Prosocial behavior in adult mice is sex-dependent

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Abstract

Prosocial behavior, defined as voluntary behavior intended to benefit another, has long been regarded as an almost uniquely human characteristic. In recent years, it was reported that laboratory animals also favor prosocial choices in various experimental paradigms, thus demonstrating that prosocial behaviors are likely evolutionarily conserved. Here, we investigated prosocial choices in adult male and female C57BL/6 laboratory mice in a task where a mouse is rewarded for either choice, but only one of the choices rewards an interaction partner. Additionally, we assessed social reward using a conditioned place preference task and empathy-like behavior in a task that measures sensitivity to the affective state of interaction partners (i.e., relieved/hungry vs. neutral). We find that female, but not male, mice had a moderate but significant preference for prosocial choices. At the same time, both male and female animals showed similar rewarding effects of social contact in the conditioned place preference test, and similarly, there was no effect of sex on the preference for interaction with a hungry or relieved partner. The observed difference between sexes is consistent with the reported higher propensity for prosocial behavior in women, although with the notable difference of similar sensitivity to the affective state of the interaction partners.

Introduction

Prosocial behavior, defined as acting to meet the need of another individual, is regarded as the highest form of empathy (de Waal, 2008; Decety et al., 2016). In humans, a major factor affecting the propensity for social behaviors is gender (Christov-Moore et al., 2014). It was observed that females have superior emotion discrimination abilities (Thompson and Voyer, 2014), are more concerned about the well-being of others (O'Brien et al., 2013), and utilize more resources to support others in need (Willer et al., 2015). It was proposed that altruistic, prosocial behavior is a uniquely human characteristic (e.g., Fehr and Fischbacher, 2003; de Waal and Suchak, 2010); however, a growing number of reports show that targeted helping is also observed in other species. Laboratory rodents and some bird species prefer actions that reward another conspecific in choice tasks or free another animal from a restraint, with whom they share a food reward afterward (Ben-Ami Bartal et al., 2011a; Brucks and von

Bayern, 2020; Hernandez-Lallement et al., 2014; Márquez et al., 2015). Unlike in humans, there are limited data on the effect of sex on prosocial behaviors in laboratory animals. Most rodent studies on empathy have focused on only one sex, usually males (for reviews, see Hernandez-Lallement et al., 2022; Puścian et al., 2022), although some studies have investigated females (e.g., Atsak et al., 2011; Jones et al., 2014; Schneeberger et al., 2012). Several studies examined both sexes, but the results appear inconclusive (Ben-Ami Bartal et al., 2011b; Du et al., 2020; Han et al., 2020; Langford et al., 2010; Mikosz et al., 2015; Pisansky et al., 2017; Scheggia et al., 2020a). Some of the reports suggest that females are more susceptible to emotional contagion (Pisansky et al., 2017), show enhanced emotion discrimination abilities in double approach paradigms (Langford et al., 2010; Rogers-Carter et al., 2018) and are more likely to perform prosocial actions (Ben-Ami Bartal et al., 2011b; Heinla et al., 2020a). However, other studies provide evidence for equal susceptibility of male and female rodents to emotional contagion (Du et al., 2020; Han et al., 2020; Keum et al., 2016; Langford et al., 2006; Smith et al., 2016), equal affective state discrimination skills (Scheggia et al., 2020a), and comparable levels of prosocial behaviors (Burkett et al., 2016; Du et al., 2020; Gachomba et al., 2022). Interestingly, some authors observed even higher levels of empathy-motivated behaviors in male rodents (Du et al., 2020; Kentrop et al., 2020a; Mikosz et al., 2015). Hence, further studies are needed to determine if, and under what circumstances, female rodents show superior emphatic and prosocial abilities.

Recently, it was proposed that prosocial behavior is directly motivated by empathy as well as the rewarding effects of social interactions, together termed the "camaraderie effect" (Lahvis, 2017). This theory predicts that if sex differences in prosocial behavior were to be found between sexes of a given species, they should also be found in social reward and empathy. To assess prosocial behavior, we have developed a food-motivated prosocial choice task based on the rat task described by Hernandez-Lallement and collaborators (Hernandez-Lallement et al., 2014), with a custom-designed apparatus and schedule adapted for mice. Empathy was tested in a paradigm where sensitivity to the emotional state of interaction partners was assayed, modified from Scheggia et al. (2020a), and social reward was tested in the social conditioned place preference task (Harda et al., 2022). We found that, similar to the case in humans, sex had a significant effect on the propensity for prosocial choices; however, females and males did not differ with respect to levels of social reward and empathy.

Results

Prosocial choices in adult male and female mice

To assess prosocial choices in adult mice, we used a custom-made maze, as shown in **Figure 1A**. In the task, a focal animal, the actor, chose to enter one of two compartments and would be rewarded with food pellets for either choice. A second animal, the partner, also received a reward, but only if the actor entered the compartment designated "prosocial". The schedule of the experiment is summarized in **Figure 1B**. First, the actor animals underwent up to 4 pretest sessions, one session per day, without partner animal (**Fig. 1C**). The number of food pellets consumed was checked after each trial, and only animals that consumed at least 85% of the pellets over two consecutive days were subjected to the actual test (**Fig. 1D**). The average number of sessions required to reach this criterion was 2.4 and 2.63 in male and

female mice, respectively (**Supplementary Fig. S1A-D, Supplementary Table S1**). The pretest was intended to train actors and to assess their inherent preference between the left and right compartments in the absence of a partner animal.

Then, the partners were introduced, and one of the compartments was designated "prosocial" (**Fig. 1D**). Four test sessions were performed. In female mice, the preference for the prosocial compartment increased significantly (**Fig. 1E**), while male animals appeared to show no change from their initial choices and no preference for either prosocial or antisocial choices (**Fig. 1F**). In the case of females, the preference for prosocial behavior changed from 44.14% initially to 54.30% (average from the 4 trials, t test, $t_7 = 4.33$, p = 0.003), while in males, these values were 47.52% and 47.83%, respectively. The difference in the prosociality score between males and females was significant (**Fig. 1G**, t test, $t_{16} = 2.47$, p = 0.025). Additionally, we examined correlations between the absolute and relative weights of the actor and partner and the prosociality score. The analysis revealed no significant association for absolute weights in any of the sexes and no significant correlation of relative weight in the case of females (**Supplementary Table S14**). However, a negative correlation between relative weight and prosociality score was observed in male mice (**Supplementary Table S14**, r = -0.73, p = 0.014). Thus, we found that female mice favored prosocial choices in the task, while males were indifferent to the rewards delivered for the interaction partner.

Social reward

An increase in the frequency of prosocial choices observed in females is evidence of reinforcing effects of their consequences and, thus, of a rewarding effect of the choice. No preference for the prosocial choices in male mice could potentially be attributed to a generally lower sensitivity to the rewarding effects of social interaction. To assess this possibility, we tested adult mice of both sexes in the social conditioned place preference task (sCPP). In this test, social contact causes an increase in time spent in the previously neutral context. Both female and male mice significantly increased the time spent in the context associated with group housing from pretest to posttest (**Fig. 2A**, t test, $t_{15} = 2.825$, p = 0.012, **Fig. 2B**, t test, $t_{11} = 4.202$, p = 0.002,). Likewise, the preference score was significantly higher than chance value in both female (t test, $t_{15} = 3.282$, p = 0.005) and male (t test, $t_{11} = 2.446$, p = 0.033) mice (**Fig. 2C**). These results indicate that social interactions with siblings were equally rewarding for male and female mice.

