



## Population Density and Diurnal Rhythm, but not Familiarity, Shape Proximity Behavior in Female Albino Mice

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### ABSTRACT

This study examined how population density affects proximity behaviour in female albino mice (*Mus musculus*) and whether familiarity with a partner influences this behaviour. Three group sizes were established with an equal sex ratio: small (10 individuals), medium (20), and large (30). Each group contained one focal female with familiar and unfamiliar marked males, as well as control females. Proximity, defined as following or remaining stationary near another individual, was recorded for 15 minutes every hour from 8:00 to 16:00. Proximity rates showed a strong diurnal pattern, peaking between 8:00 and 9:00 and declining to near zero by the afternoon across all densities. Familiarity did not affect proximity: females did not stay closer to familiar males than to unfamiliar males, nor to familiar females than to unfamiliar females. Group size, however, had a significant effect. Mean proximity rates were similar in small and medium densities but markedly lower in the largest density. Two-way ANOVA confirmed a main effect of density on proximity for both sexes, while familiarity and its interaction with density were not significant. These findings indicate that proximity behaviour in albino mice is modulated by time of day and overall group size, but not by individual familiarity. High population densities suppress social proximity, possibly through increased stress or competition. The results highlight the importance of considering social context and temporal factors when designing mate choice experiments.

### 1. INTRODUCTION

In mammals, social familiarity influences many aspects of behaviour, including mate choice. One widely accepted function of familiarity is inbreeding avoidance: individuals avoid mating with close relatives, often recognized through previous association (Pusey & Wolf, 1996; Robbers et al., 2021). Studies on house mice have shown that familiarity can affect mating decisions, with females sometimes preferring unfamiliar males to avoid inbreeding (Agbali et al., 2022; Fadao et al., 2000).

However, laboratory studies using simple two-choice tests have produced inconsistent results some find a preference for familiar mates (DeVries et al., 1997), others for unfamiliar mates (Clarke & Faulkes, 1999). This suggests that the expression of female choice may depend on the social environment. Population density is a key feature of the social environment. Theoretical models predict that density can alter male-male competition, female encounter rates, and the strength of sexual selection (Kokko & Rankin, 2006; Evans et al., 2021). Empirical work, mainly on insects and fish, has shown that density affects male courtship and aggression (Jirotkul, 1999). In albino mice, male dominance and aggression have been shown to influence female mate choice, but not always in a straightforward way (Saleh et al., 2022). Yet little is known about how density affects proximity behaviour, a non-aggressive, following or staying-near behaviour that may reflect female interest or tolerance. In rodents, proximity can be a subtle indicator of social preference, especially when direct mating is not possible (Hurst & Beynon, 2004). No study has systematically examined how population density affects proximity between familiar and unfamiliar individuals in a mammalian species, while keeping the operational sex ratio equal. The albino mouse (*Mus musculus*) is a practical model because it breeds year-round and shows clear social behaviours under laboratory conditions (Guenet et al., 2015). Previous research on albino mice has demonstrated that females can distinguish between preferred and non-preferred males using olfactory cues, and that this preference correlates with offspring fitness (Agbali et al., 2023). However, the role of density in modulating proximity behaviour remains unexplored. It was hypothesised that female albino mice would show higher proximity rates towards unfamiliar males than towards familiar males, as a mechanism to avoid inbreeding. This preference was expected to be strongest at low density, where females can exercise choice without male interference, and weakest at high density, where male-male competition may constrain female behavior. Additionally, it was predicted that proximity rates would vary with time of day, reflecting the natural activity rhythm of mice, with the highest rates during the early morning. The primary aim of this study was to compare proximity rates of familiar and unfamiliar males across three group densities (small, medium, large). A secondary aim was to determine whether density affects the proximity of females towards familiar versus unfamiliar females as a social control. Finally, the study aimed to examine diurnal changes in proximity behaviour from 8:00 am to 4:00 pm in order to identify any temporal patterns that could inform future experimental design.

