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Full Length Article

Male scent but not courtship vocalizations induce estrus in wild female house mice

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ABSTRACT

Exposure to males or male urinary scent can induce and accelerate the rate of female estrous cycling in house mice ("Whitten effect"), and this response has been replicated many times since its discovery over 60 years ago. Here, we tested whether exposing female mice to recordings of male courtship ultrasonic vocalizations (USVs) induces estrous cycling, and whether exposure to both male scent and USVs has a stronger effect than to either of these stimuli alone. We conducted our study with 60 wild-derived female house mice (*Mus musculus musculus*). After singly housing females for 14 days, we monitored estrous stages via vaginal cytology for two weeks while isolated from males or male stimuli. We continued monitoring estrus for two more weeks during experimental exposure to one of four different types of stimuli: (1) clean bedding and background noise playback (negative control); (2) recordings of male USVs (16 min per day) and clean bedding (male USV treatment); (3) soiled male bedding and background noise playback (male odor treatment; positive control); or (4) male USVs and soiled male bedding (male odor and USV treatment). Females were then paired with males to test whether any of the four treatments influenced female reproduction (especially latency to birth). We confirmed that exposure to male odor increased female cycling, as expected, but exposure to recordings of male USVs had no effect on estrus. Females exposed to both USVs and odor went through more cycles compared to controls, but did not differ significantly from exposure to male odor (and background noise). After pairing females with a male, females showing male odor-induced cycling produced their first litter sooner than controls, whereas USVs did not have such an effect. This is the first study to our knowledge to show that male odor induces estrus in wild house mice and to show functional effects on reproduction. Our results do not support the hypothesis that male vocalizations induce female estrus, although we suggest other approaches that could be used to further test this hypothesis.

1. Introduction

Rodents and many other mammals emit complex ultrasonic vocalizations (USVs), but their functions are still unclear. In adult house mice (*M. musculus*), USVs are emitted during courtship and mating [1,2], and also same-sex interactions and parent-offspring interactions [3]. When males detect a sexually mature female, or just their scent, they begin vocalizing [4,5], and males produce most (ca. 85%) of the USVs recorded during opposite-sex interactions [6]. Mice produce several types of USVs, and during sexual interactions, they increase the number and types over time [7,8], and once mounting begins, mating pairs reduce the emission of some and increase production of other types of USVs (Matsumoto & Okanoya, 2016). Studies on the functions of male courtship USVs so far have only examined their attraction to females (signaling and releaser effects). For example, females are more attracted to vocalizing than experimentally de-vocalized males [9] and to playbacks of recorded male USVs compared to no sound [10] or background noise [5]. Females show a preference for playbacks of USVs from males of the same species [11] and for unrelated males versus siblings [5]. Female preferences for male USVs develop early in life before weaning (negative sexual imprinting) [12]. Females' attraction to male USVs does not appear to depend on the stage of their estrous cycle ([10,13]; but see [12]). Yet, no studies to our knowledge have tested whether the USVs of male mice have priming effects, such as accelerating puberty or inducing estrus, or otherwise influence female sexual receptivity.

House mice are polyestrous and spontaneous estrous cyclers (the average cycle duration in laboratory mice varies from ca. four to seven days) [14,15], and exposure to a male mouse or their urine accelerates

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female growth and sexual development ('Vandenbergh effect'; [16]) and estrous cycling ('Whitten effect'; [17,18]). In his original study, Whitten [18] put male mice into a small basket and placed them into a females' cages for two days before pairing, which increased their mating frequency, as well as shortening female estrous cycles overall. Many studies have subsequently shown that estrus can be induced or accelerated after exposure to male urine (e.g., [19–24]), urinary volatiles [25], or synthetic analogs of urinary pheromones ([26,27]; reviewed in [28]). Mice and rats make up 80–95% of all laboratory animals [29,22], and estrus induction has become a tool for breeding mice and for experiments aiming to manipulate or control variation in estrous stages. Hormonal stimulation can be problematic due to having varying physiological effects [22], and thus some researchers make use of the Whitten effect to induce estrus [22,30,31]. Surprisingly little is known about the neuroendocrine mechanisms controlling estrus induction [32] or its adaptive functions [33]. Wild male mice deposit urinary scent marks in response to encountering females or their scent [34,35], which may function to influence female estrus and sexual receptivity. Yet, to our knowledge, previous studies have only investigated the Whitten effect in domesticated laboratory mice, despite calls for testing this behavior and its function in wild house mice [36].