Sensitivity to the affective state of the interaction partner

A plausible explanation for the observed effect of sex on prosocial choices would be a difference in sensitivity to social cues during interaction. To test this possibility, mice were assayed for their preference for interaction with a "neutral" vs. altered affective state ("relived" or "hungry") demonstrator in a procedure based on the task described by Scheggia et al. (2020a) and summarized in **Figure 3A**. During the main phase of the test, the animal tested (observer) was placed in the cage where demonstrators were present, both confined under wire cups. One of the demonstrators was food deprived: for 24h (hungry) or for 23 hours and then offered *ad libitum* food access for 1 h preceding the test (relieved). The second demonstrator (neutral) had constant *ad libitum* food access. Both female and male observer

mice spent significantly more time sniffing the relieved demonstrator during the first minute of the test (**Fig. 3B**, ANOVA, $F_{1.88}$= 3.25, p = 0.074, Šídák's test, p = 0.013; **Fig. 3C**, ANOVA, $F_{1.96}$ = 11.86, p = 0.001, Šídák's test, p = 0.016), and there was no effect of sex on the fraction of time spent sniffing the relived demonstrator (**Fig. 3D**). Similarly, both female and male observer mice spent significantly more time sniffing the hungry demonstrator during the first minute of the test (**Fig. 3E**, ANOVA, $F_{1.56}$ = 10.09, p = 0.002, Šídák's test, p = 0.001; **Fig. 3F**, ANOVA, $F_{1.72}$ = 1.58, p = 0.212, Šídák's test, p = 0.011), and there was also no effect of sex (**Fig. 3G**). The position of the altered affective state demonstrator (east vs. west side of the testing apparatus) was selected randomly, and no effect of the relieved or hungry demonstrator's position on the percentage of time spent sniffing this demonstrator was found (**Fig. S2A-B, Fig. S2F-G**). Additionally, to control for inherent preference of the position of the cups (east vs. west), we also analyzed the time spent sniffing the cup in which the relieved/hungry demonstrator would be placed during the last day of adaptation. The time spent sniffing the empty cup did not differ from chance level (**Fig. S2E & H**) except for females paired later with relieved demonstrators. In this case time spent sniffing the empty cup was significantly shorter than the chance level (**Fig. S2E,** t test, t_8 = 2.495, p = 0.037). Finally, we also analyzed the correlations between the fraction of time spent sniffing the relieved/hungry demonstrator and the weight of the animals. This analysis revealed a significant negative correlation for both relived demonstrator (r = -0.7, p = 0.001) and observer weight (r = -0.62, p = 0.028), as well as an observer-relived demonstrator weight difference (r = 0.53, p = 0.073), in females (**Supplementary Table S14**). These results could indicate greater empathy-like behavior toward "relief" among smaller females. No significant correlations were found in the case of hungry demonstrators, in either sex (**Supplementary Table S14**). Taken together, these results show that both female and male mice discriminate between affective states of familiar conspecifics. Thus, there is no evidence of an effect of sex on sensitivity to affective states of conspecifics.

Discussion

We found that female, but not male, C57BL/6 mice showed a preference for prosocial behavior toward a familiar partner. The preference for prosocial choices was previously observed in similar experimental settings in rats (Gachomba et al., 2022; Hernandez-Lallement et al., 2014; Kentrop et al., 2020b; Márquez et al., 2015). Interestingly, the studies performed on rats reported either no sex differences (Gachomba et al., 2022) or even male superiority in prosocial behavior (Kentrop et al., 2020b). The two pioneering studies analyzed only male behavior and revealed a robust prosocial effect (Hernandez-Lallement et al., 2014; Márquez et al., 2015). The discrepancy between mice and rats is intuitively consistent with the difference between the social systems of the two species. Wild male mice (*Mus musculus*) form territories on which they tolerate the presence of several females. Interactions between adult males are rare and usually antagonistic. In contrast, rats (*Rattus norvegicus*) form large colonies consisting of multiple males and females of different ages, in which fights rarely occur (for a review, see Kondrakiewicz et al., 2019; Lee and Beery, 2019). Nevertheless, the extent

to which laboratory animal strains retain the traits of the species they were derived from remains uncertain (Blanchard et al., 1998; Chalfin et al., 2014).

The observation that female mice are robustly more prosocial than males in a food-motivated prosocial choice task is in accordance with other findings in rats using different measures of prosociality. Ben-Ami Bartal and colleagues found that females are more likely to learn how to free a trapped cagemate, and, when the task is learned, females perform it faster than males (Ben-Ami Bartal et al., 2011a). Furthermore, Heinla and colleagues found that female, but not male, rats show consolation-like behavior directed toward cagemates that were recently attacked by another individual (Heinla et al., 2020b). These behaviors are often interpreted as evidence for empathic abilities in rodents (Meyza et al., 2017; Silberberg et al., 2014). If prosocial behavior is seen as a form of empathy, the reports cited and our findings support the Primary Caretaker Hypothesis, stating that females have evolved superior emphatic abilities due to offspring care, since greater sensitivity to nonverbal expression could increase offspring survival (Babchuk et al., 1985; Preston, 2013). The Primary Caretaker Hypothesis also predicts a female advantage in the emotion recognition task. Here, we found no sex differences in the affective state discrimination task. Similar levels of emotional discrimination in male and female mice were also observed by Scheggia et al. in a test where a positive state ("relief") was induced by deprivation and subsequent provision of water and the negative state ("stress") was induced by 15 min of restrain before the test (Scheggia et al., 2020b). Nevertheless, these results are not necessarily in disagreement with the Primary Caretaker Hypothesis and could be explained by previously reported observations. The first potential explanation is one of the interpretations of the Primary Caretaker Hypothesis, called the "fitness threat hypothesis", which states that female advantage in emotion discrimination may be limited to negative emotional expressions, as only these emotions signal a potential threat to the offspring (Hampson et al., 2006). In support of the "fitness threat hypothesis", studies using severe stress stimuli (pain or footshock) have demonstrated higher emotion discrimination abilities in female rodents (Langford et al., 2010; Rogers-Carter et al., 2018), whereas one study that induced mild stress or a relief state in demonstrator animals provided no evidence for sex differences in emotion discrimination abilities (Scheggia et al., 2020b). In our study, we managed to replicate these results, confirming that independently from valence, there are no sex differences in the recognition of other emotional states in mice.

Second, it could be speculated that mice of both sexes are equally able to recognize affective states of conspecifics but that only females act on the information on the suffering of others. This explanation is consistent with studies on empathy for pain. Specifically, Lanford and collaborators found that both male and female mice are affected by the pain of conspecifics in such a way that their own expressions of pain are enhanced by seeing a conspecific in pain (Langford et al., 2006). However, only females approach the conspecific in pain more than a conspecific in a neutral state (Langford et al., 2010).