## 2. METHOD

### **Study species and housing**

Albino mice (*Mus musculus* Linnaeus, 1758) were used. They are polyestrous and breed year-round, with females reaching sexual maturity at 3-4 weeks (Guenet et al., 2015). Mice were outbred and housed in standard cages. Males and females were kept separately on different racks. Food (seed mix with insect additives) and water were provided ad libitum. Bedding was wood shavings, and straw was given for nest construction.

### **Density treatments**

Three group sizes (different individuals' density) were established, each with an equal sex ratio of one male to one female and three replicates per treatment. A total of 60 individuals were involved in experiments. The small groups contained ten individuals (five males and five females), the medium groups had twenty individuals (ten males and ten females), and the large groups consisted of thirty individuals (fifteen males and fifteen females). Terrarium volume was constant (15 L) but base dimensions increased with group size, small (40×40 cm), medium (56.5×56.5 cm), large (69.2×69.2 cm). Thus, floor area increased proportionally, keeping spatial density roughly constant while varying the number of social partners. Before the experiment, two males and two females in each terrarium were randomly marked for individual identification using colour using non-toxic permanent markers to allow clear recognition during behavioural observations. One male was designated as familiar and the other as unfamiliar to the focal female. The same was done for two control females. Here, prior to each trial, females were given indirect olfactory exposure to the familiar male only. We used his soiled bedding for this purpose. About 100 grams of bedding that the familiar male had occupied alone for one week was transferred daily into the female's home terrarium, had a capacity of 15 litres. This transfer was repeated for six days in a row. The whole procedure was meant to mimic a natural situation where a female repeatedly picks up the scent of a nearby male without ever meeting him face to face. The unfamiliar male received no such exposure. As a result, the female had no prior experience, either by smell or by direct contact, with the unfamiliar male.

### **Behavioral definition and recording**

Proximity was defined as one animal following another while walking (not running), or remaining still for several seconds near another individual. Similar definitions have been used in rodent studies of social preference (Van Loo et al., 2001). Observations were made in the home terrarium with all individuals present in each group size.

Behaviour was recorded for 15 minutes every hour from 8:00 am to 4:00 pm (nine observation periods per day). Proximity rate was calculated as the frequency of proximity behaviour per observation. period.

### Statistical analysis

Data were tested for normality using the Anderson-Darling test. One-way ANOVA was used to test for differences in proximity rate across time points within each density. T-tests were used to compare proximity rates between familiar and unfamiliar individuals (males and females separately) within each density. Two-way ANOVA was used to examine the effects of group size (density), familiarity, and their interaction on proximity rate for males and females separately. Significance was set at  $\alpha = 0.05$  and Minitab version 17 was used in all statistical tests.

## 3. ETHIC APPROVAL

We confirm that ethical approval for this research (Principal Investigator: Muna Agbali) was granted by Al-Mukhtar Committee for Biosafety Bioethics (MCBB). The research protocol underwent review and received approval under reference number **NBC: 007. A. 26. 87**. This approval covers all work conducted at the Zoology Department, Science Collage, Omar Al-Mukhtar university, and the study was carried out in line with relevant ethical guidelines.

## 4. RESULT

### Temporal pattern of proximity rate

The proximity rate changed significantly over the course of the day in all three density treatments. This was true for both familiar and unfamiliar individuals, whether male or female. The statistical analysis confirmed a significant time-of-day effect for each group, with p-values below 0.01 in all cases. The highest proximity rates were consistently recorded during the first two observation hours, namely at 8:00 am and 9:00 am. After that, the rates steadily decreased. By the afternoon hours, specifically at 2:00 pm, 3:00 pm, and 4:00 pm, the proximity rates frequently dropped to zero. This daily pattern is illustrated in figure 1 for familiar and unfamiliar males, also the temporal changes are shown in figure 2 for familiar and unfamiliar females. In every case, the morning peak and the afternoon decline were very similar across all densities.