Estrus can also be induced by male vocalizations in several non-rodent species, but no studies have tested this hypothesis in mice or any other rodents to our knowledge. Male vocalizations have been found to have priming effects on female reproduction in some other vertebrates. In ringdoves (*Streptopelia risoria*) and budgerigars (*Melopsitacus undulatus*) male vocalizations stimulate female gonad development and activity [37–39]. The roaring of male red deer (*Cervus elaphus*) advances female ovulation [40], and similar findings have been made in goats (*Capra hircus*). Exposing solitary females to male vocalizations trigger an increase of luteinizing hormone (LH) secretion, which controls estradiol release, and more frequent displays of characteristic estrous behavior [41]. In wild house mice, increased USV emission during sexual interactions reduced the time until mated pairs produced a litter [7]. In laboratory mice, USV emission induced the activation of kisspeptin neurons, which control gonadotropin-releasing hormone (GnRH) release in female brains, and increased the number of litters produced by breeding pairs [42]. These findings are consistent with the hypothesis that male USVs influence female reproductive physiology and sexual receptivity.

In this study, we aimed to test whether male USVs influence estrous cycling in female house mice (*M. musculus musculus)*. We tested the effect of male USVs on female estrus when presented alone or simultaneously with male odor, and whether exposure to both types of stimuli has a stronger effect than either stimulus alone (multimodal signaling hypothesis). A previous study, for example, found evidence for such an interaction of pup odor and USVs on attracting maternal attention in laboratory mice [43]. To test these hypotheses, we exposed females to the following male stimuli: (1) USVs; (2) odor; or (3) both USVs and male odor; and then examined their effects on female estrous cycling compared to controls. We predicted that all of these stimulus treatments would promote estrus, and this is the first such experiment to our knowledge. Furthermore, we aimed to test whether exposing females to male odor or USVs also promotes their reproductive success. Half of the females were paired with an unfamiliar male to test whether any of these stimulus treatments affected their reproductive success. We predicted that male stimuli that promote estrus would also reduce the time for females to give birth and potentially increase their litter size, as required to show that priming effects have functional consequences.

2. Methods

2.1. Subjects

We conducted our study with wild-derived house mice (laboratory-reared F3 of wild *M. musculus musculus,* caught at nine locations in Vienna and Lower Austria; herein, 'wild mice' for brevity). Studies with wild mice are needed to generalize findings obtained from laboratory strains [36], especially given the differences in their behavior and physiology [44]. This is particularly important for studies on acoustic communication, given the auditory defects in many strains of laboratory mice [45]. We used 60 age-matched (146 ± 22 d old) and sexually mature female mice as subjects. To ensure a high number of regularly cycling females, all of the mice were 3–10 months old at the time of the experiment, which is within the range in which females cycle the most [46]. Females were raised with their father until weaning (3 weeks after birth) and then pair-housed with another female (51 females were housed with their sister, and nine were housed with an unrelated female) prior to the experiment.

2.2. Housing

Female subjects were housed in standard, open type IIL cages (36.5 × 20.7 × 14 cm, Tecniplast, Germany) containing wooden bedding (ABEDD, Austria), a plastic nest box (Tecniplast, Germany), nesting material (cotton nestlets, ABEDD, Austria), and a cardboard roll for environmental enrichment. Food (standard rodent diet 1324, Altromin, Germany) and water were provided ad libitum. After each cage change (once at the beginning of each phase during the study period), we provided each mouse with additional food as dietary enrichment, i.e., circa seven g of a mixture of wheat, sunflowers, sorghum, oats, and peanuts.

To establish a baseline for estrous cycling without male stimulation, female subjects were separated from the rest of our mouse colony and moved to a different room two weeks before the beginning of the experiment. To obtain independent measurements and avoid cycle synchronization, the mice were singly housed rather than socially, which has been shown to reduce variance in cycle length [46], so that they would continue cycling [18,46–49], despite being isolated from males (rather than estrus suppression from male isolation in socially housed females; 'Lee-Boot effect'). We allowed time for the mice to acclimatize to the new environment (14 days before beginning the study) because changing environmental factors, such as light, noise or temperature, can influence cycling [50, 51]. The animals were kept under a consistent 12 h:12 h day:night cycle (light on/off at 03:00 /15:00) and the temperature in the room was monitored daily. The mice belonging to the same olfactory treatment condition shared a rack (30 cages/rack), and the two racks were placed on opposite sides of the ventilated room to odor-isolate the groups as much as possible. There were no differences in the environmental conditions of the two sides of the room that we could detect.

2.3. Study design

We singly housed females and isolated them from males for 14 days, and then began monitoring each female's estrous stage via vaginal cytology for two weeks (exposing them to a sham treatment lacking male stimulation). Over the next two more weeks, we continued monitoring estrus during experimental exposure to one of four types of stimuli: (1) clean bedding and background noise playback ('negative control'); (2) male USVs and clean bedding ('male USV treatment'); (3) soiled male bedding and background noise ('positive control'); or (4)

Table 1
Female subjects were assigned to one of four experimental groups, and they were then exposed to one of four different types of stimuli combinations.