Finally, a methodological caveat should be considered as well, which is related to exposure time. In human studies where long exposure times were used (≥10 s), participants of both sexes displayed high accuracy scores, indicating a ceiling effect (Duhaney and McKelvie, 1993; Hampson et al., 2006; Kirouac and Dore, 1985). Accordingly, it has been

argued that studies using long exposure times lack ecological validity since real-life emotional expressions tend to change on shorter time scales (Hampson et al., 2006). Unfortunately, it is not feasible to study emotion discrimination in rodents using millisecond stimulus exposure times.

Based on the recently proposed "camaraderie effect" theory (Lahvis, 2017), we hypothesized that a female advantage in prosocial behavior may stem from higher rewarding effects of female–female, compared with male–male, social interactions. However, no sex difference in the rewarding effects of social interactions was observed. This is especially surprising, as males of the *Mus musculus* species studied in natural or seminatural conditions usually have been found to form territories and aggressively defend them from other males (Kondrakiewicz et al., 2019). Female mice, in contrast, are capable of communal nesting and nursing (Hayes, 2000). Both male and female mice disperse from their natal groups, but males do this more frequently and at younger ages (Groó et al., 2012). Taken together, these literature data suggest that the motivation for the social context preference observed here may differ between males and females. In females, amicable social interactions are the most likely cause of the increase in the preference for social context. In males, however, the opportunity for aggressive encounters may have caused the same effects. Earlier studies support this interpretation, as rewarding effects of aggression were repeatedly documented in male mice and rats (for a review, see Golden et al., 2019), but are absent in female mice (Aubry et al., 2022).

Taken together, our results show that, similar to humans, female mice tend to be more prosocial than males, but this difference may not stem from superior emphatic abilities or higher rewarding effects of social interactions. Thus, the relationships among prosociality, empathy and social reward should be reconsidered, and correlations between these traits are not indicative of causation.

Materials and methods

Animals

Experiments were performed on C57BL/6 mice bred at the Maj Institute of Pharmacology Animal Facility. Mice were housed in a 12-hr light-dark cycle (lights on at 7 AM CET/CEST) under the controlled conditions of 22 ± 2 °C temperature and 40-60% humidity. In the prosocial choice test, mice were housed as sibling pairs. For affective state discrimination, sCPP mice were housed with littermates of all the same sex or alone, depending on the phase of the experiment. Water was available *ad libitum*. Home cages contained nesting material and aspen gnawing blocks. Behavioral tests were conducted during the light phase under dim illumination (5-10 lux). Affective state discrimination and sCPP tests were video recorded with additional infrared LED illumination. The age and weight of all experimental groups are summarized in **Supplementary Table S15**.

All behavioral procedures were approved by the II Local Bioethics Committee in Krakow (permit numbers 224/2016, 34/2019, 35/2019, 32/2021) and performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September

2010 on the protection of animals used for scientific purposes. The reporting in the manuscript follows the ARRIVE guidelines.

Prosocial choice test

The method was partly based on the prosocial test for rats described by Hernandez-Lallement et al. (2015). The custom cage used in the procedure is shown in **Figure 1A**. The primary difference from the previously described apparatus is the single compartment for the interaction partner. The experimental schedule is summarized in **Figure 1B** and consisted of 4 phases**:** food restriction (5-7 days), habituation (2 days), pretest (2-4 days, depending on completion criterion; see **Supplementary Table S1**), and main test (4 days). Mice had restricted access to food throughout the experiment. Habituation was performed when animals reached 85-90% of their initial body weight. On the last day of food restriction, the larger mouse from each cage was selected as the actor, and the smaller mouse was used as the partner. The rationale was to increase the chance of observing prosocial behavior in actors, as it was shown that the number of reward portions provided to the hungry partner is negatively correlated with the partner's weight in rats (Hernandez-Lallement et al., 2014; Schneeberger et al., 2012)**.** On the two days preceding the pretest, actors and partners were placed in the assigned compartments for 10 minutes to freely explore all compartments. Reward was available *ad libitum*. During the pretest, only actors were present in the cage. The pretest session consisted of 6 forced choice trials and 16 free choice trials. The sequence of forced trials was always an alternation of right and left choices, starting with right.

At the beginning of each trial, the actor was placed in the starting compartment. Then, the doors were lifted, and the actor could access one of the reward compartments (during forced trials) or was offered a choice between the two compartments (during free choice trials). The actor received a food reward irrespective of choice (two chocolate chips, BioServ, 20 mg, #F05301). After a mouse consumed the reward, it was placed back in the starting compartment. The time mice could spend in each of the compartments was limited (**Fig. 1 A**). In case the mouse did not move to the desired compartment before the time limit, the experimenter gently pushed it. The completion criterion for the pretest was 37 out of 44 food pellets consumed in two consecutive sessions (for the number of animals excluded based on this criterion, see **Supplemental Figure S1** and **Supplementary Table S1**). Additionally, an exclusion criterion was a >70% average preference for one of the compartments (the '70% criterion'). No animals were excluded from testing based on this criterion.

During the main test phase, both the actor and partner were introduced to the cage, and testing sessions were performed daily over 4 days. Each actor's entry to the "prosocial" compartment resulted in reward delivery for both mice. Conversely, upon entrance to the "antisocial" compartment, only the actor was rewarded. The prosocial compartment was assigned as follows: in the case of mice with less than a 60% preference, the compartment was selected randomly. For the mice with an initial preference between 60 and 70%, the less preferred compartment was chosen as prosocial.

We considered the possibility that the prosocial behavior in female mice could be affected by estrous cycle phase. The estrous cycle in mice lasts for approximately four days. To minimize

the possible effect of estrous cycle phase on the differences between males and females, the four-day average of the test phase results was used for pretest-posttest comparison and for comparison between sexes.

Social conditioned place preference (sCPP)

sCPP was performed as previously described (Harda et al., 2022, 2020). The procedure consisted of three phases: pretest, conditioning phase, and posttest.

During pretest each cage compartment contained one type of context (context A and context B) that differed in bedding type and gnawing block size and shape (**Supplementary Table S16**). Both conditioning contexts were different from the home cage context, which consisted of aspen bedding (**Supplementary Table S16**) and a distinct gnawing block. Mice were allowed to freely explore the cage for 30 minutes. Two animals were tested in parallel in adjacent cages.

After the pretest, animals were returned to their home cages for approximately 24 h. Then, mice were assigned to undergo social conditioning (housing with cage mates) for 24 h in one of the contexts used in the pretest followed by 24 h of isolated conditioning (single housing) in the other context. To preserve an unbiased design, the social context was randomly assigned in such a way that approximately half of the cages received social conditioning on context A and half on context B. The conditioning phase lasted 6 days (3 days in each context, alternating every 24 h). After conditioning, the post-test was performed identically as pretest. Two sets of conditioning contexts were used (**Supplementary Table S16),** and the results from both contexts were pooled.

Affective state discrimination

The test was based on the protocol developed by Scheggia et al. (2020a). The behavior was assessed in a rectangular cage with opaque walls (see Fig. 3A; 53 cm x 32 cm x 15 cm). Demonstrators were placed on plastic platforms and confined under wire cups (diameter 9.5 cm x height 9 cm, Warmet, #B-0197). The procedure comprised two phases: habituation (3 days) and testing (1 day). The largest animal in the cage at the start of habituation was always assigned the "observer" role, the second largest was assigned the "relieved" or "hungry" demonstrator role, and the smallest was assigned the "neutral" demonstrator. This was done to match the role assignment in the prosocial choice task and to ensure that the relieved/hungry and neutral demonstrators had a similar weight such that the only characteristic that distinguished them was the affective state. The relieved/hungry demonstrator and observer were always naïve, while the neutral demonstrators were tested twice with different observers in 9 cases, always a week apart.

