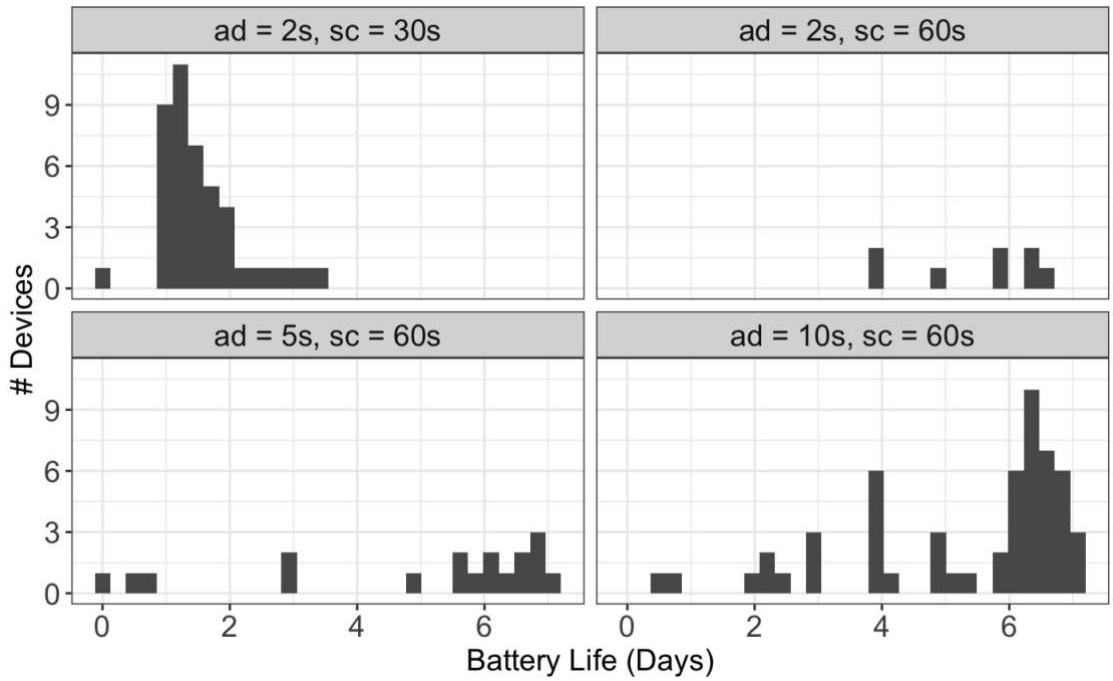

Figure 3. Battery life of Juxta bio-loggers with different combinations of advertising (ad) and scanning (sc) intervals. Large variation in battery life was observed in each combination. Devices shut down early suggested battery issues.

Table 2. Battery lifespan and number of logs with different advertising (Ad) and scanning (Sc) interval configurations for Juxta loggers. The mean and median battery life for Ad/Sc = 2/30 was underestimated by approximately 1 day.

Ad/Sc (s)	Config Start Date	# Devices	Mean Lifespan (Days) (Range)	Median Lifespan (Days)	Mean # Logs (Range)
2/30	May 5	43	1.5 (0.1 ~ 3.5)	1.4	1247 (158 ~ 3308)
2/60	May 11	8	5.5 (3.9 ~ 6.5)	5.8	1497 (1418 ~ 1575)
5/60	May 15	18	4.9 (0.1 ~ 7.2)	5.9	221 (2 ~ 315)
10/60	May 26	55	5.3 (0.5 ~ 7.1)	6.2	310 (3 ~ 2520)

Social Networks

- *Generated from Trapping Data*

Across 60 total trapping sessions from May to September 2023 (corresponding to a total of 15152 traps set), we documented 14 instances of two adult voles captured in the same trap, 1 instance of three adult voles captured in the same trap, and 28 instances of two adult voles captured in traps set at the same trapping location (same trapping stake/rebar). Social networks of voles from all four enclosures were constructed from all trapping data, depicting voles captured in the same trap or at the same rebar throughout the entire field experiment (Fig. 4). Each node represents an individual vole, with lines between each pair indicating the presence of association. The thickness of the line reflects the strength of association. For example, the thick line connecting male vole 88BD95B with female vole C01E8C8 in enclosure E suggests that these voles were caught together multiple times, possibly indicating the formation of pair bonds between them. Enclosure A exhibited the highest number of associations, whereas the other three enclosures showed relatively fewer associations. This discrepancy was probably attributed to differences in vole populations, as enclosure A maintained the highest number of adult voles throughout the field season (see Fig. 22). The majority of associations were male-female pairs (16 in A, 8 in C, 2 in E, 1 in G), with some male-male (7 in A, 3 in C, 2 in E, 0 in G) and female-female pairs (3 in A, 0 in C, 1 in E, 0 in G) also observed.

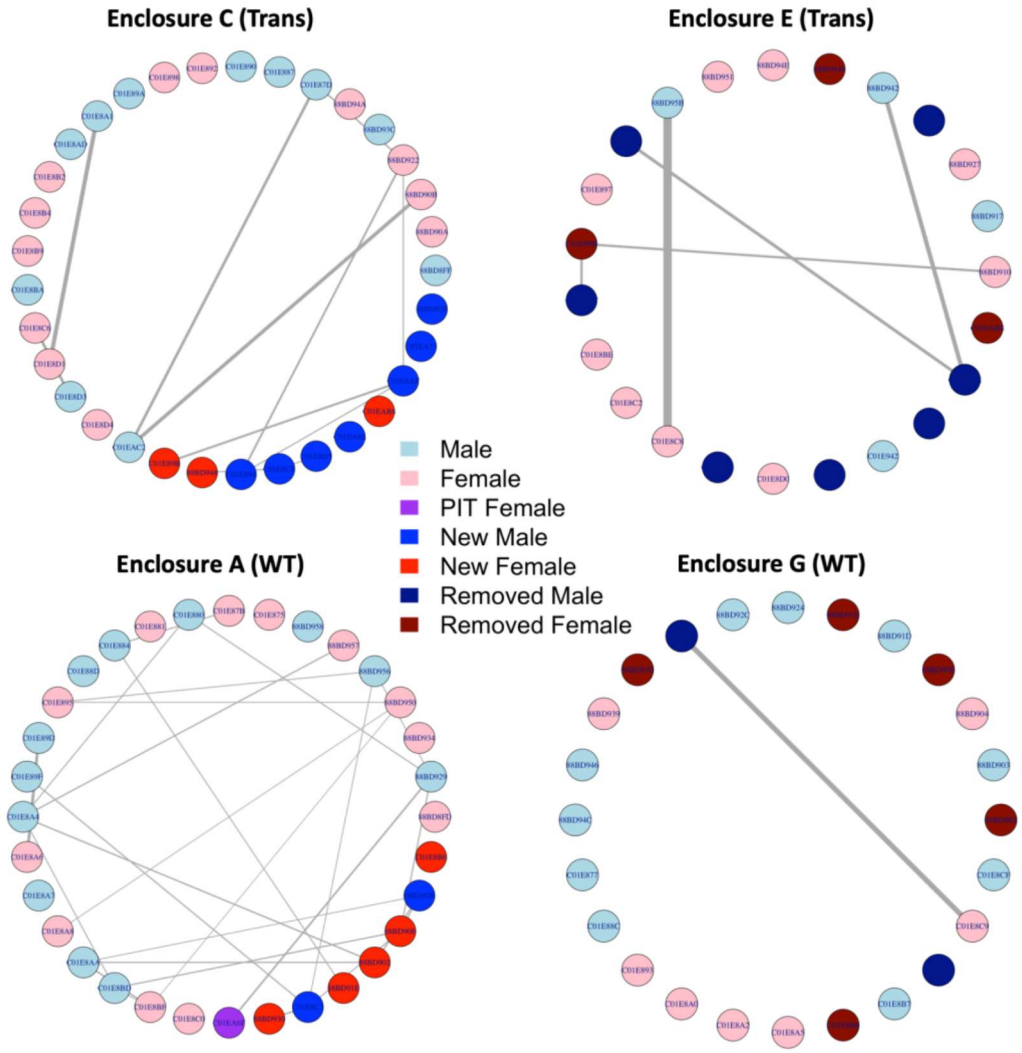

Figure 4. Vole social networks of all four enclosures constructed from trapping data throughout the experimental period. Each node represents an individual vole. A line connecting a pair of voles indicates presence of association, with thicker lines reflecting stronger associations (in this case, indicating more times for voles to be trapped together in the same trap or at the same rebar). "PIT Female" is a female vole that was inserted with a new PIT tag, but its former PIT tag identity is unknown. "New Male" and "New Female" are voles that were newly released into the enclosures during the combination period, while "Removed Male" and "Removed Female" are voles that were removed from the enclosures during the combination period.

- *Generated from Juxta Log Data*

The periods of time during which Juxta log data were successfully collected for each vole in each enclosure exhibits sparsity (Fig. 5). For the construction of social networks from Juxta log data, we segmented the data into three periods:

Period 1: From May 6 to May 8 (Time >= 2023-05-06 12:00:00 AM & Time < 2023-05-09 12:00:00 AM)

Period 2: From May 9 to June 26 (Time >= 2023-05-09 12:00:00 AM & Time < 2023-06-27 12:00:00 AM)

Period 3: From June 27 to July 28 (Time >= 2023-06-27 12:00:00 AM & Time < 2023-07-29 12:00:00 AM)

Based on data availability, we constructed social networks for Period 1 in all four enclosures, and for all three periods in enclosure A exclusively. Period 1 represents vole interactions during the initial three days after their release into the enclosures when most had functional Juxta loggers, and before our first trapping session on May 9. Period 2 captures vole interactions midway through the experiment when there was no disturbance. Period 3 illustrates vole interactions following the introduction or removal of voles during the vole combination when voles from enclosures G and E were put into enclosures A and C respectively from June 27 to July 8. The majority of data for this period were collected after July 20, coinciding with the availability of additional Juxta loggers for deploying on each trapped vole near the end of the experiment.

We constructed social networks for Period 1 of all four enclosures (Fig. 6). Enclosure E (containing transgenic voles) appeared to exhibit the highest number of associations, albeit with weak strengths, while the other three enclosures had fewer associations but with stronger strengths, particularly in the case of enclosures A and G containing wildtype voles. The majority of associations in all four enclosures were male-female pairs (6 in A, 19 in C, 33 in E, 13 in G), with some male-male (3 in A, 2 in C, 11 in E, 2 in G) and female-female pairs (4 in A, 6 in C, 18 in E, 4 in G) also observed.

We then constructed social networks for all three periods of enclosure A (containing only wild-type voles: Fig. 7). There seemed to be a notable change in vole social patterns across time, with no common associations between any two of the three periods. The majority of associations in all four enclosures were male-female pairs (6 in Period 1, 3 in Period 2, 11 in Period 3), with some male-male (3 in Period 1, 0 in Period 2, 0 in Period 3) and female-female pairs (4 in Period 1, 0 in Period 2, 1 in Period 3) also observed.