		Acoustic Stimuli: Background noise	**Male USV playback**
Olfactory Stimuli:	**Clean bedding**	(1) Control (15 females)	(2) USV (15 females)
	Male-soiled bedding	(3) Odor (14 females, one died)	(4) USV + Odor (15 females)

male USVs and soiled male bedding ('male odor and USV treatment') (Table 1). The 60 subject females were systematically assigned to one of four groups by balancing age, family (litter), and previous housing, and then to make decisions for draws, such as for females from the same litter, a die was used to make assignments. Each female was subsequently exposed to one of these treatment groups or controls for 28 consecutive days, and daily vaginal smears were taken via vaginal lavage (with a pipette) to determine the number and length of each female's completed cycles during the experiment.

The experiment was separated into two 14-day phases, the first "pre-treatment" and the next "stimulus treatment" or just "treatment" phase. In both phases, the animals underwent the exact same procedures, which included exposure to stimuli (or sham control stimuli), as well as the daily vaginal lavage. During the pre-treatment phase, females were exposed to control stimuli (background noise and clean bedding), which also allowed us to determine individual baselines for cycling activity without any male stimulation. Then, the treatment phase began in which we exposed females to one of four possible combinations of olfactory (clean vs. male soiled bedding) and acoustic (background noise vs. male USVs) stimuli for two weeks. As mentioned above, females were assigned to one of these four different groups (Table 1), while balancing age, family and previous housing conditions. To determine the effect of exposure to male stimuli, we compared changes in individual females' cycling patterns between the two different phases and between the treatment groups. On the last day of the experiment, eight females from each treatment group (32 females in total; more breeding pairs were impossible due to spatial limitations) were placed in a cage with an unfamiliar and unrelated male. These breeding pairs were monitored for two more months to track their reproductive success, and we measured the latency to the date of birth of the first litter and the litter sizes.

2.4. Preparation of male stimuli and female exposure

To prepare the olfactory stimuli, we created mixtures of soiled bedding collected from four males (unrelated and unfamiliar to the female) for each female subject. In total, an equal amount of bedding from 16 males from 16 families was used for these mixtures. We mixed bedding to reduce the variation due to differences among individual males. The soiled and the control clean bedding were packed in separate freezer bags and stored at -80 °C until use. To expose females to the olfactory stimuli, circa ten g of their assigned stimulus bedding (or control) was added to their cage each day at the time that the female was handled for the vaginal lavage. During the pre-treatment phase, all 60 females received clean bedding. Due to the sensitivity of mice to odor, special care was taken throughout the experiment to separate these groups and use different and regularly cleaned areas, surfaces, and tools to handle them.

To prepare the auditory stimuli, USV recordings of four different males were selected from a sample of recordings from 29 males (sexually mature, wild-derived male house mice) that vocalized at a high rate and were unrelated and unfamiliar to subject females. The males were recorded in the same contexts, i.e., after sexually priming, the males were presented with female urine and a sexually primed female on the oppositive side of a transparent, perforated divider (see [52]) for five minutes on two different dates. The recordings were modified using STx (Version: 5.0.6.10071; Acoustics Research Institute, Vienna, Austria) by cutting gaps without any vocalizations present to a maximum length of five seconds, as well as removing the entire sound spectrum below 20 kHz to reduce noise and squeaks. The final USV files for the playback consisted of one minute of background noise followed by seven minutes of a looped male's first recording, another minute of background noise and then seven minutes of the same male's second recording, for a total of 16 min. To simulate typical intra-individual variation in male vocalizations, we also created a second playback file from each male, in which the first and second recordings were switched. Thus, we obtained eight different USV playback files. All files started with a ramp-up to avoid startling the females. To exclude file quality as a confounding factor, the design was balanced so that each female in the USV treatment groups was exposed to each of the eight playback files at least once during the 14 days of the treatment phase. To create the sham control ("background noise"), a noise-free segment without vocalizations was taken from one of the recordings and looped for a duration of 16 min.

To expose female subjects to auditory stimuli, each mouse (in its home cage) was placed inside of an acoustic exposure box (0.6 m x 1 m base and 0.5 m deep), which had an ultrasonic speaker (Ultrasonic Dynamic Speaker Vita, Avisoft Bioacoustics, Germany) attached to the lid and directed toward the mice (Fig. 1). The lid was closed, and the playback of the assigned stimulus was initiated for four mice at the same time for 16 min. A microphone (Avisoft Bioacoustics, Germany) was placed inside of the box to confirm playbacks, and preliminary tests confirmed that the playbacks were detected inside of all four cages. To avoid volatile male odor spreading from odor-treatment and to control mice, two different boxes were used in the experiment, one for females exposed to male bedding and the other for females exposed to control bedding.

After each playback exposure was completed, a vaginal lavage was performed, and the male bedding was added to the female's cage. Order and timing of each female exposure to male stimuli were kept consistent during the experiment to keep the interval between vaginal lavages constant, and thus avoid variability due to temporal differences in sample collection [53].