On the first day of habituation, the observers were placed in the experimental cage for 12 minutes. In the experiment with relieved demonstrators the cage was empty for half of the animals, and it contained empty wire cups for the other half. No effect of cup presence during habituation was observed (**Fig. S3 C, D**). In the experiment with hungry demonstrators the cage always contained empty wire cups on the first day of habituation. On habituation Days 2 and 3, observers were placed in the experimental cage for 6 minutes, and the wire cups were

introduced for the next 6 minutes to habituate the observer to their placement during the test.

A glass jar was always placed on the top of the wired cups to prevent the observers from climbing the cups. Demonstrators were placed every day for 10 minutes in the experimental cage under the wired cups without an observer present. After the last habituation session, animals were placed in separate home cages for 24 hours, i.e., until the main test. The relieved/hungry demonstrators were deprived of food immediately after being put in a separate cage, while neutral demonstrators and observers had access to food *ad libitum*. One hour before the test, the relieved demonstrators were provided *ad libitum* access to food. Ten minutes before the test, observers were placed in the testing arena for habituation, and demonstrators were placed under wire cups on the table in the experimental room. Additionally, hungry demonstrators were presented with two chow pellets placed in unreachable distance to the wired cage to induce stress. After habituation, two demonstrators (neutral and relieved or hungry) were placed in the arena for 4 minutes (under wire cups). Observers who investigated both partners for less than 30 seconds were excluded from the analysis (experiment with relieved demonstrators: n=2 females, n=2 males; experiment with hungry demonstrators: n=1 females, n=1 males).

Data analysis

Distance moved and presence in separate cage compartments in the sCPP test were automatically analyzed using EthoVision XT 15 software (Noldus, The Netherlands). Time spent sniffing relieved/hungry and neutral demonstrators and time spent in the respective zones were scored manually using Boris software (Friard and Gamba, 2016) by the experimenter, who was blinded to the demonstrators' state. The significance level was set at $p < 0.05$. Comparisons of sample means were performed using analysis of variance (ANOVA) followed by Šídák's multiple comparisons test or Student's t test for cases with only two samples.

Acknowledgments

Author contributions

KM, JRP and ZH designed the study. KM, ZH, MK, MC, ŁS, AB performed experiments. KM, KP and ZH analyzed the data. JRP and ZH supervised the study. JRP and ZH wrote the paper with contributions from all of the authors.

Data availability statement

All data are available at https://zenodo.org/record/6988393. Raw video recordings of the tests will be made available on request.

Competing Interests Statement

The Authors declare no competing interests.