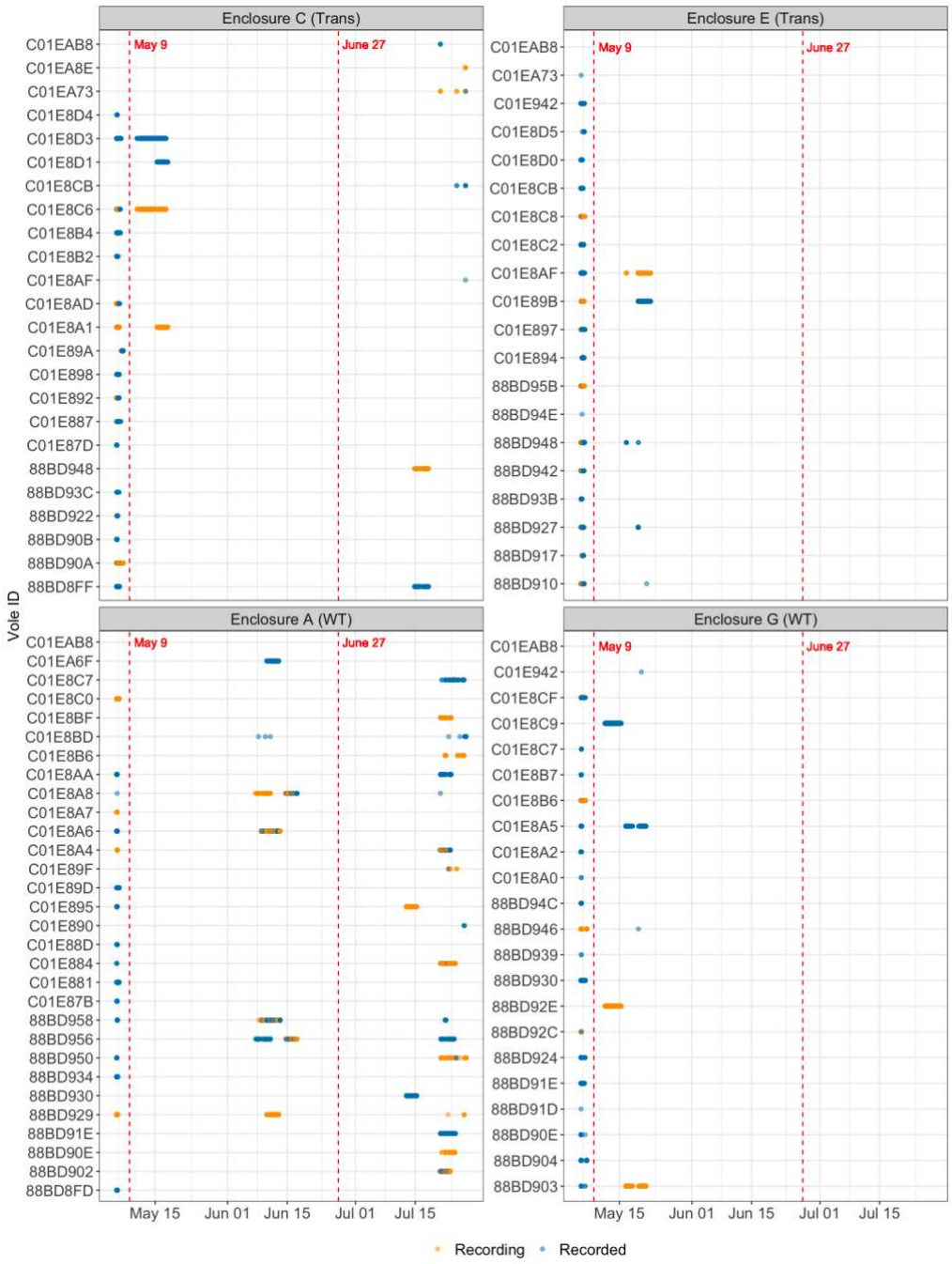

Figure 5. Periods of time when Juxta log data were successfully collected for each vole in each enclosure. “Recording” denotes voles (with devices) recording devices on other voles, while “Recorded” denotes voles (with devices) recorded by devices on other voles. The three periods of log data we used for constructing social networks were separated by the two red dashed lines (Period 1 was May 6 - 8 2023, Period 2 was May 9 - June 26 2023, Period 3 was June 27 - July 28 2023).

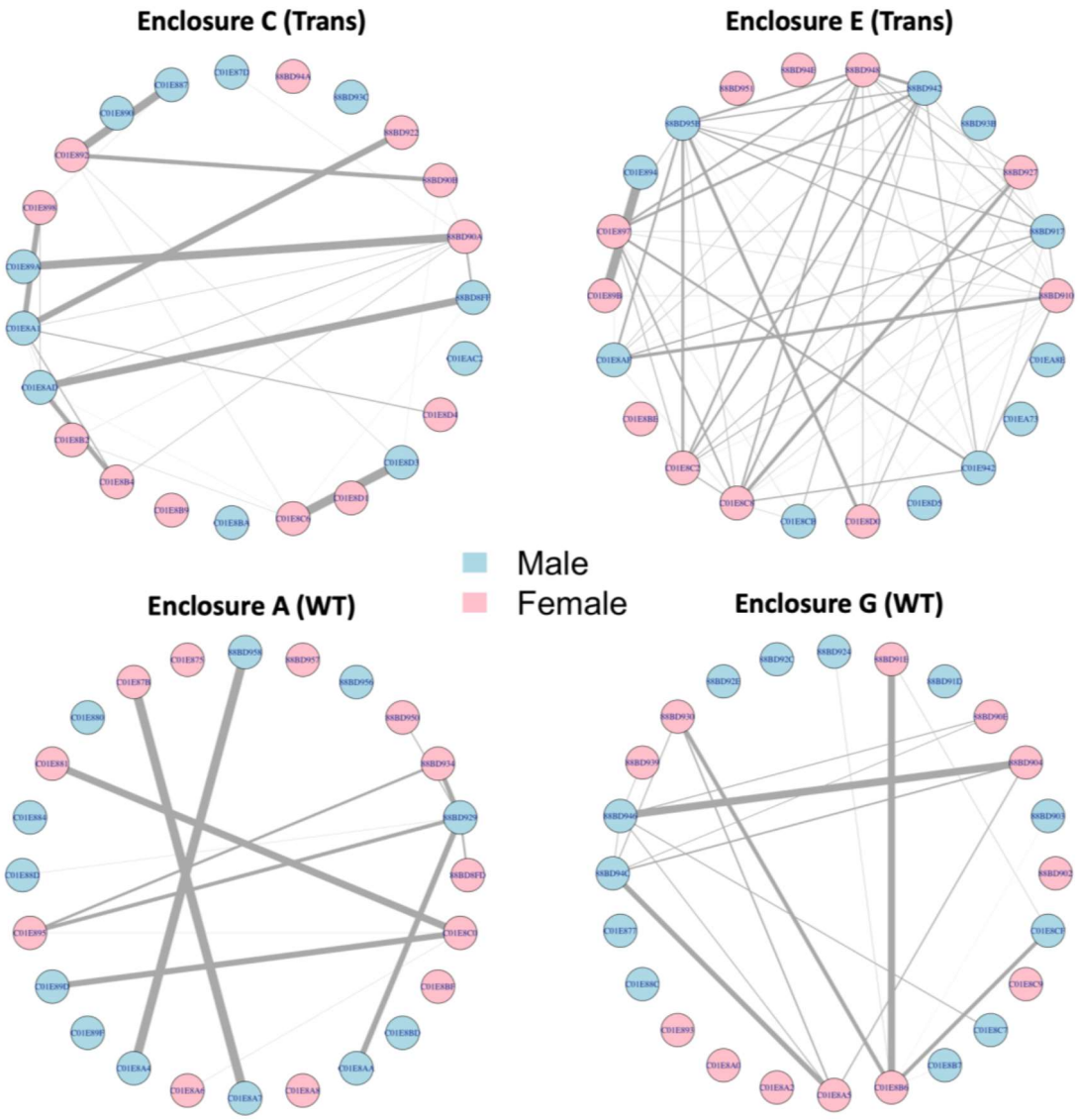

Figure 6. Vole social networks of all four enclosures constructed from Period 1 (from May 6 to May 8) of Juxta log data. Each node represents an individual vole. A line connecting a pair of voles indicates presence of association, with thicker lines reflecting stronger associations (in this case, indicating more time for voles to be recorded together by Juxta loggers).

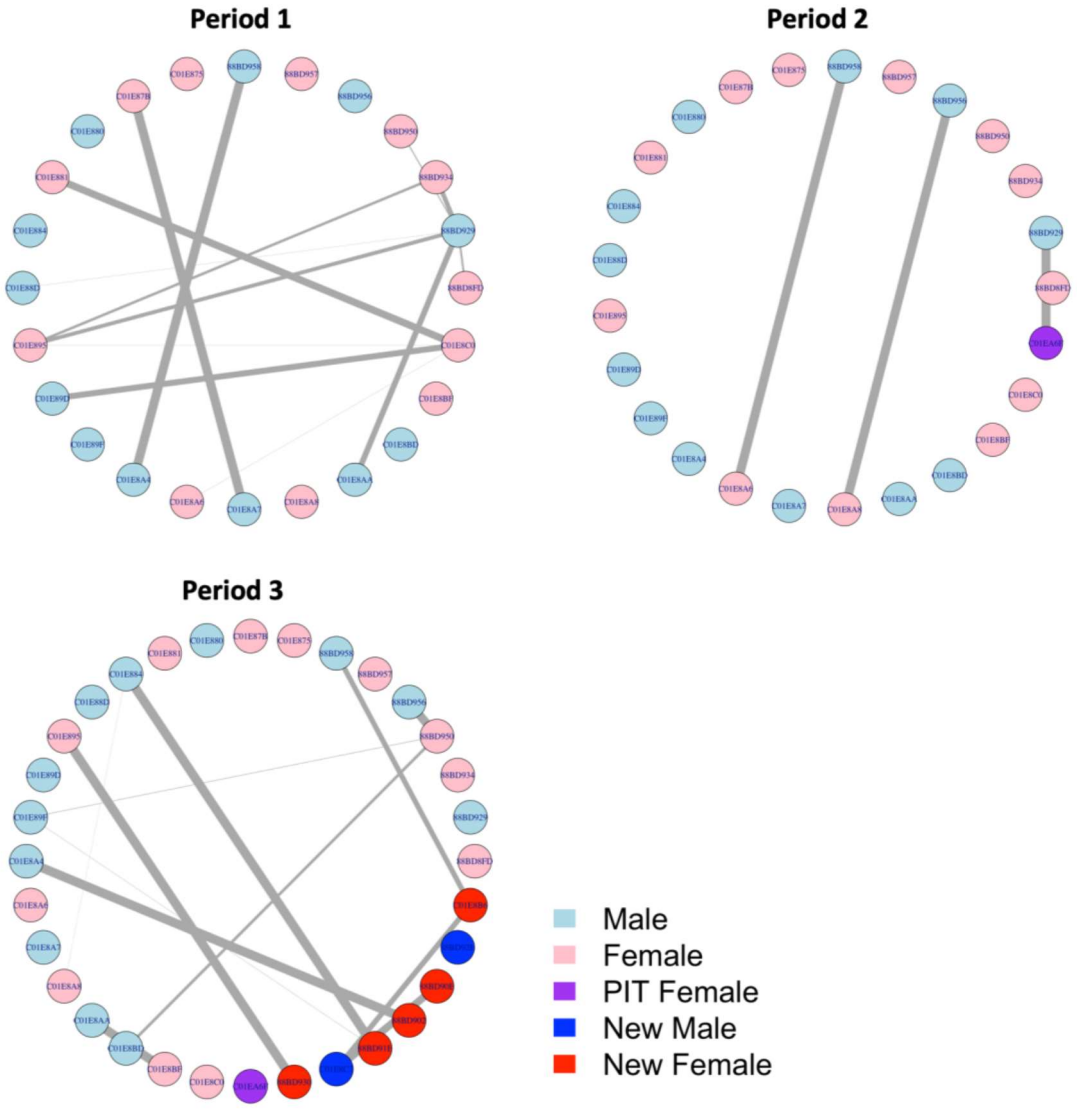

Figure 7. Vole social networks of enclosure A (containing only wild-type voles) constructed from all three periods of Juxta log data. Each node represents an individual vole. A line connecting a pair of voles indicates presence of association, with thicker lines reflecting stronger associations (in this case, indicating more time for voles to be recorded together by Juxta loggers).

Interaction Duration

The interaction duration estimated from the Juxta log data ranged from 2 to 783 s, with a mean of 92.4 s and a median of 60 s. The mean of the interaction duration recorded in transgenic voles was significantly shorter than that recorded in the wild-type voles ($t = -2.02$, $df = 3010.1$, $p = 0.044$ in the t-test). Interaction duration also increased

as the date increased (t = 4.86, df = 1176.0, p < 0.0001). The type of sex combination (e.g., female-male vs. male-male) and the interaction between strain and type of sex pair both significantly affected the interaction duration (Table 3). However, post-hoc comparisons showed that transgenic and wild-type voles did not differ significantly in the interaction duration for each type of sex pair. (Fig. 8; Table 4).

Table 3. Results of the linear mixed-effects model for vole interaction duration, with enclosure included as a random intercept (n = 37 transgenic voles, comprising 56 male & female pairs, 15 male & male pairs, and 24 female & female pairs; n = 40 wild-type, comprising 35 male & female pairs, 7 male & male pairs, and 9 female & female pairs). Intercept represents wild-type male-female interaction.

	Estimate	SE	df	t	p-value
Log (Interaction duration)					
Intercept	-0.01	0.11	4.43	-0.13	0.90
Strain (Transgenic)	0.08	0.16	4.39	0.53	0.62
Sex pair (Female-Female)	0.19	0.07	3117.61	2.64	**0.0085**
Sex pair (Male-Male)	0.08	0.17	2999.39	0.51	0.61
Date	0.13	0.03	1175.98	4.86	**< 0.0001**
Strain (Transgenic) × Sex pair (Female-Female)	-0.33	0.10	3114.18	-3.27	**0.0011**
Strain (Transgenic) × Sex pair (Male-Male)	-0.39	0.18	3043.32	-2.15	**0.032**

Table 4. Post-hoc comparisons of interaction duration between transgenic and wild-type voles within each category of sex pairs.