2.5. Estrous staging: vaginal cytology

To monitor estrous cycles, a daily vaginal lavage was performed [54] after each exposure. To perform the lavage, each mouse was transferred from its home cage by allowing it to enter a plastic bottle, as we never hold mice by their tails. They were then transferred into a small cotton bag, which allows quick handling, as wild mice are smaller and more agile than laboratory mice. The lavage was performed by gently placing a pipette tip with 0.3 mL of room temperature tap water on the vaginal opening, making sure not to penetrate $>$ 1 mm, as to avoid tissue damage or inducing pseudopregnancy [53,55], and gently flushing the liquid 4–6 times into the vagina. This method is considered non-invasive [53], and mice become accustomed to the procedure [56]. The entire procedure lasted ca. 10 to 15 s and was always performed by two experimenters, one male and one female. After the lavage, the liquid was pipetted onto a microscope slide to dry and then evaluated with a light microscope (Nikon Optiphot-2). We determined the stage of the estrous cycle (following [54]): Proestrus, estrus, metestrus, diestrus, and their transitional stages were based on the proportion of visible cells, such as leukocytes, cornified epithelial cells, and nucleated epithelial cells. Daily monitoring improves classification, but if the stage of a sample could not be unambiguously classified due to a low number of cells, then diestrus was assumed [18,53]. This assumption did not change our results, as for statistical purposes, we only counted occurrences of the very distinctive estrus/metestrus stages. All the smears were evaluated by a single person to ensure consistency [46,53], and the evaluator was blind to the treatment group of the sample. As we address in the Discussion, a lack of regular cycling baselines during the pre-treatment phase prevented comparing cycle lengths between the phases. Instead, we used the difference in the number of detected cycles for a female in each phase as an indicator of cycling induction. One cycle was defined as a female entering and leaving estrus – the most distinguishable phase – one time. If no estrus but only metestrus was detected, we assumed that estrus must have passed on the same or previous day.

2.6. Statistical analyses

To test for changes in the number of cycles between the two phases within a group, Wilcoxon signed-rank tests were performed. General Linear models (package: "lme4" V.1.1–27.1; [57]) were used to test for

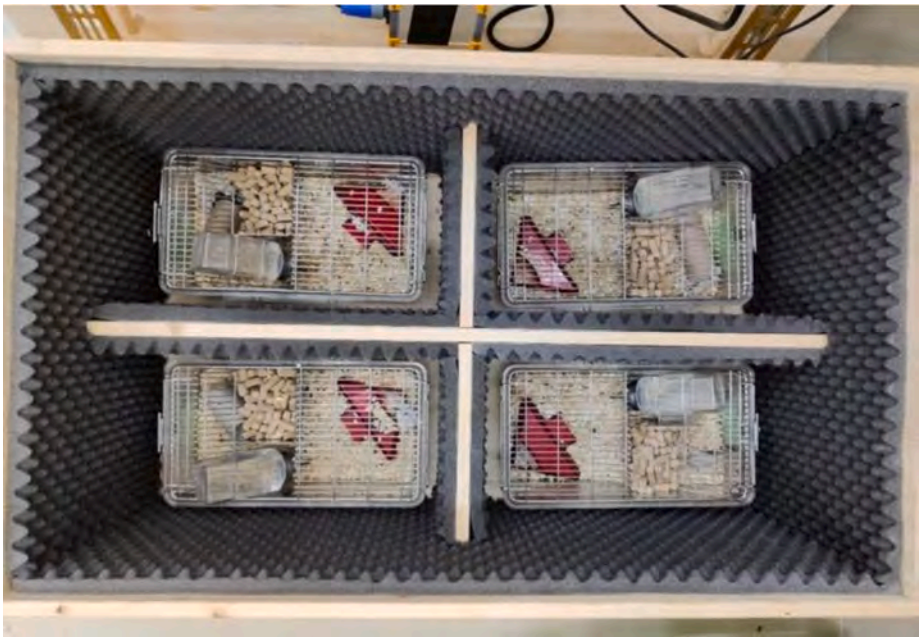

Fig. 1. View inside of one of the acoustic exposure boxes, showing four cages with subjects placed into four acoustically isolated compartments. The walls and compartments of the wooden box were covered with foam material to prevent sound distortion, and the speaker was fixed to the lid to face downwards towards the mice when closed.

treatment effects on cycling (GLMM: number of observed cycles by stimulus treatments as fixed effects, with individual ID and litter ID as random effects; Poisson distribution) as well as breeding (GLM: latency to birth by stimulus treatments as fixed effects; negative binomial distribution). Spearman rank correlations were used to test for effects of age on cycling and reproduction. All statistical tests and plots (package:

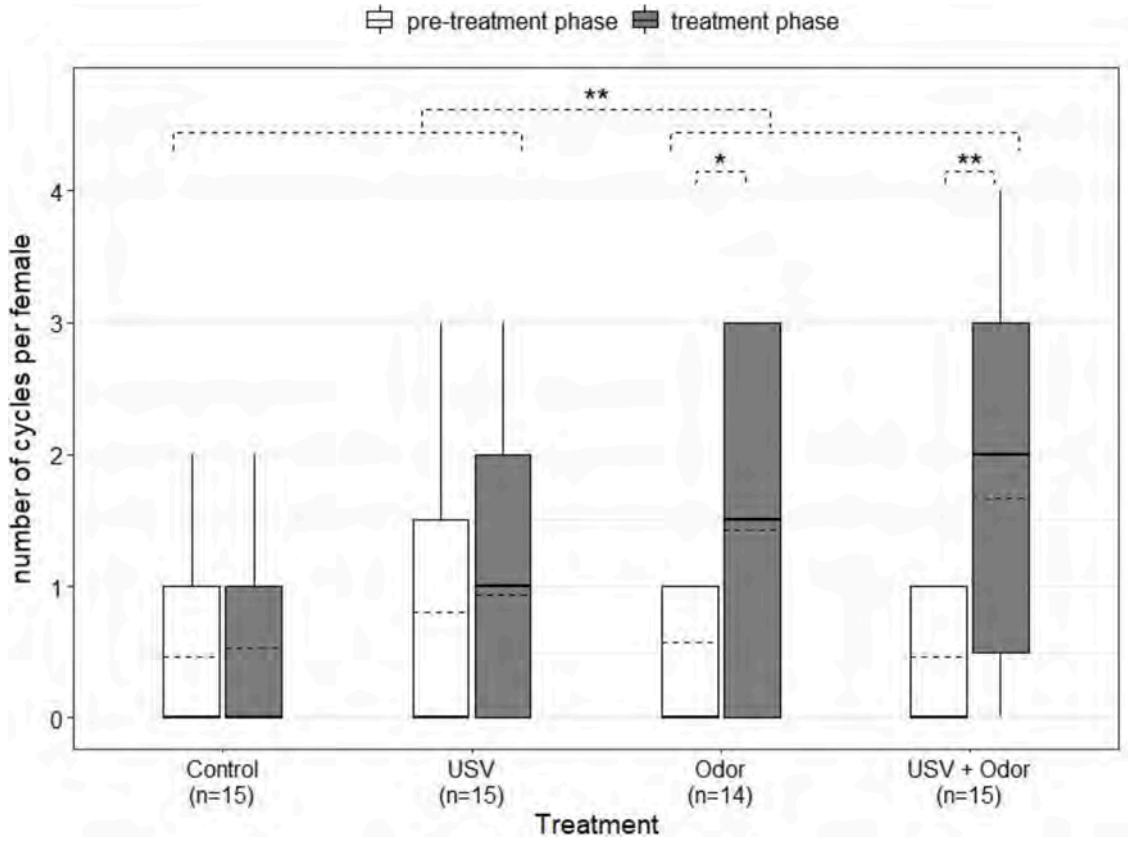

Fig. 2. Boxplot showing the average number of estrous cycles of females in the treatment and control groups during the pre-treatment phase without any male stimulation versus the subsequent treatment phase with male stimulation. Solid lines indicate medians, dashed lines the means, and vertical lines represent interquartile ranges.

"ggplot2" V. 3.3.5; [58]) were performed in R (V.1.2.5001).

3. Results

3.1. Estrus induction

Out of the 60 female subjects, one died for unknown reasons, and 16 females remained in diestrus during the entire 28 d A total of 1633 vaginal lavage samples were taken, which were used to identify 101 cycles. Significantly more cycles were observed in the treatment phase (67) than in the pre-treatment phase (34) (Wilcoxon signed-rank test: $V = 70$, $p = 0.0003$), which shows that the stimulus treatments overall effectively induced estrus. When analyzing the four treatments, a significant change in cycling activity between pre-treatment phase and treatment phase was only found in the odor (Wilcoxon signed-rank test: $V = 2$, $p = 0.0226$) and USV plus odor treatment (Wilcoxon signed-rank test: $V = 3$, $p = 0.0068$). A generalized linear mixed model (GLMM) was performed to test for differences in the number of observed cycles in both phases. Cycling activity was affected by the olfactory ($z = 2.810$, $p = 0.0049$) but not by the auditory stimuli ($z = 0.916$, $p = 0.356$). There was no interaction between the olfactory and auditory stimuli ($z = -0.167$, $p = 0.8674$) (Figs. 2 and 3).

The four treatment groups followed the same cycling activity pattern over time, although the extent of cycling induction differed among these groups. During the pre-treatment phase, overall cycling activity declined from the first week to the second week. After male stimuli treatments began on day 15, however, cycling activity increased across all groups in the third and fourth week, with the odor exposed groups showing a faster increase than others (Table 2). No significant effects on cycling were found for female age (Spearman's rank correlation: $S = 27{,}904$, $\rho = 0.184564$, $p = 0.1617$) or previous housing of the females (Wilcoxon signed-rank test: $V = 148.5$, $p = 0.0857$).