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Figures
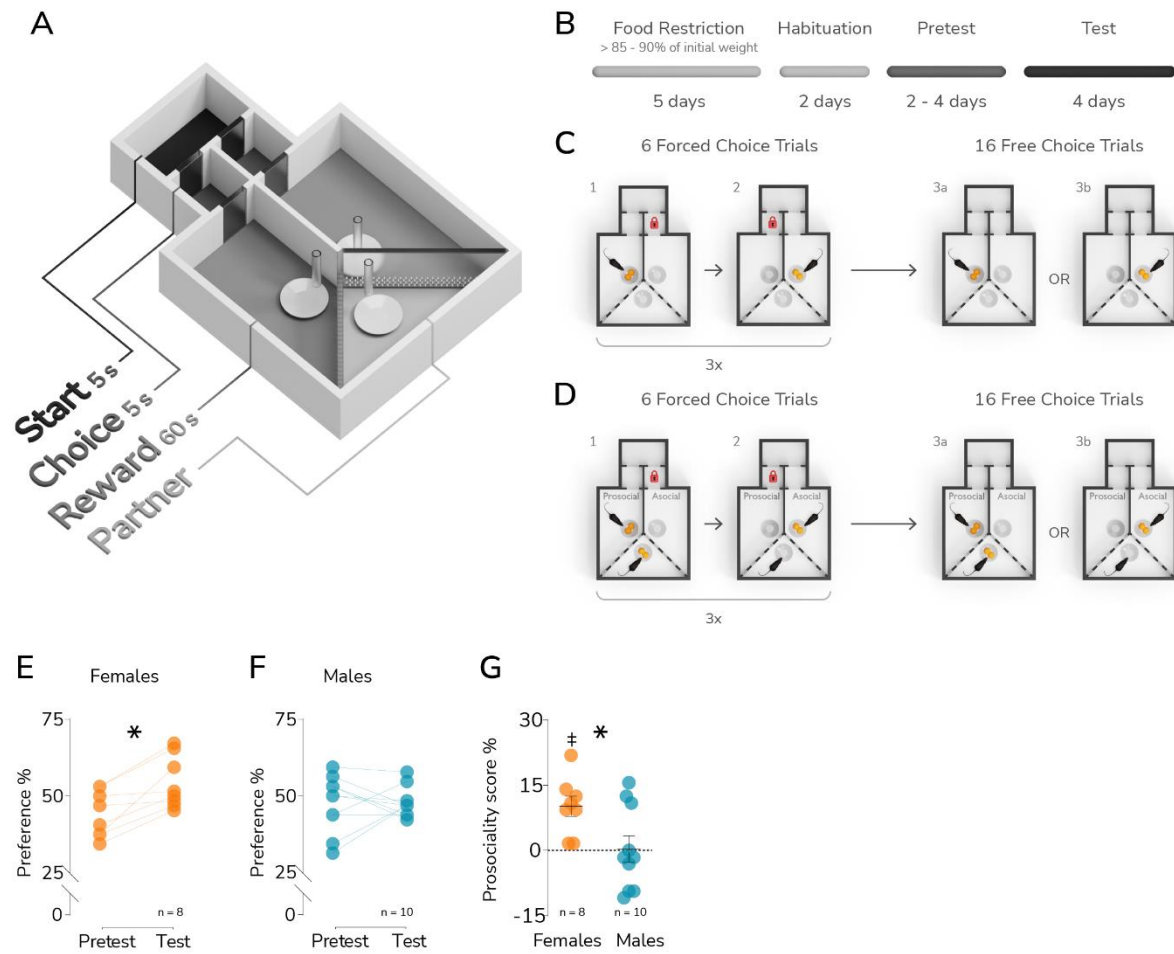
Figure 1. Prosocial choices in male and female mice. (**A**) A schematic representation of the testing apparatus. (**B**) Experimental schedule. (**C**, **D**) A diagram summarizing the pretest and test phases of the experiment, respectively. (**E, F**) The change in preference for the 'prosocial' compartment between the pretest and test phases in female and male mice, respectively. The results shown are the mean values from all sessions in the pretest and test phases. A significant difference between the phases is marked with a "*" (paired t test p < 0.05). Respective group sizes are indicated below the graphs. (**G**) The prosociality score, defined as the change in preference from pretest to test. The bar and whiskers represent the mean and s.e.m. values. A significant difference between means is marked with a "*" (t test p<0.05), and a significant difference vs. 0 is indicated by a "‡" (one-sample t test p<0.05). The group sizes are indicated below the graph.
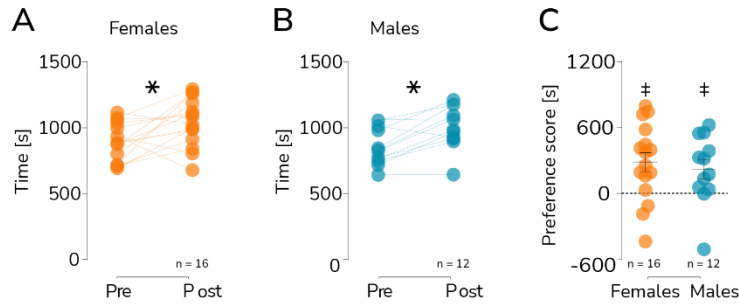
Figure 2. Social conditioned place preference. (**A, B**) The change in preference for the social context after conditioning in female and male mice, respectively. Each pair of points represents an individual animal, and the group sizes are indicated below the graphs. A significant difference between the pre- and posttests is marked with a "*" (paired t test, $p < 0.05$). (**C**) The preference for social context during the posttest. Each point represents an individual female or male mouse, with the mean and s.e.m. shown in black and the group sizes indicated below. A significant preference (greater than 0) is indicated by a "‡" (one-sample t test $p < 0.05$).
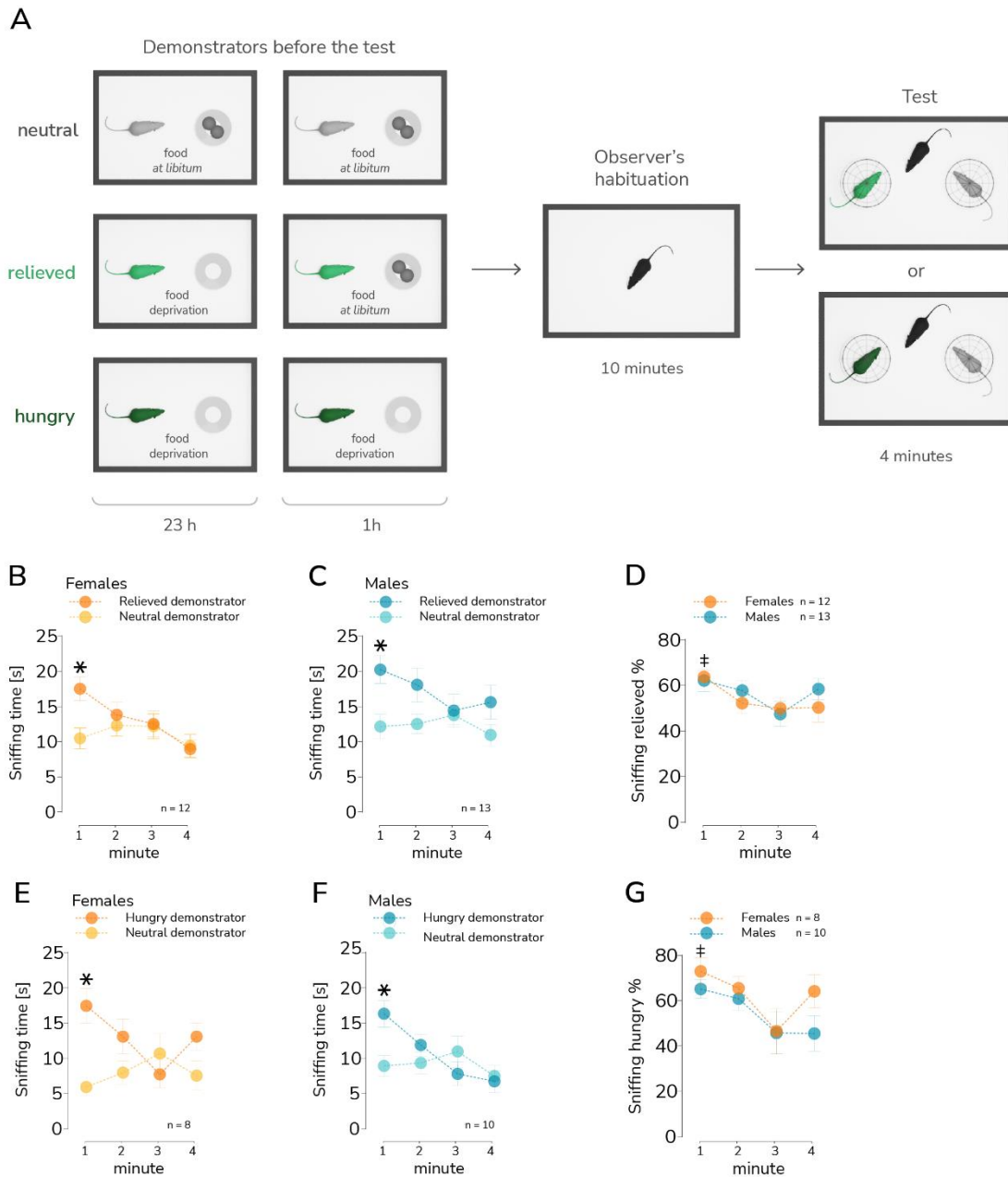
Figure 3. Affective state discrimination. (**A**) A schematic representation of the task. (**B, C**) The time spent by the demonstrator sniffing the relieved (darker points) and neutral (lighter points) female and male demonstrators, respectively. Each point represents the mean time spent sniffing respective partners during a 1-minute interval (bin), and the group sizes are indicated below the graph. The whiskers represent s.e.m. values, and significant differences between the mean time spent sniffing in the first one-minute interval are shown with a "*" (p < 0.05, post hoc Bonferroni-corrected t test). (**D**) Preference for sniffing the relieved demonstrator over the neutral demonstrator. The points represent mean values over 1-minute intervals, whiskers represent the s.e.m. Significant differences vs. 50% during the first 1-minute interval in both female and male mice are indicated by a "‡" (one-sample t test p<0.05). (**E, F**) The time spent by the demonstrator sniffing the hungry (darker points) and neutral (lighter points) female and male demonstrators, respectively. Each point represents the mean time spent sniffing respective partners during a 1-minute interval (bin), and the group sizes are indicated below the graph. The whiskers represent s.e.m. values, and significant differences between the mean time spent sniffing in the first one-minute interval are shown with a "*" (p < 0.05, post hoc Bonferroni-corrected t test). (**D**) Preference for sniffing the relieved demonstrator over the neutral demonstrator. The points represent mean values over 1-minute intervals, whiskers represent the s.e.m. Significant differences vs. 50% during the first 1-minute interval in both female and male mice are indicated by a "‡" (one-sample t test p<0.05).