Transgenic - Wild-type	Estimate	SE	z-ratio	p-value
Male & Female	0.08	0.16	0.53	0.60
Male & Male	-0.30	0.23	-1.34	0.18
Female & Female	-0.25	0.18	-1.40	0.16

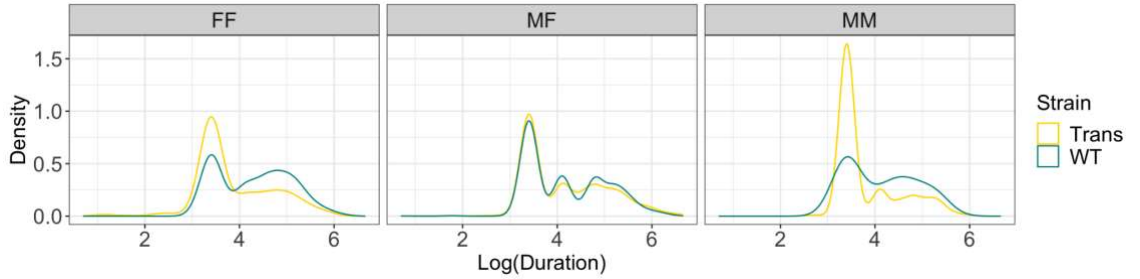

Figure 8. Density plot of interaction durations (in seconds) for voles of different strains for each type of sex pair (female-female, male-female and male-male) involved in interaction. On average, interaction durations recorded in the transgenic voles were significantly shorter than those in the wild-type voles, but no significant differences were observed within each type of sex pair (Table 4).

Interaction Distance

The RSSI values recorded by the Juxta log data ranged from -101 to -39 dBm, with a mean of -81.9 dBm and a median of -83 dBm. The mean of the RSSI values recorded in transgenic voles were significantly lower than that recorded in the wild-type voles ($t = -29.084$, $df = 12752$, $p < 0.0001$ in the t-test), indicating that the social interactions between transgenic voles occurred at greater distances than in the wild-type voles. RSSIs also increased as the date increased ($t = 20.97$, $df = 20300$, $p < 0.0001$). The type of sex pair and the interaction between strain and type of sex pair both significantly affected the interaction distance (Table 5). Post-hoc comparisons showed that transgenic female pairs exhibited significantly lower RSSIs than wild-type female pairs (z-ratio = -2.18, $p = 0.030$, Table 6; Fig. 9), while transgenic male pairs also displayed marginally significantly lower RSSIs than wild-type male pairs (z-ratio = -1.88, $p = 0.061$, Table 6; Fig. 9). These findings suggest that transgenic pairs of the same sex, particularly female-female pairs, interacted at significantly greater distances than their wild-type counterparts.

Table 5. Results of the linear mixed-effects model for RSSI values, with enclosure included as a random intercept (n = 39 transgenic voles, comprising 68 male & female pairs, 26 male & male pairs, and 19 female & female pairs; n = 45 wild-type, comprising 112 male & female pairs, 34 male & male pairs, and 54 female & female pairs). Intercept represents wild-type male-female interaction at hour 0/24.

	Estimate	SE	df	t	p-value
Log (Interaction duration)					
Intercept	-0.05	0.11	4.36	-0.43	0.69
Strain (Transgenic)	-0.10	0.15	4.07	-0.68	0.54
Sex pair (Female-Female)	-0.09	0.03	22280	-3.30	**0.0010**
Sex pair (Male-Male)	0.02	0.06	22260	0.32	0.75
Date	0.18	0.01	20300	20.97	< **0.0001**
Hour1	0.12	0.03	22280	3.91	< **0.0001**
Hour2	0.21	0.03	22280	6.28	< **0.0001**
Hour3	0.32	0.03	22280	10.05	< **0.0001**
Hour4	0.31	0.03	22280	10.42	< **0.0001**
Hour5	0.38	0.03	22280	12.69	< **0.0001**
Hour6	-0.13	0.04	22280	-3.35	**0.0008**
Hour7	0.19	0.03	22280	6.29	< **0.0001**
Hour8	0.31	0.03	22280	9.74	< **0.0001**
Hour9	0.25	0.03	22280	7.28	< **0.0001**
Hour10	0.33	0.04	22280	9.04	< **0.0001**
Hour11	0.28	0.04	22280	7.15	< **0.0001**
Hour12	0.11	0.04	22280	2.77	**0.0056**
Hour13	0.18	0.04	22280	4.37	< **0.0001**

Hour14	0.15	0.05	22280	3.03	**0.0025**
Hour15	0.31	0.05	22280	5.92	**< 0.0001**
Hour16	0.30	0.06	22280	5.37	**< 0.0001**
Hour17	0.18	0.06	22280	3.03	**0.0025**
Hour18	0.21	0.06	22280	3.48	**0.0005**
Hour19	0.67	0.05	22280	12.93	**< 0.0001**
Hour20	0.43	0.06	22280	7.34	**< 0.0001**
Hour21	0.22	0.06	22280	3.88	**0.0001**
Hour22	0.51	0.05	22280	10.44	**< 0.0001**
Hour23	0.56	0.05	22280	10.75	**< 0.0001**
Strain (Transgenic) × Sex pair (Female-Female)	-0.23	0.03	22280	-6.97	**< 0.0001**
Strain (Transgenic) × Sex pair (Male-Male)	-0.20	0.06	22270	-3.28	**0.0010**

Table 6. Post-hoc comparisons of RSSI values between transgenic and wild-type voles within each category of sex pairs.

Transgenic - Wild-type	Estimate	SE	z-ratio	p-value
Male & Female	-0.10	0.15	-0.67	0.50
Male & Male	-0.31	0.16	-1.88	0.061
Female & Female	-0.34	0.15	-2.18	**0.030**

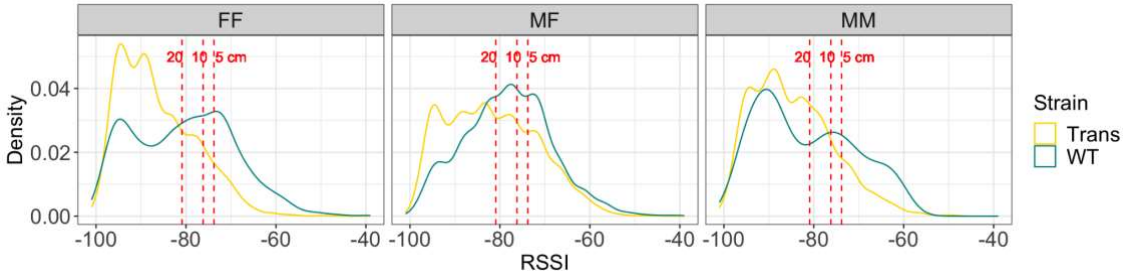

Figure 9. Density plot of RSSI values (in dBm) for voles of different strains for each type of sex pair (female-female, male-female, male-male) involved in interaction. On average, RSSIs recorded in the transgenic voles were significantly lower than those in the wild-type voles, primarily due to differences in female-female interactions (Table 6). Red dashed lines indicated estimated distances in centimeters.

Activity Level

Vole activity levels exhibited a bimodal pattern, with peaks observed around 0600 h at dawn and 2000 h at dusk (Fig. 10). Both female and male voles showed low activity levels during the night, followed by a notable increase from hours 0500 to 0600 h. This increase was succeeded by a slight decrease and subsequent rise in activity during the afternoon. Finally, a significant decrease in activity was observed from 2000 to 2200 h.

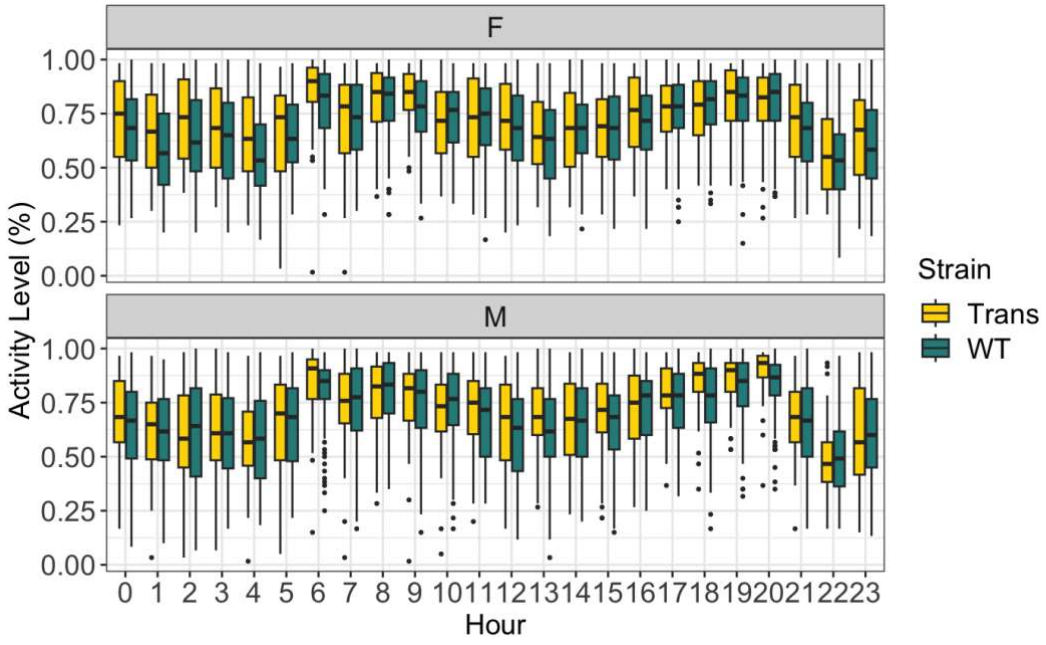

Figure 10. Vole activity levels throughout the day. Activity levels for each hour were measured as the percentage of time when we had "xl" records (i.e., count of "xl" per hour divided by 60).

Statistical analysis revealed significant differences in activity levels for females at hours 1000, 1100, and 1800, where transgenic females were significantly less active than wild-type females (Table 7, Fig. 10). Similarly, for males, significant differences were observed at hours 1000, 1800, 1900, and 2000, where transgenic males were significantly less active at 1000 h, but significantly more active at 1800, 1900, and 2000 h than wild-type males (Table 8, Fig. 10).

Table 7. Post-hoc comparisons of activity levels between female voles of different strains for each hour of the day, derived from the results of the generalized linear mixed-effects model fitted to female activity levels (n = 28 females; comprising 14 transgenic and 14 wild-type individuals).

Transgenic / Wild-type	Odds ratio	SE	z-ratio	p-value
Hour = 0/24	0.95	0.13	-0.37	0.71
Hour = 1	1.10	0.15	0.72	0.47
Hour = 2	1.24	0.17	1.60	0.11
Hour = 3	0.98	0.13	-0.12	0.91
Hour = 4	1.16	0.16	1.09	0.27
Hour = 5	0.87	0.12	-1.05	0.30
Hour = 6	1.13	0.16	0.90	0.37
Hour = 7	0.80	0.11	-1.65	0.099
Hour = 8	0.83	0.11	-1.39	0.17
Hour = 9	1.27	0.18	1.71	0.087
Hour = 10	0.73	0.10	-2.31	**0.021**
Hour = 11	0.75	0.10	-2.11	**0.035**
Hour = 12	0.89	0.12	-0.86	0.39
Hour = 13	0.92	0.12	-0.59	0.55
Hour = 14	0.83	0.11	-1.38	0.17

Hour = 15	0.79	0.11	-1.72	0.086
Hour = 16	0.97	0.13	-0.20	0.84
Hour = 17	0.82	0.11	-1.43	0.15
Hour = 18	0.73	0.10	-2.25	**0.024**
Hour = 19	0.93	0.13	-0.56	0.58
Hour = 20	0.79	0.11	-1.68	0.093
Hour = 21	1.11	0.15	0.73	0.47
Hour = 22	0.99	0.13	-0.11	0.92
Hour = 23	1.03	0.14	0.21	0.83

Table 8. Post-hoc comparisons of activity levels between male voles of different strains for each hour of the day, derived from the results of the generalized linear mixed-effects model fitted to male activity levels (n = 30 males; comprising 14 transgenic and 16 wild-type individuals).