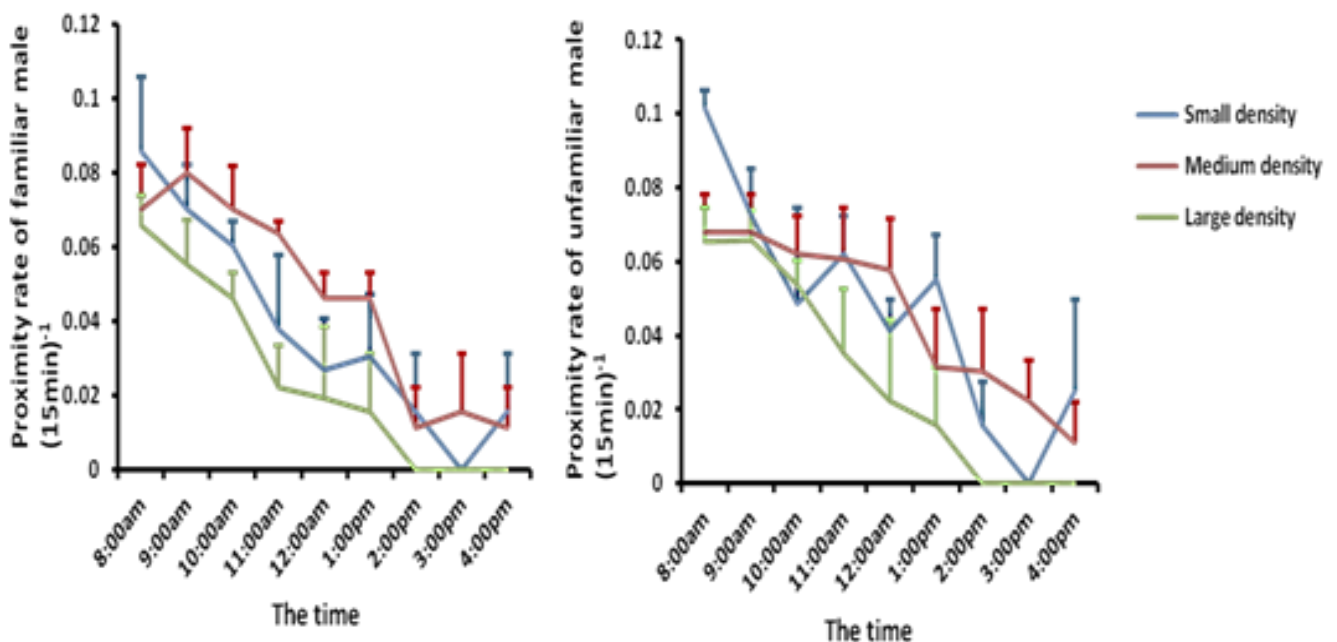


Fig.1 Proximity rate of familiar and unfamiliar males means ( $\pm$ se) in different groups densities from 8:00 am to 4:00 pm.