Supplemental Figure S1. Rewards consumed during the pretest phase of the prosocial task. (A, B) The number of food pellets consumed by the female and male actors during the first and final days of the pretest, respectively. Each point represents an individual female or male mouse, with the mean and s.e.m. shown in black and the group sizes indicated below. **(C, D)** The number of female and male mice that reached the inclusion criterion of 37 out of 44 food pellets consumed on two consecutive sessions. As shown in **(C)**, two female animals did not meet the required criterion.
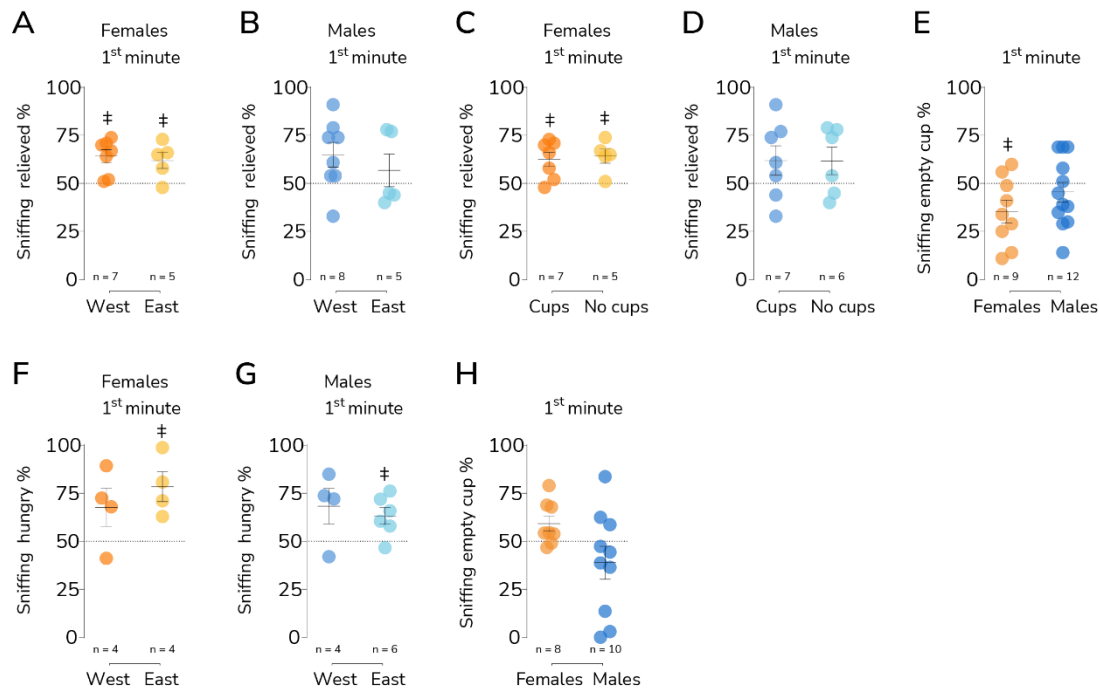
Supplemental Figure S2. Context effects in the affective state determination task. (**A, B**) The effect of the position of the relieved demonstrator (West or East) on the proportion of the time the observer spent sniffing him in male and female mice, respectively. (**C, D**) The effect of the presence or absence of the cups during habituation. (**E**) Final day of habituation. Time spent sniffing the empty cup in which the relieved demonstrator was placed during the test. Recordings of final day of habituation of 3 females and 1 male have been lost. (**F, G**) The effect of the position of the hungry demonstrator (West or East) on the proportion of the time the observer spent sniffing him in male and female mice, respectively. **H**) Final day of habituation. Time spent sniffing the empty cup in which the hungry demonstrator was placed during the test.

Supplementary Tables

Table S1. Prosocial choice test. Number of mice excluded based on predefined criteria.

Sex	Fig.	Initial n	Number of mice that did not meet criterion		Tested n
			criterion A	criterion B	
females	S1C	10	2	0	8
males	S1D	10	0	0	10

Criterion A: two days in a row consumed >84% of the reward pellets delivered during the pretest session (37 out of 44)
Criterion B: average pretest side preference below 70%

Table S2. Prosocial choice test. Average pretest and test preference for prosocial compartment.

Fig.	Sex	n	Preference during pretest %		Preference during test %		Mean difference	Paired Student's t-test		
			M	SEM	M	SEM		t	df	p
1E	females	8	44,10	2,70	54,30	3,00	10,16	4,33	7,00	**0,00**
1F	males	10	47,50	2,90	47,80	1,50	0,31	0,10	9,00	0,92

Table S3. Prosocial choice test. Prosociality score. Females and males comparison.

Fig.	Sex	Prosociality score %[a]						Mean difference	Unpaired Student's t-test		
		n	M	SEM	One sample t test. Comparison with hypothetical mean (0)				t	df	p
					t	df	p				
1G	females	8	10,20	2,30	4,35	7	**0,00**	9,88	2,47	16,00	**0,03**
	males	10	0,30	3,00	0,10	9	0,92				

[a] Mean % of prosocial choices of the last two days of pretest subtracted from mean % of prosocial choices of 4 days of test.

Table S4. Social conditioned place preference test. Number of mice excluded based on predefined criteria.

Sex	Initial n	Number of mice that did not meet criterion A	Tested n
females	17	1	16
males	12	0	12

Criterion A: Initial preference to any of the context not exceeding 70% in pretest.

Table S5. Social conditioned place preference test. Average pretest and test preference for social context.

Fig.	Sex	n	Preference during pretest [s]		Preference during test [s]		Mean difference	Paired Student's t-test		
			M	SEM	M	SEM		t	df	p
2A	females	16	893,70	34,85	1040,00	43,56	146,30	2,83	15,00	**0,01**
2B	males	12	855,80	40,59	1010,00	45,28	154,20	4,20	11,00	**0,00**

Table S6. Social conditioned place preference test. Preference score. Females and males comparison.

Fig.	Sex	Prosociality score [s][a]									
		n	M	SEM	One sample t test. Comparison with hypothetical mean (0)			Mean difference	Unpaired Student's t-test		
					t	df	p		t	df	p
2C	females	16	285,80	87,07	3,28	15,00	**0,01**	64,20	0,50	26,00	0,62
	males	12	221,60	90,59	2,45	11,00	**0,03**				

[a] Time spent in social context pretest [s] subtracted from time spent in social context posttest [s]

Table S7. Affective state discrimination test. Number of mice excluded based on predefined criteria.

Demonstrators state	Sex	Initial n	Number of mice that did not meet criterion A	Tested n
relieved	females	14	2	12
	males	15	2	13
hungry	females	9	1	8
	males	11	1	10

Criterion A: Cumulative time sniffing demonstrators not exceeding 30s during test.

Table S8. Affective state discrimination test. Anova table for sniffing neutral and relieved demonstartors [s]

Demonstrators state	Fig.	Sex	Source of variation															
			Time					Demonstrator's state					Interaction				
			SS	DF	MS	F	p	SS	DF	MS	F	p	SS	DF	MS	F	p
relieved	3B	females	315,50	3,00	105,20	3,20	**0,03**	106,90	1,00	106,90	3,25	0,07	208,30	3,00	69,44	2,11	0,10
	3C	males	129,50	3,00	43,16	0,88	0,46	584,30	1,00	584,30	11,86	**0,00**	185,90	3,00	61,98	1,26	0,29
hungry	3E	females	49,48	3,00	16,49	0,45	0,72	370,30	1,00	370,30	10,09	**0,00**	425,00	3,00	141,70	3,86	**0,01**
	3F	males	323,10	3,00	107,70	3,74	**0,01**	45,52	1,00	45,52	1,58	0,21	314,80	3,00	104,90	3,64	**0,02**

Table S8. Continued.