Transgenic / Wild-type	Odds ratio	SE	z-ratio	p-value
Hour = 0/24	1.13	0.13	1.07	0.29
Hour = 1	1.06	0.12	0.54	0.59
Hour = 2	0.94	0.10	-0.55	0.58
Hour = 3	0.93	0.10	-0.65	0.52
Hour = 4	0.89	0.10	-1.09	0.28
Hour = 5	0.89	0.10	-1.00	0.32
Hour = 6	1.25	0.15	1.94	0.053
Hour = 7	0.94	0.11	-0.51	0.61
Hour = 8	0.86	0.10	-1.29	0.20
Hour = 9	1.12	0.13	0.99	0.32

Hour = 10	0.78	0.09	-2.21	**0.027**
Hour = 11	1.17	0.13	1.40	0.16
Hour = 12	1.15	0.13	1.24	0.21
Hour = 13	1.24	0.14	1.90	0.058
Hour = 14	0.95	0.11	-0.48	0.64
Hour = 15	1.11	0.12	0.94	0.35
Hour = 16	0.93	0.10	-0.65	0.52
Hour = 17	1.11	0.13	0.90	0.37
Hour = 18	1.59	0.18	3.98	**0.0001**
Hour = 19	1.30	0.15	-0.56	**0.024**
Hour = 20	1.69	0.20	-1.68	**< 0.0001**
Hour = 21	1.00	0.11	0.73	1.00
Hour = 22	0.83	0.09	-0.11	0.10
Hour = 23	0.87	0.10	0.21	0.20

Device Temperature

Both female and male voles displayed a significant decrease in mean device temperature from 0500 to 0600 h (likely reflecting exit from the burrow/nest), elevated temperatures around noon and in the afternoon (likely reflecting increases in temperature during the day), and a subsequent increase after 2000 h (likely reflecting when voles entered into their burrow/nest) (Fig. 11). On average, transgenic females consistently exhibited lower device temperatures than wild-type females throughout the entire day.

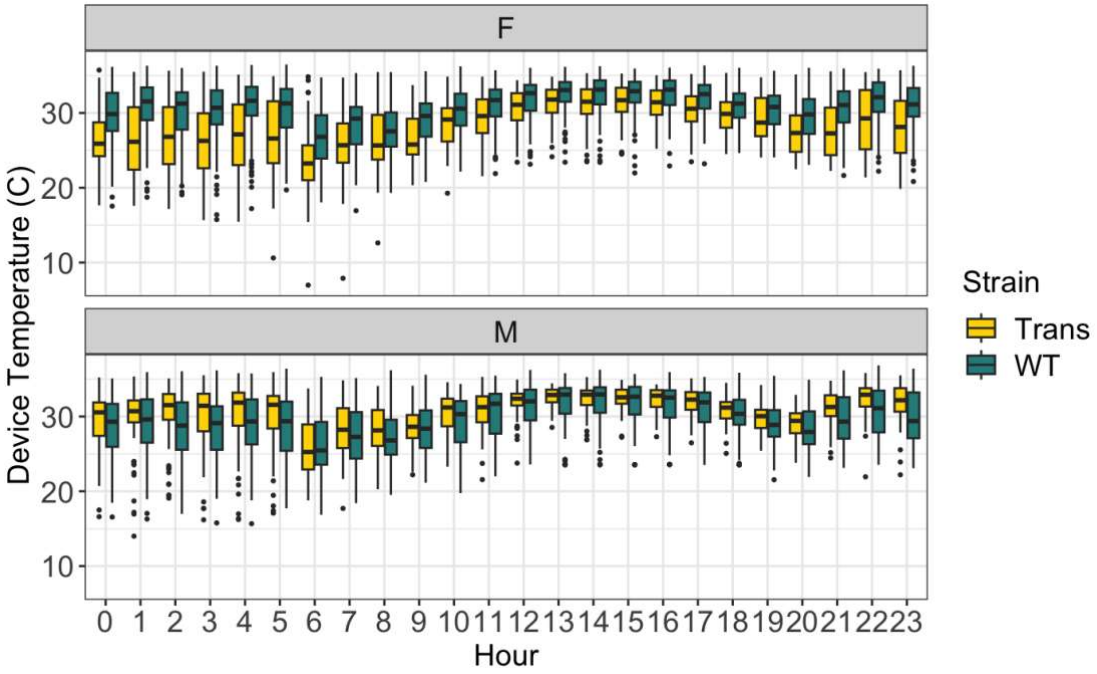

Figure 11. Device temperature throughout the day. While the device temperature did not directly represent true vole body temperature, it likely reflected certain behavioral aspects through temperature changes such as an increase in device temperature when the vole entered an insulated nest or burrow.

Statistical analysis revealed significant differences in device temperature for females at hours 2100, 2200, 2300, 0000, 0100, 0200, 0300, 0400, 0500, and 0600, where transgenic females had significantly lower device temperatures than wild-type females (Table 9). However, for males, there was no significant difference in device temperature between the two strains (Table 10).

Table 9. Post-hoc comparisons of device temperature between female voles of different strains for each hour of the day, derived from the results of the linear mixed-effects model fitted to female device temperature (n = 27 females; comprising 13 transgenic and 14 wild-type individuals).

Transgenic - Wild-type	Estimate	SE	z-ratio	p-value
Hour = 0/24	-0.46	0.16	-2.83	**0.0046**
Hour = 1	-0.64	0.16	-3.92	**0.0001**
Hour = 2	-0.53	0.16	-3.23	**0.0012**

Hour = 3	-0.53	0.16	-3.28	**0.0011**
Hour = 4	-0.64	0.16	-3.93	**0.0001**
Hour = 5	-0.53	0.16	-3.21	**0.0013**
Hour = 6	-0.38	0.16	-2.31	**0.021**
Hour = 7	-0.26	0.16	-1.56	0.12
Hour = 8	-0.03	0.16	-0.19	0.85
Hour = 9	-0.17	0.16	-1.07	0.29
Hour = 10	-0.05	0.16	-0.32	0.75
Hour = 11	-0.06	0.16	-0.40	0.69
Hour = 12	0.03	0.16	0.20	0.84
Hour = 13	0.06	0.16	0.40	0.69
Hour = 14	-0.01	0.16	-0.09	0.93
Hour = 15	0.09	0.16	0.57	0.57
Hour = 16	0.00	0.16	0.00	1.00
Hour = 17	-0.09	0.16	-0.55	0.58
Hour = 18	0.02	0.16	0.15	0.88
Hour = 19	-0.03	0.17	-0.18	0.86
Hour = 20	-0.17	0.17	-1.04	0.30
Hour = 21	-0.44	0.17	-2.63	**0.0086**
Hour = 22	-0.44	0.17	-2.60	**0.0092**
Hour = 23	-0.49	0.17	-2.89	**0.0038**

Table 10. Post-hoc comparisons of device temperature between male voles of different strains for each hour of the day, derived from the results of the linear mixed-effects model fitted to male device temperature (n = 29 males; comprising 13 transgenic and 16 wild-type individuals).

Transgenic - Wild-type	Estimate	SE	z-ratio	p-value
Hour = 0/24	1.40	1.71	0.82	0.41
Hour = 1	1.34	1.71	0.79	0.43
Hour = 2	1.60	1.71	0.94	0.35
Hour = 3	1.57	1.71	0.92	0.36
Hour = 4	1.47	1.71	0.86	0.39
Hour = 5	1.54	1.71	0.90	0.37
Hour = 6	1.15	1.71	0.67	0.50
Hour = 7	1.41	1.71	0.82	0.41
Hour = 8	1.41	1.71	0.82	0.41
Hour = 9	1.27	1.71	0.75	0.46
Hour = 10	1.47	1.71	0.86	0.39
Hour = 11	1.31	1.71	0.77	0.44
Hour = 12	1.33	1.71	0.78	0.44
Hour = 13	1.41	1.71	0.83	0.41
Hour = 14	1.26	1.71	0.74	0.46
Hour = 15	1.30	1.71	0.76	0.45
Hour = 16	1.33	1.71	0.78	0.43
Hour = 17	1.38	1.71	0.81	0.42
Hour = 18	1.32	1.71	0.77	0.44

Hour = 19	1.34	1.71	0.79	0.43
Hour = 20	1.32	1.71	0.77	0.44
Hour = 21	1.58	1.71	0.92	0.36
Hour = 22	1.62	1.71	0.95	0.34
Hour = 23	1.68	1.71	0.99	0.32

Device Temperature vs. Activity Levels

By plotting device temperature and activity levels together with respect to hours in a day (Fig. 12), we observed that at 0600 h, there was a lot of movement but low device temperature, likely suggesting that the voles were coming out of their nests around this time. Conversely, at 2200 h, there was less movement but relatively high device temperature, likely suggesting that the voles were staying in their nests where their body heat elevated the device temperatures.

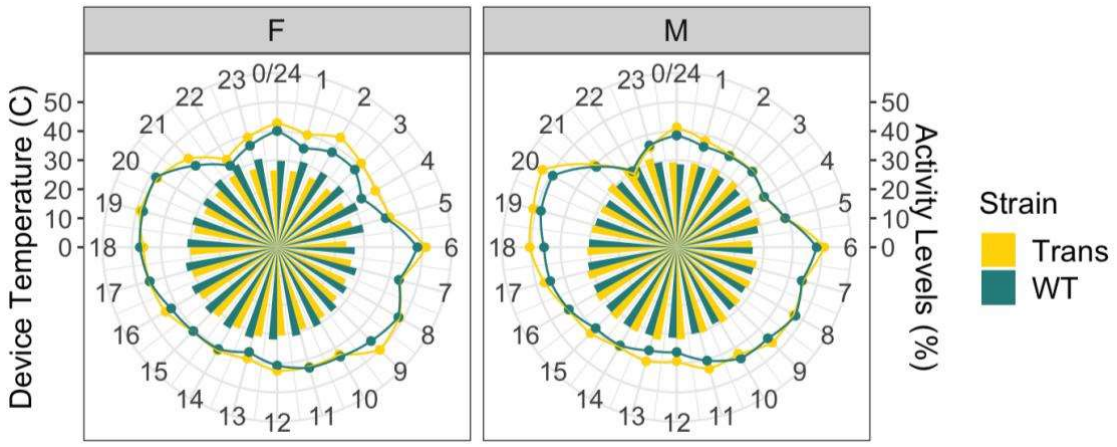

Figure 12. Device temperature (represented by bars) vs. vole activity levels (represented by lines) throughout the day. Simultaneous shifts in both measures around 0600 h and 2200 h suggest coming out of and into the burrow/nest respectively.

Trapping Effort

Throughout the field season, trapping efforts were consistent across the four enclosures where we typically aimed for 2 trapping sessions each enclosure per week (Fig. 13). From May 5 to September 11 2023, we conducted a total of 60 trapping sessions in total, comprising 57 overnight sessions (typically setting traps at dusk and checking them at dawn) and 3 morning sessions (typically setting at 0630 h and checking two hours later). Enclosures A & C were trapped a total of 38 times, while enclosures E & G were trapped a total of 39 times. The total number of rebar traps and nest traps set in each trapping session ranged from 75 to 448, with an average of 257 ± 15.4 SE traps set per trapping session (Fig. 14). The total number of unique adult voles captured each trapping session ranged from 0 to 23, with an average of 8 ± 0.9 SE voles per session. We removed all adult voles and any pups captured from the enclosures from August 2 to 5 2023 when we ended data collection and no adult voles were captured after August 5 2023 (indicated by the red dashed line in Figs. 13-16, 22).