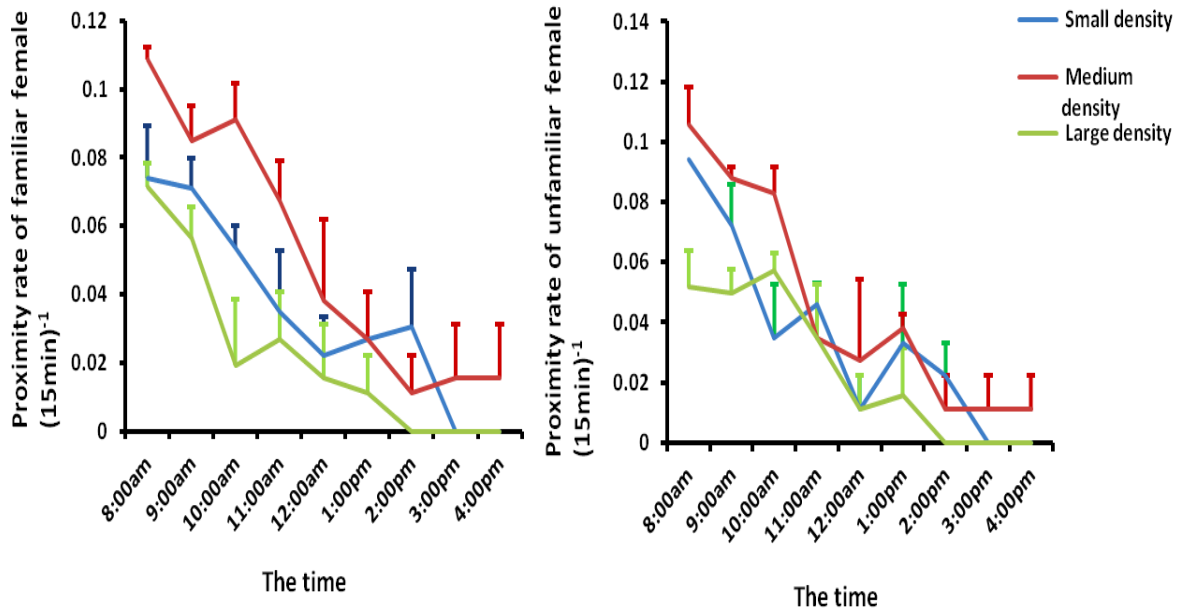


Fig.2 Proximity rate of familiar and unfamiliar females mean ( $\pm$ se) in different groups densities from 8:00 am to 4:00 pm.

#### Comparison between familiar and unfamiliar males

When proximity rates of familiar and unfamiliar males were compared within each density, no statistically significant difference emerged. In the small density group, the t-test gave a value of  $t_{51} = -0.91$  with a p-value of 0.366. In the medium density group, the t-test result was  $t_{51} = 0.03$  with  $p = 0.976$ . In the large density group, the t-test was  $t_{51} = -0.46$  with  $p = 0.650$ . In all three cases, the p-values were well above the significance threshold of 0.05. This indicates that females did not show a proximity-based preference for either familiar or unfamiliar males, regardless of whether the group size was small, medium, or large figure 3. The control tests using female stimuli produced the same null result. In the small density treatment, the t-test comparing proximity rates towards familiar versus unfamiliar females gave  $t_{51} = -0.01$  with  $p = 0.988$ . In the medium density treatment, the result was  $t_{51} = 0.49$  with  $p = 0.624$ . In the large density treatment, the t-test yielded  $t_{51} = 0.29$  with  $p = 0.776$ . None of these differences approached statistical significance. Therefore, females did not stay closer to familiar females than to unfamiliar females, nor the reverse, in any of the three density groups. These findings are shown in figures 4.