Demonstrators state	Fig.	Sex	Comparison of time sniffing relieved vs neutral demonstrators [s]							
			Time [minute]	$M_{relieved}$	$M_{neutral}$	Mean difference	Šídák's multiple comparisons test			
							SE	t	DF	p
relieved	3B	females	1st	17,53	10,46	7,07	2,34	3,02	88,00	**0,01**
			2nd	13,83	12,32	1,52	2,34	0,65	88,00	0,95
			3rd	12,53	12,22	0,31	2,34	0,13	88,00	1,00
			4th	8,95	9,40	-0,45	2,34	0,19	88,00	1,00
	3C	males	1st	20,25	12,17	8,09	2,75	2,94	96,00	**0,02**
			2nd	18,09	12,51	5,59	2,75	2,03	96,00	0,17
			3rd	14,44	13,78	0,65	2,75	0,24	96,00	1,00
			4th	15,61	10,97	4,64	2,75	1,69	96,00	0,33
hungry	3E	females	1st	17,44	5,92	11,53	3,03	3,81	56,00	**0,00**
			2nd	13,10	7,96	5,14	3,03	1,70	56,00	0,33
			3rd	7,72	10,69	-2,97	3,03	0,98	56,00	0,80
			4th	13,09	7,55	5,54	3,03	1,83	56,00	0,26
	3F	males	1st	16,34	8,93	7,41	2,40	3,09	72,00	**0,01**
			2nd	11,88	9,33	2,55	2,40	1,06	72,00	0,75
			3rd	7,79	10,98	-3,19	2,40	1,33	72,00	0,57
			4th	6,72	7,45	-0,73	2,40	0,31	72,00	1,00

Table S9. Affective state discrimination test. Anova table for sniffing relieved demonstartors %

Demonstrators state	Fig.	Source of variation														
		Time					Sex					Interaction				
		SS	DF	MS	F	p	SS	DF	MS	F	p	SS	DF	MS	F	p
relieved	3D	2577,00	3,00	859,00	2,93	**0,04**	138,70	1,00	138,70	0,47	0,49	521,40	3,00	173,80	0,59	0,62
hungry	3G	5341,00	3,00	1780,00	3,78	**0,01**	1134,00	1,00	1134,00	2,41	0,13	784,60	3,00	261,50	0,56	0,65

Table S9. Continued.

Demonstrators state	Fig.	Comparison of time sniffing relieved demonstrators females vs males %							
		Time [minute]	$M_{females}$	M_{males}	Mean difference	Šídák's multiple comparisons test			
						SE	t	DF	p
relieved	3D	1st	63,83	62,15	1,68	6,86	0,25	92,00	1,00
		2nd	52,25	57,77	-5,52	6,86	0,81	92,00	0,89
		3rd	50,00	47,46	2,54	6,86	0,37	92,00	0,99
		4th	50,33	58,46	-8,13	6,86	1,19	92,00	0,66
hungry	3G	1st	73,10	65,22	7,88	10,30	0,77	64,00	0,91
		2nd	65,59	60,97	4,62	10,30	0,45	64,00	0,99
		3rd	46,61	45,80	0,81	10,30	0,08	64,00	1,00
		4th	64,24	45,59	18,65	10,30	1,81	64,00	0,27

Table S10. Affective state discrimination test. One sample t-test for sniffing relieved demonstrators %

Demonstrators state	Fig.	Sex	Prosociality score %[a]						
			n	M	SEM	Time [minute]	One sample t test. Comparison with hypothetical mean (50%)		
							t	df	p
relieved	3D	females	12	63,83	2,72	1st	5,08	11,00	**0,00**
				52,25	4,61	2nd	0,49	11,00	0,64
				50,00	4,76	3rd	0,00	11,00	1,00
				50,33	6,30	4th	0,05	11,00	0,96
		males	13	62,15	4,96	1st	2,45	13,00	**0,03**
				57,77	4,265	2nd	1,82	13,00	0,09
				47,46	5,629	3rd	0,45	13,00	0,66
				58,46	4,635	4th	1,83	13,00	0,09
hungry	3G	females	8	73,10	6,18	1st	3,74	7,00	**0,01**
				65,59	5,32	2nd	2,93	7,00	**0,02**
				46,61	10,05	3rd	0,34	7,00	0,75
				64,24	7,33	4th	1,94	7,00	0,09
		males	10	65,22	4,261	1st	3,57	9,00	**0,01**
				60,97	5,238	2nd	2,09	9,00	0,07
				45,80	9,404	3rd	0,45	9,00	0,67
				45,59	7,961	4th	0,55	9,00	0,59

Table S11. Affective state discrimination test. One sample t-test for sniffing relieved demonstrators %. Demonstrators position (east vs west) comparison.

Demonstrators state	Fig.	Sex	Demonstrator's position	Sniffing relieved/hungry demonstrators %						Mean difference	Unpaired Student's t-test		
				n	M	SEM	One sample t test. Comparison with hypothetical mean (50%)						
							t	df	p		t	df	p
relieved	S2A	females	west	7	64,14	3,47	4,07	6,00	**0,01**	2,14	0,39	10,00	0,70
			east	5	62,00	4,23	2,84	4,00	**0,05**				
	S2B	males	west	8	65,00	6,43	2,33	7,00	0,05	8,20	0,78	11,00	0,45
			east	5	56,80	8,49	0,80	4,00	0,47				
hungry	S2F	females	west	4	67,76	9,97	1,78	3,00	0,17	10,67	0,85	6,00	0,43
			east	4	78,43	7,74	3,67	3,00	**0,03**				
	S2G	males	west	4	68,24	9,20	1,98	3,00	0,14	5,03	0,56	8,00	0,59
			east	6	63,21	4,32	3,06	5,00	**0,03**				

Table S12. Affective state discrimination test. One sample t-test for sniffing relieved demonstrators %. Demonstrators position (cups vs no cups) comparison.

Demonstrators state	Fig.	Sex	Demonstrator's position	Sniffing relieved/hungry demonstrators %						Mean difference	Unpaired Student's t-test		
				n	M	SEM	One sample t test. Comparison with hypothetical mean (50%)						
							t	df	p		t	df	p
relieved	S2C	females	cups	7	62,57	3,75	3,35	6,00	**0,02**	1,63	0,30	10,00	0,77
			no cups	5	64,20	3,73	3,80	4,00	**0,02**				
	S2D	males	cups	7	62,00	7,62	1,57	6,00	0,17	0,33	0,03	11,00	0,98
			no cups	6	61,67	7,13	1,64	5,00	0,16				

In the experiments with hungry demonstrators during the first day of habituation there were always cups in test cage.

Table S13. Affective state discrimination test. One sample t-test for sniffing relieved/hungry to be demonstrators %.

Demonstrators state	Fig.	Sex	Sniffing relieved/hungry to be demonstrators %						Mean difference	Unpaired Student's t-test		
			n	M	SEM	One sample t test. Comparison with hypothetical mean (50%)						
						t	df	p		t	df	p
relieved	S2E	females	9	35,44	5,83	2,50	8,00	**0,04**	10,06	1,28	19,00	0,22
		males	12	45,50	5,19	0,87	11,00	0,40				
hungry	S2H	females	8	59,32	3,98	2,34	7,00	0,05	20,46	2,01	16,00	0,06
		males	10	38,86	8,50	1,31	9,00	0,22				

Table S14. Descriptive Statistics and Correlations.