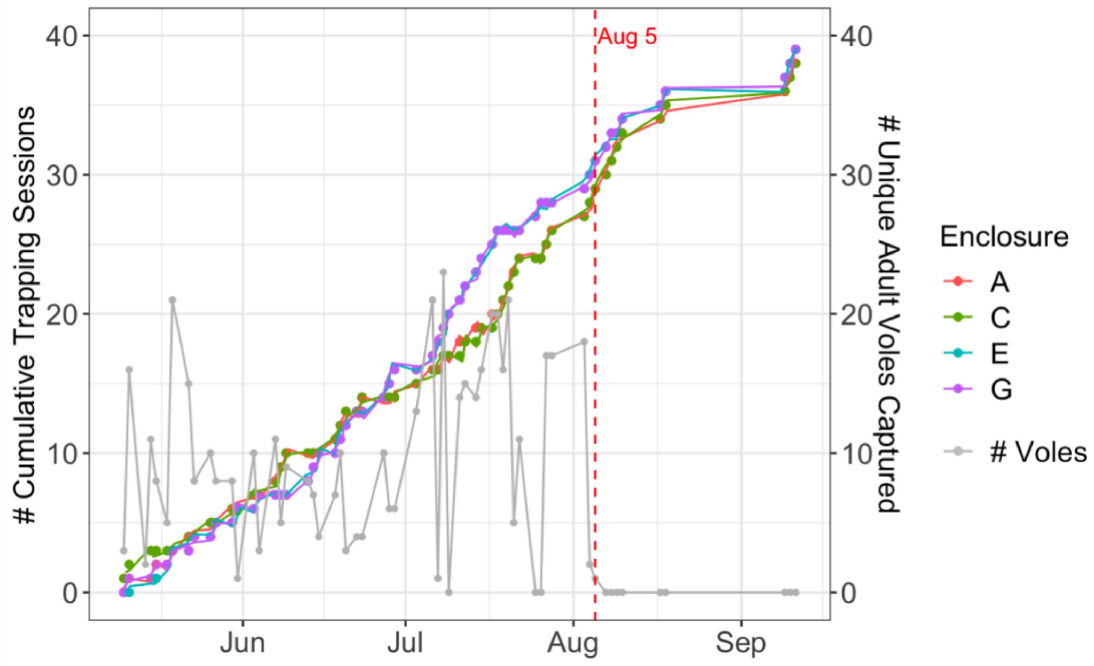

Figure 13. Cumulative trapping sessions conducted and number of unique adult voles captured in each of the four enclosures over the course of the field experiment. On average, 8 ± 0.9 SE voles were captured per trapping session. The red dashed line indicates the end of our experimental period on August 5 2023 when we removed all adult voles from the enclosures.

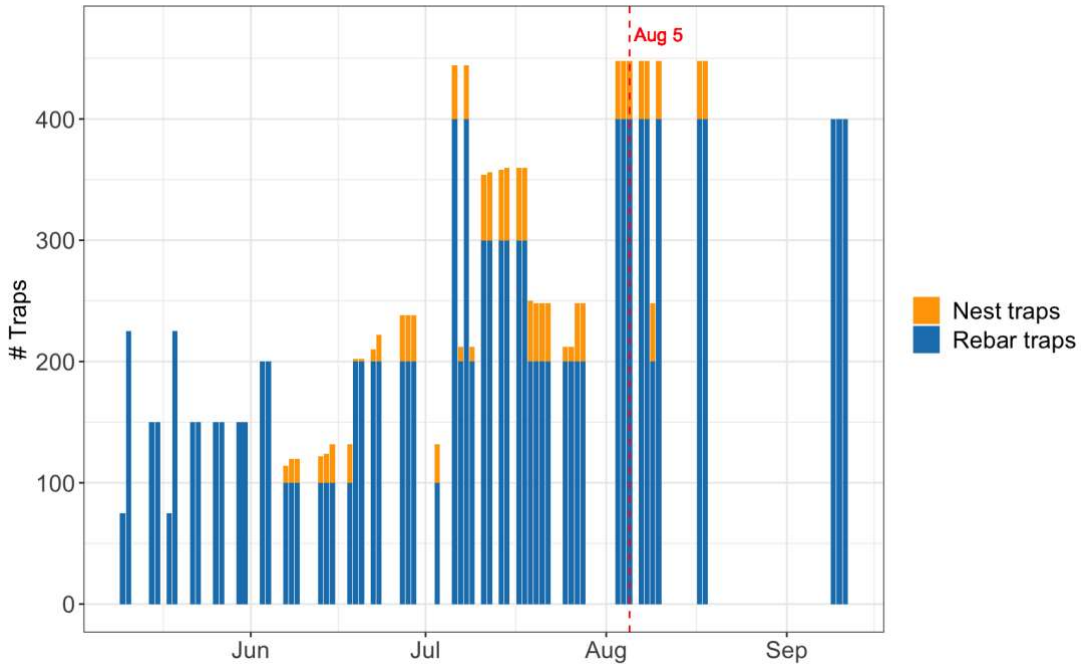

Figure 14. Number of traps set either at trapping stakes (rebar) or at vole nests in each trapping session. On average, 257 ± 15.4 SE traps were set per session with nearly all of them being overnight trapping sessions. The red dashed line indicates the end of our experimental period on August 5 2023 when we removed all adult voles from the enclosures. 9 additional trapping sessions were conducted afterward to ensure the removal of all voles.

Out of the 93 PIT tags assigned to the founding voles, 22 (3 females out of the 12 females and 1 male out of the 12 males in enclosure A; 4 males out of the 11 males in enclosure C; 3 females out of the 11 females in enclosure E; 4 females out of the 12 females and 4 males out of the 12 males in enclosure G) were not recorded again after release. Given the intensity of our monitoring using live trapping, their disappearance was likely due to voles not surviving in the enclosures. Because 4 new PIT tags were later inserted, with 2 of them having unknown correspondence to the founding tags, we conclude that 20 voles were never recaptured after release. This likely reflects death rather than disappearance or an inability to capture these voles. Before the end of June, we tended to capture more females than males for both transgenic and wild-type voles, but after that, we tended to capture more males than females (Fig. 15). The first vole pups emerged in enclosure A on June 23, which corresponds to 7 weeks after we put the voles into the enclosures. From that point onward, we captured a total of 39 unique pups in enclosure A, 13 in enclosure C, 12 in enclosure E, and 8 in enclosure G (Fig. 16).

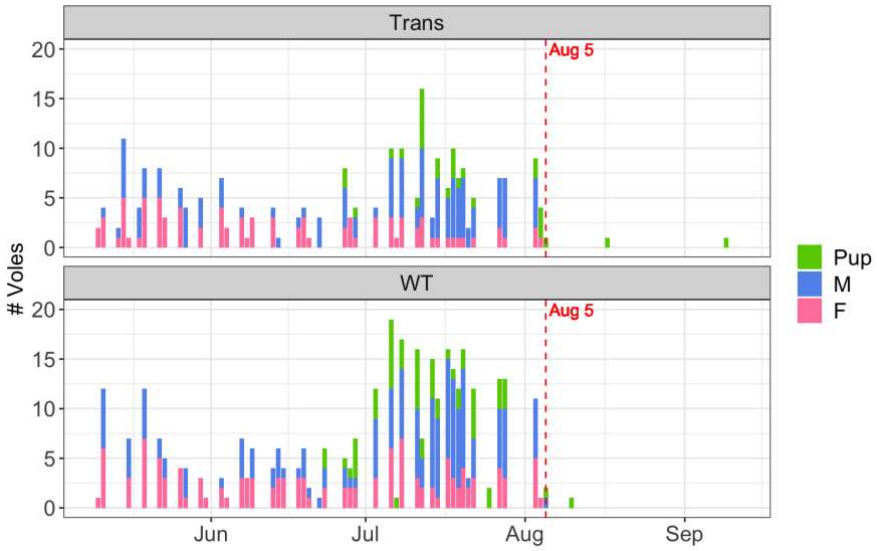

Figure 15. Number of male, female voles and pups captured per trapping session, categorized by strain (transgenic or wild-type). The red dashed line indicates the end of our experimental period on August 5 2023 when we removed all adult voles from the enclosures and then followed by trapping several more times (Fig. 13) after this date to confirm the absence of any voles.

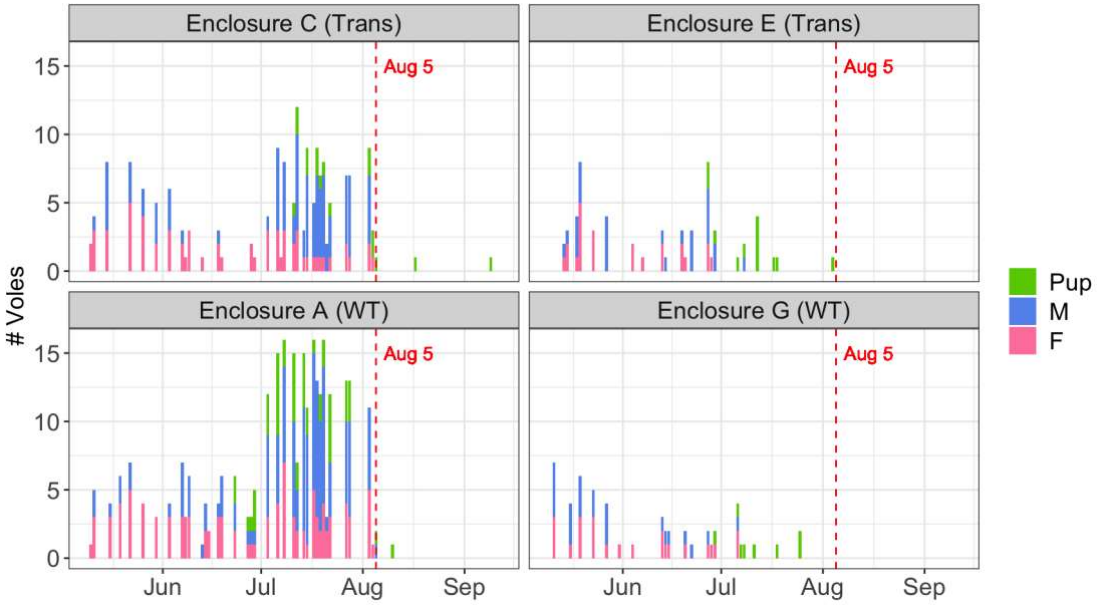

Figure 16. Number of male, female voles and pups captured per trapping session, categorized by enclosure (enclosures C and E are transgenic, enclosures A and G are wild-type). The red dashed line indicates the end of our experimental period on August 5 2023 when we removed all adult voles from the enclosures.

Trappability

The number of times each individual was trapped ranged from 0 to 22, with a mean of 5.1 ± 0.6 SE times. Voles that experienced more trapping sessions during the experimental period were trapped more times, which is consistent with our expectations ($t = 10.06$, $df = 91.83$, $p < 0.0001$, Table 11). Individual female voles were trapped fewer times than males, although this difference is marginally significant ($t = -1.73$, $df = 88.09$, $p = 0.086$, Table 11). Neither strain nor the interaction between sex and strain, significantly affected the number of times individuals were trapped (Table 11). The lack of interaction between sex and strain indicates that the difference in trapped times between wild-type and transgenic voles did not depend on sex. In other words, the effect of being wild-type or transgenic on the voles' willingness to enter traps or trappability is similar for both male and female voles (Fig. 17).

Table 11. Results of the linear mixed-effects model for the number of times each vole was trapped, with enclosure included as a random intercept (n = 39 females, n = 36 males; n = 39 transgenic, n = 36 wild-type; among which 17 voles had lived in two enclosures before and after we combined voles from enclosures G and E into enclosures A and C). Intercept represents a wild-type male.

	Estimate	SE	df	t	p-value
Number of times trapped					
Intercept	0.19	0.22	6.97	0.90	0.40
Sex (F)	-0.31	0.18	88.09	-1.73	0.086
Strain (Transgenic)	-0.29	0.29	8.21	-0.99	0.35
Number of trapping sessions	0.69	0.07	91.83	10.06	**< 0.0001**
Sex (F) × Strain (Transgenic)	0.24	0.24	88.11	1.00	0.32

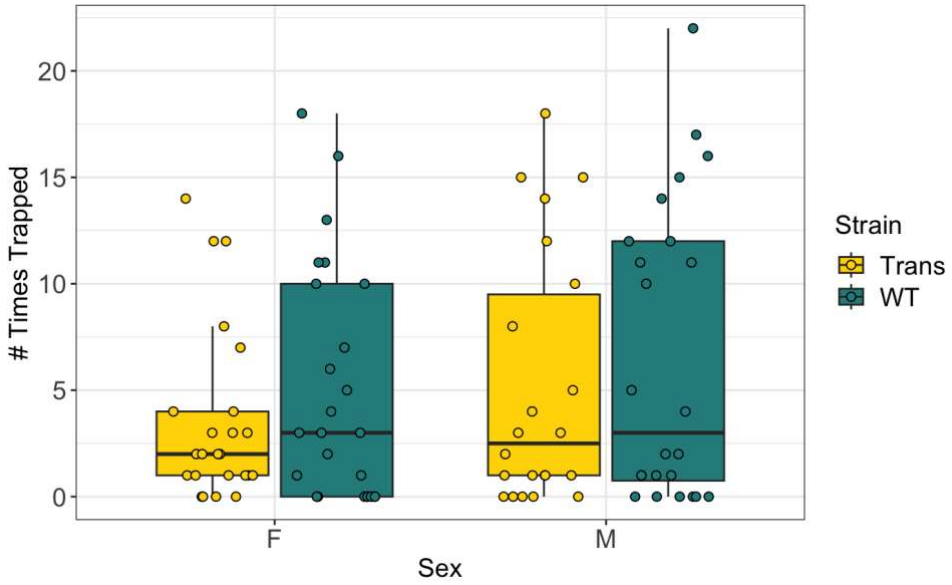

Figure 17. Boxplot of the number of times each individual vole was trapped. Individual female voles were trapped fewer times than males with marginal significance. Transgenic voles did not significantly differ from wild-type voles in terms of trappability, regardless of sex (Table 11).