Table 2
Total number of cycles identified in each treatment group during each week of the study.

Treatment	*Week 1*	*Week 2*	*Week 3*	*Week 4*
Control (n = 15)	6	1	4	4
USV (n = 15)	9	3	5	8
Odor (n = 14)	7	1	8	12
USV + Odor (n = 15)	6	1	10	15

3.2. Effects of treatments on reproductive success

Most (24/32 or 75%) of the females that were assigned to males for breeding at the end of the treatment phase gave birth. Two females were included in the analyses for the birth latency data but not the litter size, as they gave birth but then their pups died for unknown reasons. The latency until birth was affected by the odor treatment (GLM: $z = -2.219$, $p = 0.02651$), but not the auditory treatment ($z = -1.055$, $p = 0.29121$) (Table 3, Fig. 4). The age of females had a negative effect on latency to give birth, with older females taking longer (Spearman's rank correlation: $S = 1313.4$, $\rho = 0.4289533$, $p = 0.0365$). Stimulus treatment did not affect successful mating (Kruskal-Wallis $\chi^2 = 3.875$, df = 3, $p = 0.2753$) or litter size (Kruskal-Wallis $\chi^2 = 0.81959$, df = 3, $p = 0.8448$).

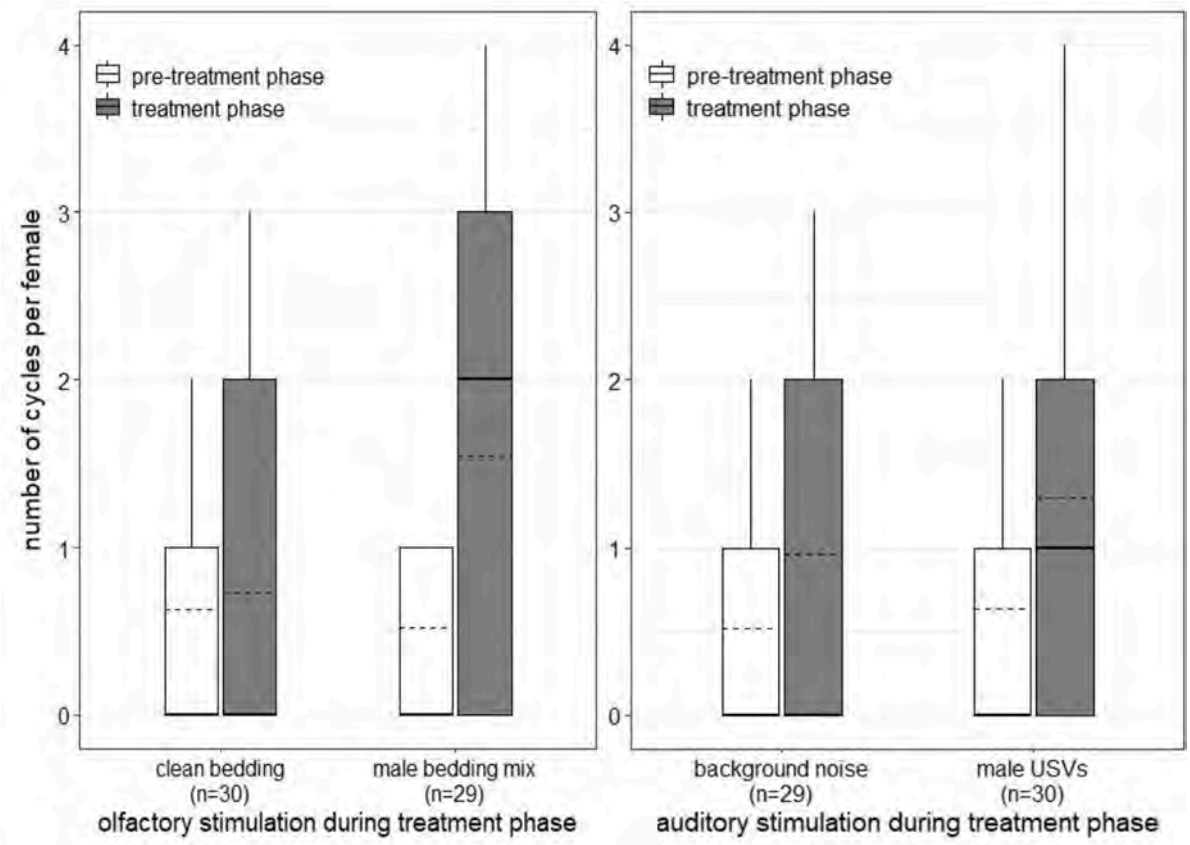

Fig. 3. Boxplot showing the average number of estrous cycles in females in both phases of the experiment. Instead of separating by treatment groups, this boxplot solely serves to visualize the effects of olfactory and auditory stimulation independently. Solid lines indicate medians, dashed lines the means, vertical lines represent interquartile ranges.