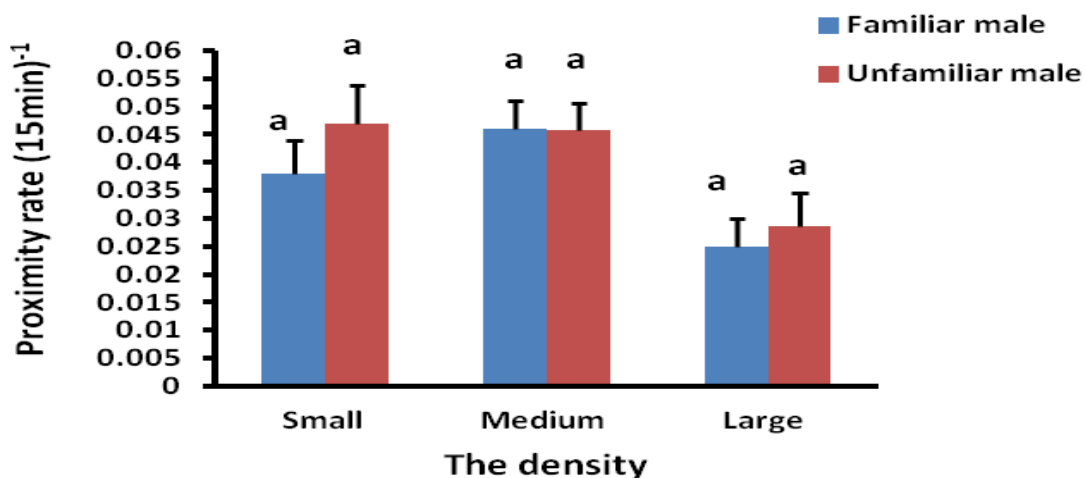


Fig.3 Proximity rate of familiar males and unfamiliar males mean ( $\pm$ se) in different groups densities. Values with the same letter did not differ significantly.

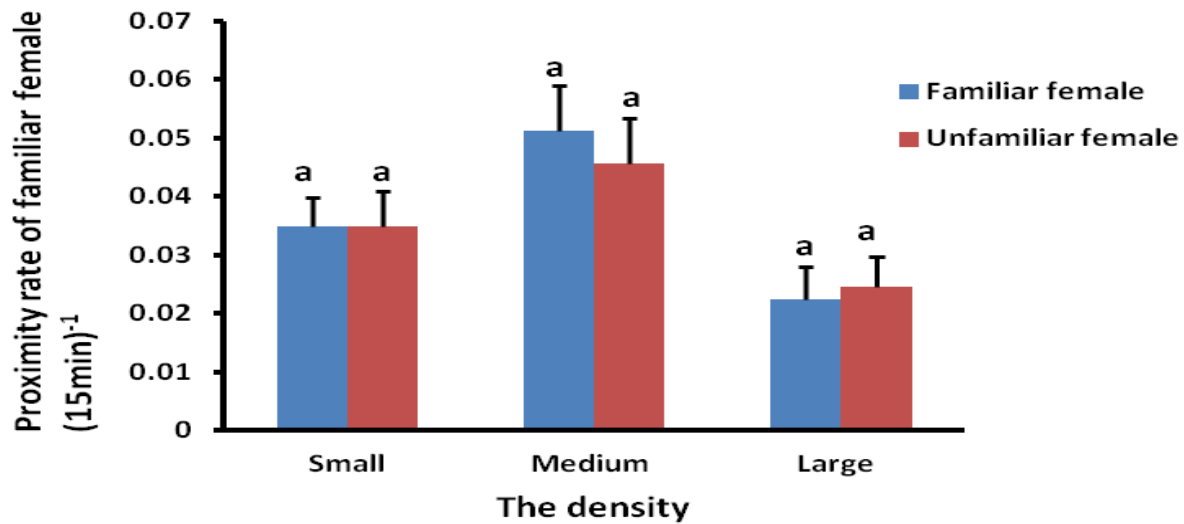


Fig.4 Proximity rate of familiar females and unfamiliar females mean ( $\pm$ se) in different groups densities. Values with the same letter did not differ significantly.

#### Effect of density on mean proximity and rate

When proximity rates were averaged across familiarity categories and across all time points within each density, a clear effect of group size became apparent. For males, a one-way ANOVA revealed a significant difference among the three densities ( $F_2 = 5.62$ ,  $p = 0.004$ ). The mean proximity rate in the small density was 0.0425, and in the medium density it was 0.0458. These two values were very similar. In contrast, the mean proximity rate in the large density was much lower, at 0.0268. Thus, the large density group had a significantly reduced proximity rate compared to the two smaller groups. This pattern is depicted in figure 5. A similar effect was observed for females in the control tests. The one-way ANOVA showed that density significantly affected mean proximity rate ( $F_2 = 7.22$ ,  $p = 0.001$ ). The mean proximity rate was 0.0348 in the small density, 0.0483 in the medium density, and 0.0234 in the large density. Again, the large density group exhibited the lowest proximity rate, while the small and medium groups had higher and comparable rates. These results are illustrated in figure 6.