Home Range Size

Vole home ranges were constructed from their trapping locations using minimum convex polygons (Fig. 18).

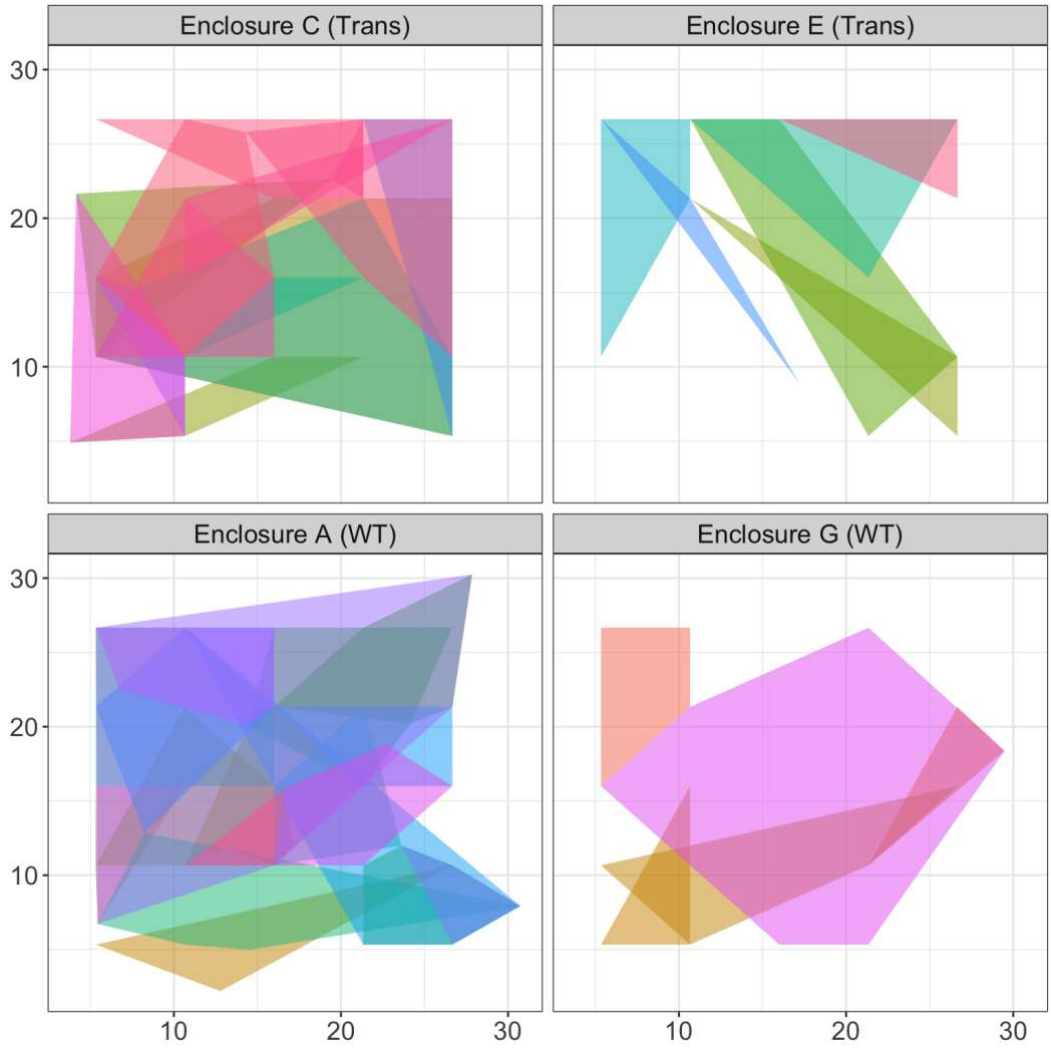

Figure 18. Vole home ranges in all four enclosures constructed by minimum convex polygon. Each color represents an individual vole captured a minimum of three times. Voles captured three times at the same place had a home range of 0 assigned to them.

The estimated vole home range sizes ranged from 0 to 346.0 m^2, with a mean of 63.3 ± 9.9 m^2 and a median of 51.3 m^2. Voles that were trapped more times had larger home ranges ($t = 4.71$, $df = 31.23$, $p = 0.0003$, Table 12), which is consistent with expectations. Neither sex nor strain, nor the interaction between sex and strain, significantly affected individual home range sizes (Table 12). The lack of interaction between sex and strain indicates that the difference in the home range sizes between wild-type and transgenic voles did not depend on sex. In other words, the effect of being wild-type or transgenic on the voles' home range sizes is similar for both male and female voles (Fig. 19).

Table 12. Results of the linear mixed-effects model for vole home range sizes, with enclosure included as a random intercept (n = 22 females, n = 20 males; n = 19 transgenic, n = 23 wild-type; among which 11 voles had lived in two enclosures before and after we combined voles from enclosures G and E into enclosures A and C). Home range sizes were estimated from trapping locations using minimum convex polygons. Intercept represents a wild-type male.

	Estimate	SE	df	t	p-value
Log (Home range sizes)					
Intercept	-0.38	0.31	45	-1.28	0.21
Sex (F)	-0.01	0.34	45	-0.36	0.72
Strain (Transgenic)	0.41	0.37	45	0.94	0.35
Number of times trapped	0.60	0.13	45	3.93	**0.0003**
Sex (F) × Strain (Transgenic)	-0.50	0.46	45	-0.03	0.98

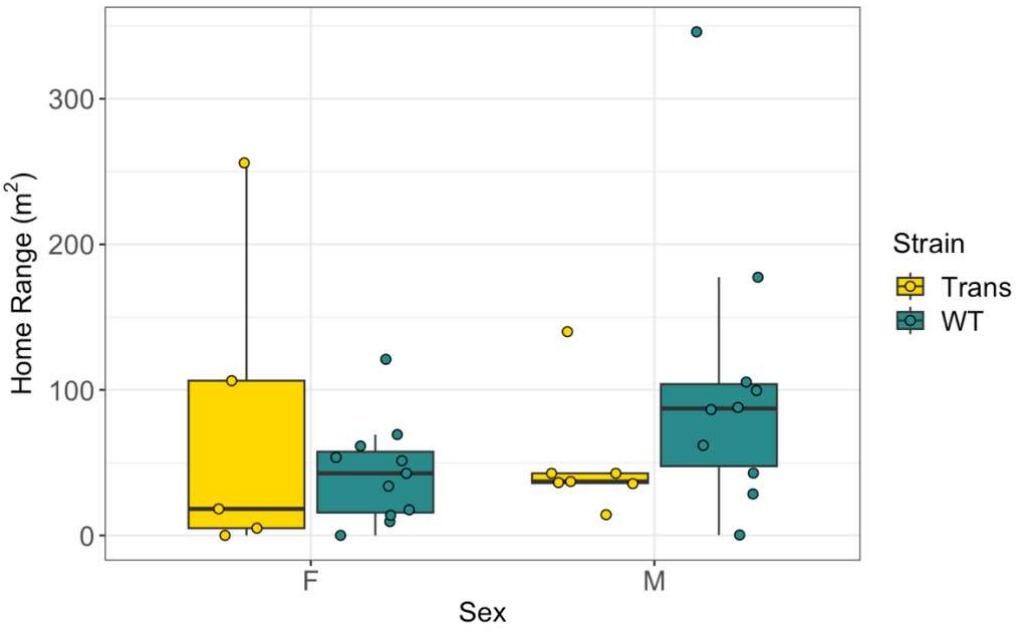

Figure 19. Boxplot of vole home range sizes. Transgenic voles did not significantly differ from wild-type voles in terms of home range sizes, regardless of sex (Table 12). Voles with a home range size of 0 m^2 were trapped three or more times at the same location.

Vole Body Weight

- *Mean Vole Body Weight Over Entire Experimental Period*

The body weight of adult female voles ranged from 24.5 to 61.3 grams, with a mean of 41.4 ± 1.1 grams. The weight of adult male voles ranged from 25.5 to 63.1 grams, with a mean of 43.2 ± 1.2 grams. For both females and males, weight increased with date, but at a decreasing rate (as indicated by the negative coefficient for Date^2 in Table 13, 14). For both females and males, individuals that were heavier at initial release into the enclosures were also heavier throughout the experimental period ($t = 8.04$, $df = 9.49$, $p < 0.0001$, Table 13; $t = 8.21$, $df = 32.47$, $p < 0.0001$, Table 14). For females, there was no statistically significant difference in weight between different strains, and no interaction effect between strain and date or strain and date^2, indicating no time-dependent differences in the effect of strain on mean vole weight (Table 13). However, transgenic males tended to weigh less than wild-type males throughout the experimental period, although this difference is marginally significant ($t = -1.85$, $df = 23.07$, $p = 0.078$, Table 14; Fig. 20). Still, there was no interaction effect between strain and date or strain and date^2 for males.

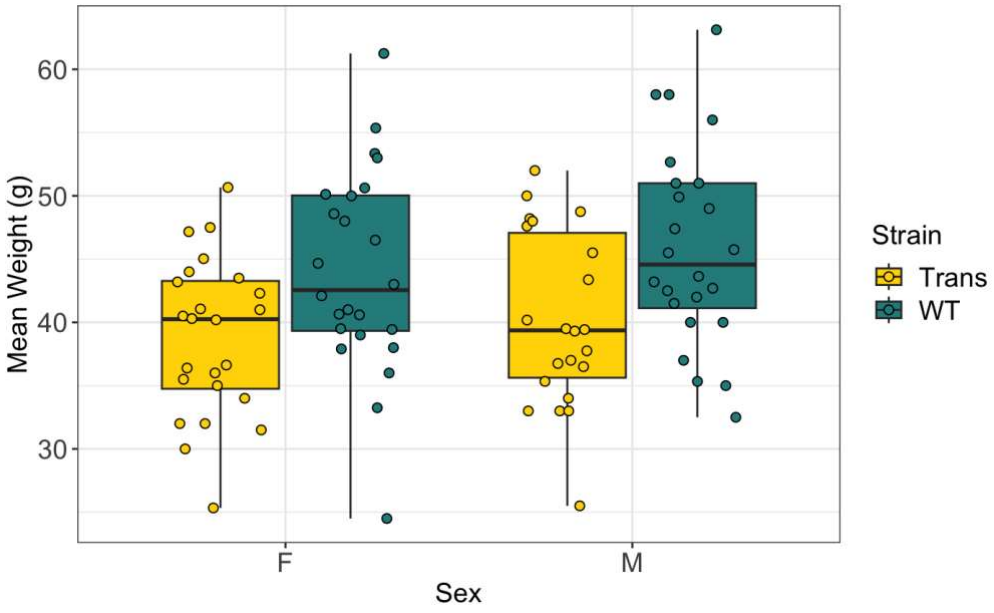

Figure 20. Boxplot of mean vole weights over the entire experimental period. Transgenic females and males tended to weigh less than their wild-type counterparts. However, only the difference between transgenic and wild-type males was marginally significant (Table 14).

Table 13. Results of the linear mixed-effects model for mean female vole weights over the entire experimental period, with PIT tag ID included as a random intercept, date and date^2 as random slopes (n = 36 females; comprising 19 transgenic and 17 wild-type individuals). Intercept represents a wild-type female.

	Estimate	SE	df	t	p-value
Log (Weight)					
Intercept	0.70	0.15	10.11	4.61	**0.0009**
Strain (Transgenic)	0.05	0.24	12.68	0.23	0.82
Date	0.61	0.09	9.78	6.53	**< 0.0001**
Date^2	-0.48	0.09	6.37	-5.37	**0.0014**
Initial weight	0.61	0.08	9.49	8.04	**< 0.0001**
Strain (Transgenic) × Date	-0.19	0.15	8.34	-1.27	0.24
Strain (Transgenic) × Date^2	-0.06	0.14	7.26	-0.41	0.70

Table 14. Results of the linear mixed-effects model for mean male vole weights over the entire experimental period, with PIT tag ID included as a random intercept, date and date^2 as random slopes (n = 35 males; comprising 17 transgenic and 18 wild-type individuals). Intercept represents a wild-type male.