Sex	Behavioral test	experiment variant	Variable	n	M	SEM	Subject's weight [g][a]			Stimulus' weight [g][b]			Weight difference %[c]		
							r^2	r	p	r^2	r	p	r^2	r	p
females	Prosocial choice test[1]		Prosociality score %	8	10,18	2,34	0,00	0,06	0,88	0,09	0,30	0,46	0,05	-0,22	0,59
	Affective state discrimination test[2]	"relieved"	Sniffing relieved %	12	63,83	2,72	0,40	-0,62	**0,02**	0,50	-0,70	**0,00**	0,28	0,53	0,07
		"hungry"	Sniffing hungry %	8	73,10	6,18	0,19	-0,44	0,28	0,00	0,04	0,92	0,48	-0,69	0,06
	Social conditioned place preference test[3]		Preference score [s]	16	285,80	87,07	0,09	0,31	0,24	NA	NA	NA	NA	NA	NA
males	Prosocial choice test[1]		Prosociality score %	10	0,30	3,03	0,13	-0,36	0,38	0,09	0,30	0,30	0,54	-0,73	**0,01**
	Affective state discrimination test[2]	"relieved"	Sniffing relieved %	13	62,15	4,96	0,07	0,26	0,37	0,05	-0,24	0,42	0,00	0,02	0,93
		"hungry"	Sniffing hungry %	10	65,22	4,26	0,00	-0,03	0,93	0,04	-0,19	0,59	0,09	0,30	0,39
	Social conditioned place preference test[3]		Preference score [s]	12	221,60	90,59	0,04	-0,12	0,54	NA	NA	NA	NA	NA	NA

1a. Actor's weight on the first day of test; **1b.** Partner's weight on the first day of test; **1c.** Percentage of weight difference between actor and partner on the first day of test. 2a. Observer's weight on the first day of adaptation; 2b. Relieved/hungry demonstrator's weight on the first day of adaptation; 2c. Percentage of weight difference between observer and relieved/hungry demonstrator on the first day of adaptation. 3a. Subject's weight on the day of posttest.

Table S15. Groups of animals used in Figures 1-3. Only mice that met predefined ctriteria.

Sex	Behavioral test	experiment variant	n	Subject's age at the start of the procedure (weeks)[a]			Subject's age at test (weeks)[b]			Subject's weight at the start of the procedure [g][a]		
				M	Range	SEM	M	Range	SEM	M	Range	SEM
females	Prosocial choice test[1]		8	10,30	9,90-10,40	0,06	11,70	11,30-11,90	0,07	19,85	18,60-21,00	0,28
	Affective state discrimination test[2]	"relieved"	12	12,62	10,90-14,60	0,31	13,09	11,30-15,90	0,36	20,38	18,50-22,20	0,37
		"hungry"	8	11,49	9,90-13,40	0,52	11,94	10,30-13,90	0,53	21,28	19,40-23,20	0,41
	Social conditioned place preference test[3]		16	10,73	10,60-10,90	0,03	11,56	11,40-11,90	0,05	18,69	17,40-19,60	0,28
males	Prosocial choice test[1]		10	10,30	9,90-11,00	0,14	11,70	11,00-12,40	0,15	26,65	24,10-28,60	0,45
	Affective state discrimination test[2]	relieved	13	11,40	9,90-12,60	0,24	11,95	10,30-14,00	0,30	26,05	23,30-29,10	0,49
		hungry	10	11,52	10,00-14,10	0,43	12,07	10,70-14,60	0,43	26,69	24,80-31,00	0,58
	Social conditioned place preference test[3]		12	10,60	10,00-11,00	0,14	11,60	11,00-12,00	0,14	23,90	21,30-26,00	0,40

Table S15. Continued.

Sex	Behavioral test	experiment variant	n	Subject's weight at test [g][b]			Stimulus' weight at the start of the procedure [g][c]			Stimulus' weight at test [g][d]		
				M	Range	SEM	M	Range	SEM	M	Range	SEM
females	Prosocial choice test[1]		8	17,01	15,80-18,40	0,34	18,25	16,70-19,90	0,34	15,50	13,8-16,90	0,34
	Affective state discrimination test[2]	"relieved"	12	19,83	17,20-21,50	0,62	19,68	17,40-21,30	0,42	16,53	15,40-18,70	0,49
		"hungry"	8	21,70	21,20-22,50	0,22	20,65	19,40-21,60	0,25	17,68	17,20-18,30	0,20
	Social conditioned place preference test[3]		16	19,82	17,40-21,50	0,31	NA	NA	NA	NA	NA	NA
males	Prosocial choice test[1]		10	22,81	20,60-25,40	0,47	25,24	20,60-25,40	0,60	21,40	22,90-27,40	0,45
	Affective state discrimination test[2]	relieved	13	25,42	23,20-28,10	0,51	24,65	21,00-28,20	0,57	20,95	17,30-26,80	0,73
		hungry	10	27,23	24,80-30,20	0,74	25,21	23,40-28-60	0,49	21,56	19,90-23,10	0,65
	Social conditioned place preference test[3]		12	23,74	21,60-25,50	0,34	NA	NA	NA	NA	NA	NA

1a. Actor's age/weight at start of the food deprivation; **1b.** Actor's age/weight on the first day of test; **1c.** Partner's weight on the start of the food deprivation; **1d.** Partner's weight on the first day of test. **2a.** Observer's age/weight on the first day of adaptation; **2b.** Observer's age/weight on test day; **2c.** Relieved/hungry demonstrator's weight on the first day of adaptation. 3a. Subject's age/weight on the day of pretest; **3b.** Subject's age/weight on the day of posttest.

Table S16. Social conditioned place preference test. Conditioning cues.

	Name	Full name	Description/size [mm]	Manufacterer	Website
Bedding types	Aspen 1	ABEDD aspen animal bedding	cubic granulates	Abedd SIA Jelgavas iela 29 Kalnciems, LV-3016, Latvia	https://www.abedd.com/
	Beech 1	Trociny bukowe przesiane gat. 1	shavings	P.P.H. "WO-JAR", Kopernika 3/30, 32-100 Proszowice, Poland	NA
	Beech 2	Trocinka bukowa Facimiech	shavings	PPHU Natur-Drew A. Czaja, os. Kopernika 5/57, 34-100 Wadowice, Poland	NA
	Cellulose	Biofresh Performance Bedding. 1/8' Pelleted Cellulose	pellets	ABSORPTION CORP 6960 Salashan Parkway Ferndale, WA 98248, USA	https://scottpharma.net/product/biofresh-performance-bedding/
Gnawing block types	Block 1	Long thin gnawing block	99 × 19 × 19	Urszula Borgiasz Zoolab, Zielona 14, 28-340 Sedziszow, Poland	http://zoolab.pl/en/enrichment-elements/
	Block 2	Long big gnawing block ("for rats").	99 × 39 × 39		
	Block 3	Cube gnawing block	49 × 39 × 39		