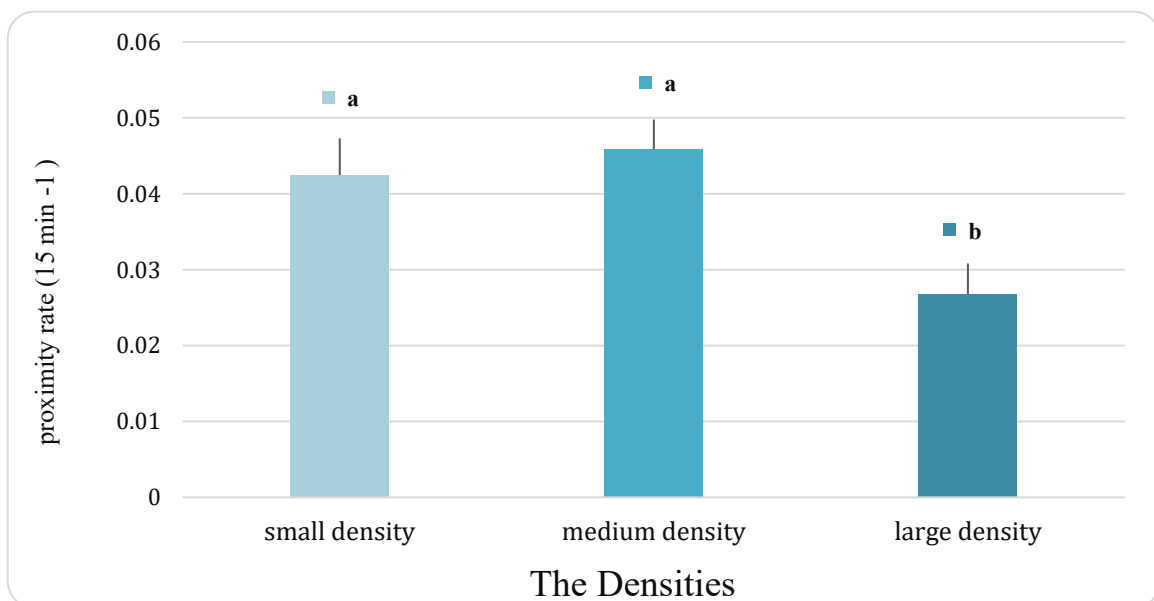


Fig.5 Proximity rate of males means ( $\pm$ se) through different groups densities. Values with the same letter did not differ significantly.