Table 3
Reproductive success of the females from the four treatment groups measured as the mean latency to give birth in the first litter, mean number of offspring (± standard deviation) in the first litter, and the number of successful breeding pairs.

Reproductive success:	Treatment groups *Control*	*USV*	*Odor*	*USV + Odor*
Mean latency to birth (d)	26.8 ± 6.4	23.6 ± 5.5	21.5 ± 0.6	22.1 ± 1.3
Mean litter size	4 ± 2	5 ± 3	6 ± 3	4 ± 2
Number of successful pairs	6/8	7/8	4/8	7/8

4. Discussion

We found that exposing females to male odor induced estrus, as expected from studies on laboratory mice [18], and these are the first such results from wild house mice to our knowledge. We also found that females exposed to male odor decreased the latency to give birth, as predicted. However, we found no evidence that exposing females to male USVs was sufficient to induce estrous cycling, and exposure to both types of male stimuli, odor and USVs, did not have a significantly stronger effect on estrus than exposure to male odor alone. Below we address our main findings in more detail.

4.1. Unexpected estrus suppression

After separating the females to establish a baseline in cycling, we expected a decrease in cycling based on previous studies [24,48], but the almost complete lack of cycling that we observed across all females was surprising, as cycling does not usually cease completely in laboratory mice (but see [24]), unless females are group housed and isolated from males ("Lee-Boot effect"). The 'Lee-Boot' effect is suspected to provide a mechanism for females to conserve energy and resources in the absence of potential mates. It is also thought to require socially housing females, or exposing singly housed females to the odor of other females [47]. The spread of volatiles among females cannot be ruled out in our study, as their cages were not isolated from each other. Why females would require stimuli from other females to conserve energy is unclear, but regardless, our result suggests a potential difference between domesticated versus wild house mice (or between *M. musculus domesticus* and *M. muscuslus musculus)*. Also, most females were cycling for the first few days before going into prolonged diestrus in our study (Table 2), suggesting that handling during our pre-treatment phase might have also suppressed cycling, either through spreading female-specific odorants, or by inducing pseudopregnancy (prolonged diestrus for 12–14 days [56]). It has been suggested that vaginal stimulation with the pipette tip can induce pseudopregnancy, although we were careful not to insert the tip > 1 mm, as recommended [46,53]. Another non-exclusive hypothesis for the suppressed cycling, albeit seemingly far-fetched, is the presence of a novel male human odor during the experiment [59]. Previous studies on estrus induction in mice used cycle length as the primary measure, but this was not possible in our study due to the unexpectedly low number of observed cycles during the pre-treatment phase. Thus, future studies are needed to better understand whether exposure to female odor is necessary to induce the Lee-Boot effect, and if so, then why, and to also test the effects of handling. Nevertheless, this unpredicted response allowed us to compare the actual induction of estrus in terms of the number of cycles for each female during each phase, which is a more conservative measure of cycling activity and less prone to errors than the cycle length.