	Estimate	SE	df	t	p-value
Log (Weight)					
Intercept	0.26	0.11	21.85	2.37	**0.027**
Strain (Transgenic)	-0.29	0.16	23.07	-1.85	0.078
Date	0.33	0.05	19.19	7.01	**< 0.0001**
Date^2	-0.29	0.05	22.36	-6.41	**< 0.0001**
Initial weight	0.65	0.08	32.47	8.21	**< 0.0001**
Strain (Transgenic) × Date	0.03	0.07	22.25	0.40	0.70
Strain (Transgenic) × Date^2	0.06	0.07	24.07	0.87	0.39

- *Within-individual Changes in Weight across Experimental Period*

8 out of 24 wild-type males, 6 out of 24 wild-type females, 6 out of 22 transgenic males, and 2 out of 23 transgenic females survived until or after July 27 2023. This allowed us to assess the change in body mass within these individuals from the start of the field experiment on May 5 2023 to the end after July 27 2023 (Fig. 21).

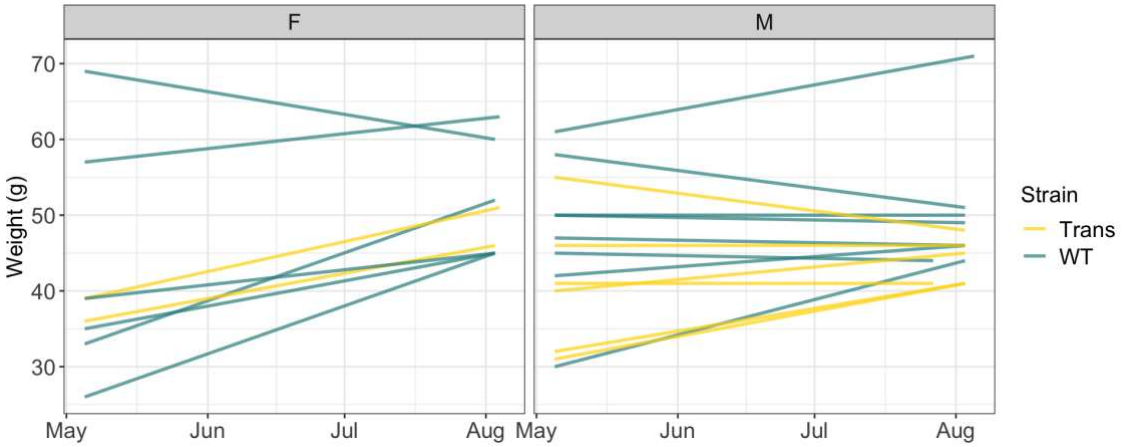

Figure 21. Within-individual vole weight changes from their initial weights recorded on the day of release (on May 5 2023) to their last weights recorded at the end of the experiment (on or after July 27 2023). Transgenic voles did not significantly differ from wild-type voles in within-individual weight changes, regardless of sex (Table 15).

The within-individual weight change of voles ranged from -9 to +19 grams, with a mean of +4.9 ± 1.7 grams, indicating that most voles gained weight during the experimental period. Neither sex nor strain, nor the interaction between sex and strain, significantly affected individual weight change (Table 15). The lack of interaction between sex and strain indicates that the difference in within-individual weight changes between wild-type and transgenic voles did not depend on sex. In other words, the effect of being wild-type or transgenic on within-individual weight changes is similar for both male and female voles.

Table 15. Results of the general linear model for within-individual vole weight changes across the experimental period (n = 8 females, n = 14 males; n = 8 transgenic, n = 14 wild-type; df = 18). Voles in these models were weighed at the beginning and end of the field experiment. Intercept represents a wild-type male.

	Estimate	SE	t	p-value
Within-individual weight change				
Intercept	-0.34	0.35	-0.97	0.34
Sex (F)	0.79	0.53	1.50	0.15
Strain (Transgenic)	0.07	0.53	0.14	0.89
Sex (F) × Strain (Transgenic)	0.24	0.96	0.25	0.80

Survival Days and Population Size

According to trapping data, the estimated adult vole population sizes decreased in all four enclosures during the experimental period, with particularly rapid declines observed among both males and females in enclosure C, females in enclosure E, and males in enclosure G (Fig. 22). Adult vole populations increased in enclosures A and C but decreased in enclosures E and G between June 27 and July 8 (between the purple lines in Fig. 22), as we moved all adult voles from enclosure G into enclosure A and all adult voles from enclosure E into enclosure C during that time. The adult vole populations in enclosures A and C continued to decrease thereafter until the end of the experimental period on August 5 2023. Only 7 out of 24 wild-type males, 6 out of 24 wild-type females, 5 out of 22 transgenic males and 3 out of 23 transgenic females that were placed into the enclosures on May 5 survived until the end of the experiment on August 5 2023.

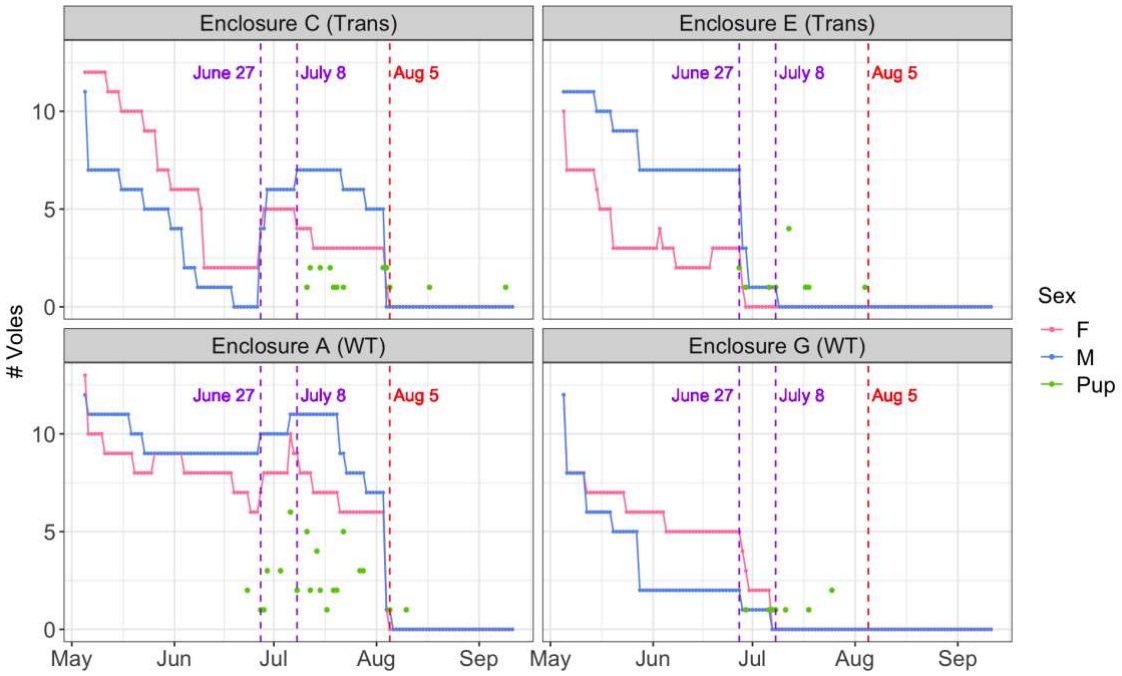

Figure 22. Population estimation of adult voles. The increase in vole population in enclosures C and A and the decrease in enclosures E and G corresponded to the period (between purple dashed lines) when we took the wild-type voles from enclosure G and put them into A and when we took the transgenic voles from E and put them into C starting on June 27 2023. No adult voles were captured in enclosures E and G after July 8 2023. The red dashed line indicates the end of our experimental period on August 5 2023 when we removed all adult voles from the enclosures.

The survival days (total number of days alive in the field enclosures) of voles ranged from 0 to 92 days, with a mean of 38.4 ± 3.6 SE days. Neither sex nor strain, nor the interaction between sex and strain, significantly affected individual survival days (Table 16). The lack of interaction between sex and strain indicates that the difference in survival days between wild-type and transgenic voles did not depend on sex. In other words, the effect of being wild-type or transgenic on the voles' survival days is similar for both male and female voles (Fig. 23).

Table 16. Results of the linear mixed-effects model for vole survival days, with enclosure at release included as a random intercept (n = 47 females, n = 46 males; n = 45 transgenic, n = 48 wild-type). Survival days were estimated as the difference between the vole's last captured date and the date of release. Intercept represents a wild-type male.

	Estimate	SE	df	t	p-value
Survival days					
Intercept	0.19	0.24	9.24	0.76	0.46
Sex (F)	-0.16	0.35	10.44	-0.48	0.64
Strain (Transgenic)	-0.12	0.28	89.06	-0.44	0.66
Sex (F) × Strain (Transgenic)	-0.18	0.40	89.31	-0.45	0.65

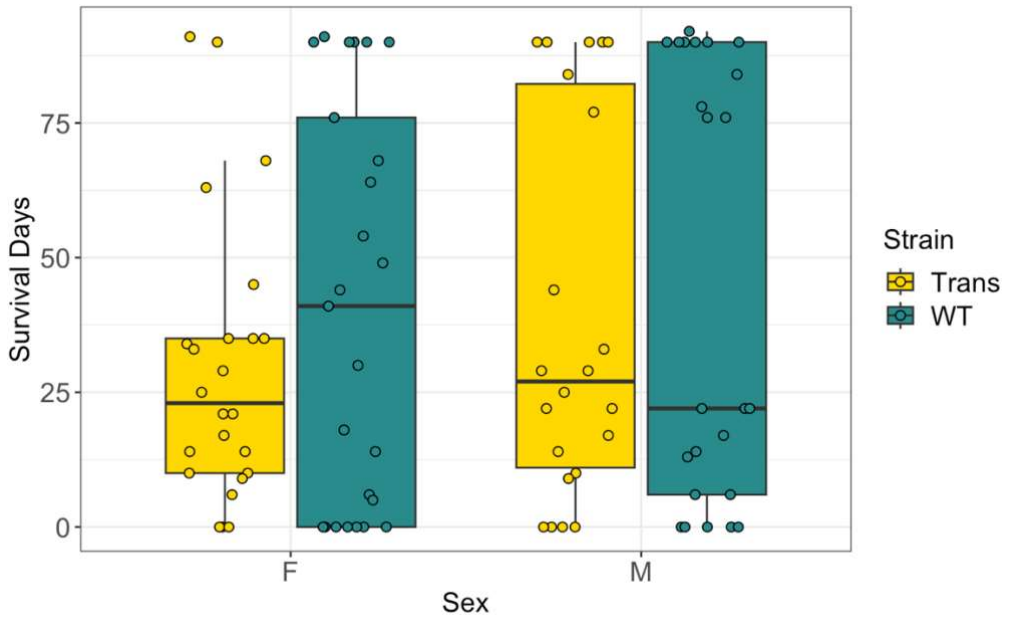

Figure 23. Boxplot of estimated individual vole survival days. Transgenic voles did not significantly differ from wild-type voles in survival days, regardless of sex (Table 16). Survival days were estimated as the length of time voles spent in the enclosure from release until removal, based on the lifespan of recorded PIT tags.

Discussion

In this study, we employed our self-developed Juxta bio-logging system to explore individual behavior variation in prairie voles attributed to mutations in the *Shank3* gene. During the field experiment, we encountered a notable loss rate of the Juxta devices. The lost collars that were recovered from the enclosures appeared intact, albeit with some showing signs of chewing and biting. This suggests that the collars might have been too loose, leading them to slip off the voles' necks as they attempted to remove them. Achieving the delicate balance between ensuring the collar is snug enough to avoid suffocation yet not too loose to risk loss presents a significant challenge in deployment, especially in fossorial animals like voles. This underscores the importance of the deployment methods used to attach instruments to animals. For birds, backpack and leg-loop harnesses are commonly used to attach research devices (Anderson et al., 2020). While Mallory & Gilbert (2008) argued that the backpack style may cause discomfort and irritation under the wing and designed the leg-loop harness, Mott et al. (2015) found that leg-loop harnesses trialed on Brown (*Sula leucogaster*) and Masked (*Sula dactylatra*) boobies were unsuitable and hence unsuccessful, suggesting species-specific deployment methods taking into account their morphological and behavioral characteristics. Despite their intrusiveness, implantable devices have been actively explored as alternatives to address the challenges associated with external bio-loggers (White et al., 2013) and to facilitate the acquisition of neurophysiological data (Gaidica & Dantzer, 2022).