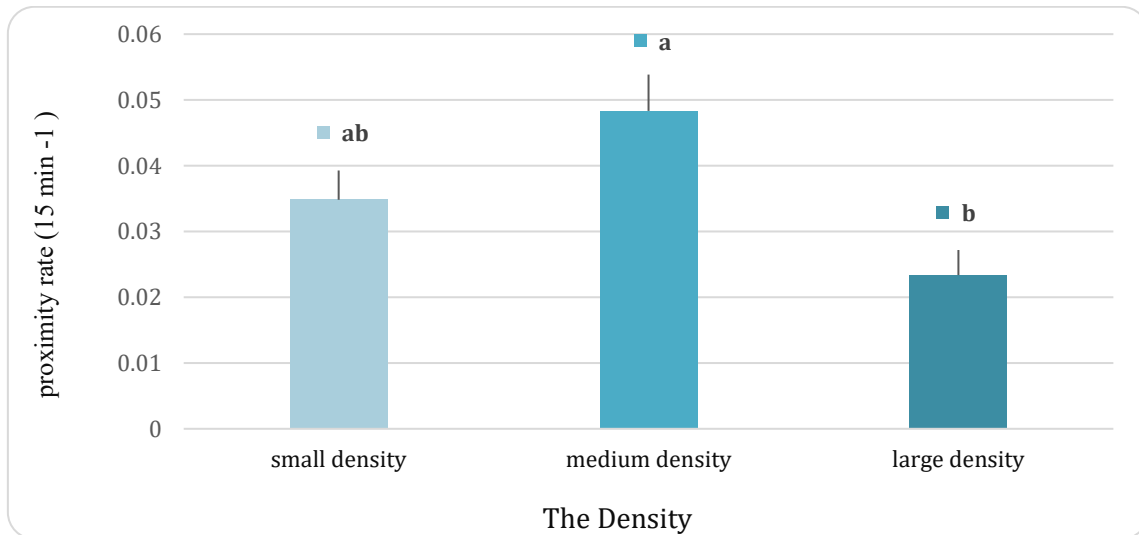


Fig.6 Proximity rate of females means ( $\pm$ se) through different groups densities. Values with the same letter did not differ significantly.

### Combined effects of group size and familiarity on proximity rate

A two-way analysis of variance was conducted to examine the independent and interactive effects of group size (density) and familiarity on proximity rate. For males, group size had a significant main effect ( $F_{2,156} = 5.56, p = 0.005$ ). Familiarity, on the other hand, did not show a significant main effect ( $F_{1, 156} = 0.68, p = 0.411$ ). Moreover, the interaction term between group size and familiarity was not significant ( $F_{2, 156} = 0.28, p = 0.757$ ). This means that density influenced proximity behaviour in males, but whether the stimulus male was familiar or unfamiliar made no difference, and the effect of density did not depend on the familiarity status. For females in the control tests, the two-way ANOVA gave similar outcomes. Group size had a significant main effect ( $F_{2,156} = 7.11, p = 0.001$ ). Familiarity was not significant ( $F_{1,156} = 0.04, p = 0.846$ ), and the interaction between group size and familiarity was also not significant ( $F_{2,156} = 0.18, p = 0.835$ ). Thus, density affected female proximity behaviour as well, but familiarity with the stimulus female did not, and there was no combined effect. All these statistical results are summarised in Table 1.

**Table 1:** Two-Way ANOVA mean of proximity rates of males, females mice and interactions between these factors (familiarity and group size).

Source	Df	SS	MS	F-value	P-value
Proximity rate for males					
Group size(G)	2	0.011186	0.0055931	5.56	0.005
Familiarity(f)	1	0.000684	0.0006844	0.68	0.411
Familiarity $\times$ group size (f $\times$ g)	2	0.000562	0.0002810	0.28	0.757
Error	156	0.157017	0.0010065		
proximity rate for females					
Group size(G)	2	0.016861	0.0084303	7.11	0.001
Familiarity(f)	1	0.000045	0.0000450	0.04	0.846
Familiarity $\times$ group size (f $\times$ g)	2	0.000427	0.0002136	0.18	0.835
Error	156	0.185080	0.0011864		

## 5. DISCUSSION

In this study, proximity rate was strongly time-dependent. In all densities, proximity peaked in the early morning (8:00-9:00 am) and dropped to near zero by the afternoon. This matches the known crepuscular/nocturnal activity pattern of house mice (Hut & van der Zee, 2011). Similar diurnal rhythms in social behaviour have been reported in other rodent species, including golden hamsters and Norway rats (Gattermann et al., 2008; Gaskill et al., 2012).

These findings imply that studies measuring social proximity should standardise observation times; otherwise, results may be confounded by time of day. Moreover, the timing of behavioural testing can influence the detection of mate preferences, as female receptivity and male courtship also follow circadian patterns (Frynta et al., 2010; Stein, 2022).