Animals wearing bio-loggers can induce negative effects on their physiology and natural behavior (Portugal & White, 2018). For example, Robstad et al. (2021) found that untagged beavers on average gained daily weight whilst tagged beavers on average lost weight daily. The deployment procedure can also cause animals pain, suffering and distress (Hawkins, 2004). To mitigate the impact of bio-logging on animal behavior and ensure animal welfare, a commonly acknowledged guideline for using bio-logging technologies is that the weight of the device should not exceed 5% of the total body weight for terrestrial animals (Williams et al., 2019). In our study, the average body weight of prairie voles was 44.2 grams. The total weight of a Juxta collar was approximately 3 grams, accounting for 6.79% of the average vole body weight. The Juxta collars could have had negative impacts on the voles' body condition, reproduction, and survival, though this seems unlikely given that most voles gained weight (though this might reflect some type of survivorship bias). Researchers are recommended to reduce the relative mass of the devices borne by animals (Portugal & White, 2018). However, this presents significant engineering challenges, particularly for devices designed for smaller animals.

The miniaturization of Juxta bio-loggers is primarily hindered by battery size, because ensuring an adequate running time in the field necessitates large enough batteries.

The battery life of Juxta devices was highly dependent on the advertising and scanning intervals, with the scanning interval having a greater impact due to its higher power consumption. Longer advertising and scanning intervals result in lower temporal resolution of proximity data. With a rechargeable 40mAh 3.7V LiPo battery, a 10-second advertising interval, and a 60-second scanning interval, Juxta devices were expected to have a battery life of ideally 6 to 7 days. Therefore, the tradeoff between device weight, battery life, and data resolution is a critical consideration in the application of bio-logging technologies. In addition to advocating for advancements in battery technologies, Gaidica et al. (2024) proposed the use of more intelligent sampling modes or alternative proximity detection technologies to enhance power efficiency. For example, extremely low-power ferromagnetic or capacitive sensors can be added on collars to activate the devices only when necessary, rather than running a fixed sampling routine continuously.

Social networks constructed from Juxta log data were far more informative than the ones constructed from trapping data in terms of the number of associations observed. Nevertheless, several challenges existed in using Juxta log data to construct social networks. Firstly, the log data exhibited sparsity (Fig. 20). This stemmed from two main factors: the low trapping rate of voles, which present difficulty in keeping all devices functioning simultaneously and led to unrecorded interactions, and the loss of many devices that were unable to be retrieved, resulting in the loss of data. Secondly, the log records were not symmetric because the advertising and scanning schedules of the devices were not synchronized. Consequently, logs could be missed if one device was scanning while the other was not advertising, or vice versa. Additionally, occasional but unreliable detection of devices at far distances, as demonstrated in the grid validation, could also have contributed to this asymmetry. Thirdly, varying advertising and scanning intervals used throughout the experimental period resulted in different data resolutions. Fourthly, unlike voles trapped in the same trap or at the same trapping stake/rebar indicating a high possibility of interactions (Sabol et al., 2018), the detection of devices and recording of logs did not necessarily indicate individual interactions. All of these could lead to biased and incomplete social networks. To address these challenges, several strategies were employed. Converting the number of logs to interaction durations partially resolved asymmetry in logs and inconsistency in data resolutions. Setting a threshold on the Received Signal Strength Indicator (RSSI) enabled us to limit logs recorded within a certain distance, thereby linking proximity logs to vole interactions and filtering out unreliable logs. Possible efforts to avoid device loss and increase battery lifetime were also discussed above. However, the observed variation in detection range and the relationship between RSSI and distance among different devices, along with the unreliable logs at far distances revealed in the grid validation results, underscored the presence of inter-logger variation. Additionally, intra-logger variation was evident

through the differing RSSIs recorded for the same device at identical distances. These emphasize the importance of conducting pre-experiment calibration and quantifying biases to ensure data accuracy and reliability.

Both social networks, constructed from trapping data and Juxta log data, revealed that the majority of associations were between voles of opposite sexes, while some voles also formed associations with individuals of the same sex. This supports the general understanding that prairie voles form social bonds, primarily between opposite-sex pairs. However, it is important to note that certain associations, particularly those weaker ones, may reflect agnostic rather than affiliative interactions. Future research may explore classifying different types of social behavior based on interaction duration and distance. Remarkably, transgenic voles on average interacted less and at significantly farther distances, particularly driven by same-sex pairs, notably female-female pairs, which implies a reduced level of sociality in female voles due to the *Shank3* mutation. However, no significant differences in interaction duration or distance were observed between transgenic and wild-type male and female pairs, which suggests the continued presence of pair bonding between males and females, and contradicts the laboratory findings that transgenic females interacted less with novel males and huddled less with their partners (Larios, 2021). This discrepancy implies that the *Shank3* mutation may result in different behavioral variation under naturalistic conditions, possibly influenced by the interplay of more complex factors, including social elements such as population density and sex ratio, as well as abiotic factors like precipitation and temperature. Further investigation is necessary to better understand these dynamics. Moreover, it underscores the imperative of investigating animal behavior within ecologically relevant settings, as highly controlled laboratory settings may oversimplify crucial environmental factors, obscuring their interaction and impact on variation in individual behavior.

The social networks constructed from Period 1 (from May 6 to May 8 2023) of the Juxta log data illustrate how voles interacted with unfamiliar individuals and formed social bonds in a new environment. The transgenic enclosure E exhibited numerous associations with weak strengths compared to the other three enclosures, where fewer associations were observed but with stronger connections between specific vole pairs. This suggests that voles in enclosure E might not form strong bonds as readily or rapidly as those in the other three enclosures. Social bonding has been found to be beneficial in protecting against stress in prairie voles (McNeal et al., 2017), and its emergence or disappearance is subject to various physical and social factors. As social bonds are essentially behavioral strategies of individuals aiming to better adapt to the environment and maximize their reproductive success (Sachser, 2005), it is possible that the environmental conditions, such as the vegetation in enclosure E, provided better shelter and food resources initially, thereby reducing stress levels and potentially eliminating the

need for the voles in enclosure E to form social bonds hastily. However, there were not obvious differences in vegetation among the four enclosures that would suggest the voles in enclosure E had more food (Davidson, 2024). Moreover, the fact that no one pair was observed consistently across all three periods of social networks in enclosure A may be attributed to limited sampling during Period 2, coupled with high mortality rates, as well as the introduction of new individuals during Period 3. This highlights the key role of demographic changes in shaping animal social networks (Shizuka & Johnson, 2019).

The rhythm in the activity levels was consistent with the findings of Calhoun (1945), who found a bimodal pattern of activity in prairie voles with intense activity periods peaking at the time of transition from darkness to light and between 1800 h and 2400 h. We found that transgenic females were less active than wild-type females at 1000, 1100, 1800 h, and transgenic males were less active than wild-type males at 1000 h but more active at 1800, 1900, 2000 h. These differences may imply altered behavioral strategies in foraging, nesting or socializing. It would be intriguing to further investigate whether activity levels were associated with the frequency of social encounters. Additionally, we found that transgenic females consistently displayed lower device temperatures than wild-type females throughout the entire day, with significant differences observed at night from hours 2100 to 0600. This may suggest that transgenic females huddled less with their partners or offspring, which supports the findings from the laboratory study conducted by Larios (2021). Alternatively, it is possible that they exhibited distinct nest types or nesting behavior, leading to reduced warmth compared to wild-type females. Furthermore, the oscillations in both the activity levels and the device temperatures, particularly the coincidence of peaks and troughs at dawn and dusk respectively, offered insights into the behavior of the voles. Specifically, high levels of activity coupled with low device temperatures around 0600 h suggests emergence from nests, while low levels of activity but high device temperatures around 2200 h suggests voles staying in nests and getting warm. The movement and temperature data collected by the Juxta loggers hold promise for investigating nest use or nest attendance patterns and for classifying behavior. Similar methodologies have been successfully employed in previous studies, such as those conducted by Skudd et al. (2016, 2018) on red squirrels.

In terms of other behavioral traits, our findings revealed no difference in trappability or home range sizes between transgenic and wild-type voles. Given that trappability and home range sizes have been used as measures of boldness and exploratory tendency (Johnstone et al., 2021; Schirmer et al., 2019; Santicchia et al., 2020; Eccard et al., 2022), these results suggest that the mutation in the *Shank3* gene or reduced sociality did not influence the boldness or exploratory tendency of the voles, though future work measuring boldness and exploratory behavior using additional measures would be necessary to examine this possibility. Additionally, we found no

significant difference in mean weights and survival days between transgenic and wild-type voles, suggesting no impact of the mutation in the *Shank3* gene or reduced sociality on their body condition or survival. The discrepancy between these findings and our initial hypotheses may arise from the inhibition of *Shank3* mutation expression in realistic environments, or the compensation or overshadowing of social deficits by other physical and social factors, or the absence of a clear correlation between sociality and other behavioral traits or consequences.

Conclusion

Overall, our study represents an experimental endeavor in using an advanced bio-logging system to quantify variability in the social behavior of prairie voles by leveraging *Shank3* gene manipulations. We conducted a thorough field evaluation of our self-developed bio-logging system, Juxta, assessing its failure rates, battery life, and the relationship between RSSI values and distances. The quantity and quality of the collected data were validated through the construction of social networks from spatial-temporal co-occurrence proximity data, as well as the behavioral information derived from the movement and temperature data. We also identified challenges and proposed future improvements related to deployment methods, the tradeoff between device weight, battery life, and data resolution, along with the complexities involved in constructing social networks using proximity loggers. We concluded that our Juxta bio-logging system successfully captured high-resolution data for studying animal behavior and constructing social networks in secretive free-ranging small animals, though future improvements are necessary.

Furthermore, through the integration of the Juxta system with traditional live-trapping methods, we conducted a comprehensive investigation into the behavioral variation of prairie voles with and without the *Shank3* mutation. This encompassed various aspects including sociality, activity levels, device temperature, trappability, home range sizes, as well as the potential consequences in terms of body weight and lifespan. Our results imply reduced sociality in female prairie voles with the *Shank3* mutation, as evidenced by increased interaction distances between female pairs, but not between male and female pairs, and significantly reduced local temperatures at night, possibly due to decreased huddling with other individuals. Additionally, we found no significant difference in trappability, home range sizes, body weight and lifespan between transgenic and wild-type voles, suggesting that voles with the *Shank3* mutation behaved typically when put into a semi-natural environment. While our findings partially reflect the laboratory observations of Larios (2021) regarding the social deficit exhibited by *Shank3* mutant female prairie voles in their natural habitat, the underlying mechanisms may differ due to the complex interplay of multiple factors present in the naturalistic environment. This highlights the importance of understanding the interplay of genetic and environmental factors shaping individual behavior.

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Supplemental Material

Table S1. Trapping schedule throughout the entire experimental period from the release of voles on May 5 2023 to the conclusion of the experiment on September 11 2023. Trapping sessions conducted after August 5 2023 were aimed at ensuring the complete removal of all voles from the enclosures.

May 2023

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
	1	2	3	4	5 Release	6
7	8	9 C	10 ACG	11	12	13
14 CE	15 AG	16	17 E	18 AEG	19	20
21 AC	22 EG	23	24	25 AC	26 EG	27
28	29 AC	30 EG	31			

June 2023

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
				1	2 AC	3 EG
4	5	6 AC	7 AC	8 AC	9	10
11	12 EG	13 EG	14 EG	15	16	17 AC
18 ACEG	19 ACEG	20	21 EG	22 AC	23	24
25	26 EG	27 EG	28 EG	29	30	

July 2023

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
						1

2 AC	3	4	5 ACEG	6 EG	7 ACEG	8 EG
9	10 AEG	11 CEG	12	13 AEG	14 CEG	15
16 AEG	17 CEG	18	19 AC	20	21 AC	22
23	24 EG	25 EG	26 AC	27 AC	28	29
30	31					

August 2023

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
		1	2 ACEG	3 ACEG	4 ACEG	5 Removal
6 ACEG	7 ACEG	8 AC	9 ACEG	10	11	12
13	14	15	16 ACEG	17 ACEG	18	19
20	21	22	23	24	25	26
27	28	29	30	31		

September 2023

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
					1	2
3	4	5	6	7	8 ACEG	9 ACEG
10 ACEG	11 End	12	13	14	15	16
17	18	19	20	21	22	23
24	25	26	27	28	29	30