Familiarity did not affect proximity rate at any density in the present study. Neither males nor females showed higher proximity towards familiar or unfamiliar individuals. This contrasts with the hypothesis that females would prefer unfamiliar males as an inbreeding avoidance mechanism (Pusey & Wolf, 1996). However, other studies have also failed to find a familiarity effect on spatial proximity in rodents. For instance, female meadow voles did not differ in time spent near familiar versus unfamiliar males when given free access (Ferkin, 1988; Theil et al., 2020). Similarly, in house mice, familiarity reduced aggression but did not increase affiliative proximity (Hurst & Beynon, 2004). In albino mice, a recent study using odour and live-encounter tests found that females clearly discriminated familiar from unfamiliar males when only olfactory cues were available, but this preference disappeared or became inconsistent during direct live encounters (Agbali et al., 2026). Also in mice, previous work has shown that females can discriminate between familiar and unfamiliar males using olfactory cues, but this discrimination does not always translate into differential proximity in group settings (Agbali et al., 2023; Agbali et al., 2022). One possible explanation is that proximity is a less sensitive measure of preference compared to scent investigation or mating frequency (Roberts & Gosling, 2004). Another possibility is that in mixed-sex groups with multiple individuals, females may use other cues (e.g., male behaviour or dominance status) that override familiarity (Qvarnström & Forsgren, 1998). The absence of a familiarity effect in control tests with female stimuli further suggests that proximity in this context is not primarily driven by kin recognition or social bonding, but rather by general activity and crowding (Banks et al., 1979; Kavaliers et al., 2022).

Our findings revealed that density significantly affected mean proximity rate, but in a non-linear way. Proximity rates were similar in small and medium groups, then declined sharply in the largest density. This pattern is unlikely to be explained by spatial crowding alone, because the floor area was scaled up to keep individuals per cm<sup>2</sup> roughly constant. Instead, social factors may be at play. At very high group sizes (30 individuals), increased male-male aggression or general social stress might reduce the frequency of following and staying behaviours. Similar reductions in social contacts under high density have been reported in bank voles (Koskela et al., 1999) and in laboratory mice (Van Loo et al., 2001). Increased population density can activate stress responses (e.g., elevated corticosterone levels) and suppress non-aggressive social behaviours (Christian, 1971; Creel et al., 2013). In wild house mice, high density leads to increased aggression and reduced social tolerance, which could explain the drop in proximity (Singleton & Krebs, 2007). Moreover, the presence of many individuals may create a confusion effect that reduces the likelihood of sustained following (Lima & Dill, 1990; Rimbach & Pillay, 2022).

The lack of any interaction between density and familiarity indicates that density affects proximity behaviour generally, not specifically towards familiar or unfamiliar individuals. Both types of stimuli were responded to similarly across all group sizes. This suggests that in high-density environments, animals may adopt a general avoidance strategy rather than a fine-tuned discrimination based on identity (Gillis & Nams, 1998). A similar pattern has been observed in other social animals: when group size increases, individuals rely more on simple rules (e.g., avoid all others) to reduce conflict, rather than on individual recognition (Ward & Webster, 2016).

These findings have implications for captive breeding and conservation programmes. Housing rodents at very high densities may reduce social interactions, including those necessary for pair bonding and successful mating (Swaigood et al., 2018). Therefore, moderate group sizes may be optimal for maintaining natural social behaviours. Future studies should manipulate both total density and the operational sex ratio independently, as these factors can have opposing effects on mate competition (Jirotkul, 1999; Head et al., 2008; Miller et al., 2023). Additionally, using automated tracking systems could provide finer-scale measures of proximity and association patterns (Ohayon et al., 2013). Finally, combining proximity data with direct mating observations would clarify whether proximity is a reliable proxy for mate preference.

## 6. CONCLUSION

Proximity behaviour in female albino mice depends on time of day and group density, not on whether a partner is familiar. Females came closest in the early morning. Large groups lowered proximity, maybe because of stress or more competition. One limitation is that proximity alone might not capture all sides of female choice; females could tell familiar from unfamiliar males by smell, but that didn't make them stay closer. Findings may be useful for captive breeding, keeping groups too big could reduce social contact, so moderate sizes work better. Any experiment on behaviour should watch the clock – the morning peak is strong. Next studies could mix proximity records with direct observations of mating, try wild mice, and change density and sex ratio separately. If researchers control for time of day, they are less likely to miss or misread preferences.

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