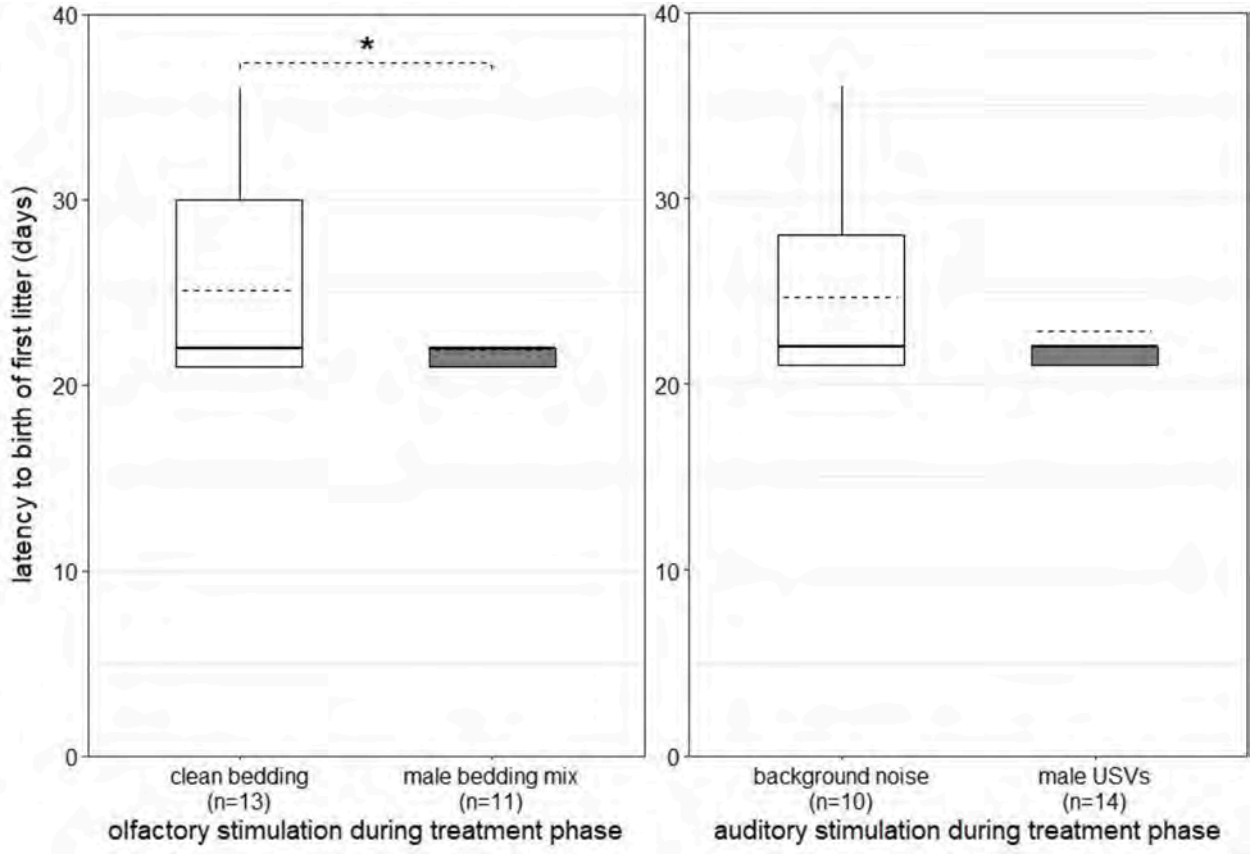

Fig. 4. Boxplots showing the observed birth latency in breeding females ($n = 24$) by treatment conditions. Solid lines indicate medians, dashed lines means, vertical lines represent interquartile ranges. Previous exposure to male odor significantly affected the females' latency to give birth.

4.2. Odor stimulus (positive control)

As we detected no change in cycling activity across the two phases in the control group, the increase in cycling activity in the three experimental treatment groups can be attributed to exposure to male stimuli. We found significant differences in estrus between control versus odor-treatment groups (Figs. 2 and 3), which shows that male odor induced estrus cycling, as expected [18]. Our results extend previous findings on laboratory mice to wild *M. musculus* (and to *M. musculus musculus)*. It is often assumed that the Whitten effect occurs in socially housed females, and yet our results confirm that male odor also influences cycling solitarily housed females [18]. Studies are needed to test whether odor-induced estrous cycling occurs in wild house mice living in more natural social contexts and whether socially dominant males are more effective than subordinates [60].

4.3. Acoustic stimulus

In contrast to male odor, we found no evidence that male USVs induced estrus, either when females were exposed only to USVs alone, or to both USVs and odor (contrary to the multimodal hypothesis). We found a somewhat larger increase in cycling activity among females exposed to both male odor and USVs than the other treatments; however, this difference was not statistically significant. These results are surprising – assuming that male USV emission functions to influence female sexual receptivity, and that females adjust cycling depending upon the availability of potential mates, as generally assumed.

It is unclear why the females responded to male odor but not to USVs; however, there are several reasons why we may have failed to detect an actual effect from USVs or significant evidence for the multimodal hypothesis. First, females were exposed to USV playbacks for 16 min per day, whereas they were chronically exposed to male odor and estrous induction might require a longer duration of exposure (e.g., more calling, like scent marking, might provide a more reliable indicator of social dominance and ability to defend a territory). We considered exposing females to playbacks of USVs chronically, but such exposure would be artificial (male mice do not continuously vocalize) and potentially stressful. We also considered reducing the duration of olfactory exposure, but we opted for continuous exposure, the method previously shown to induce estrus. Second, it is possible that only recordings of particular males, such as familiar individuals or dominant males, emitting specific types of calls or sequences of USV types, influence estrous cycling. Third, it is possible that USV playbacks are too artificial and not perceived by females as male USVs. Fourth, females might respond to male USVs if previously exposed to a male mouse or another type of sensory stimulus than urine. Thus, further studies are needed to explore other types of male stimuli.

If male USVs do not induce female estrous, as our results suggest, then this finding still needs explaining. We expected male courtship USVs would influence female estrus because they appear to provide reliable indicators of a male's species identity [11], health [61], and social status ([62,63]; but see [64]) – much like male urinary odor [60, 65]. – and sexual arousal [8]. However, the USVs of male and female mice are very similar [52,66,67], and may not be sufficiently sexually dimorphic to enable sex recognition. In contrast, mouse urine is highly sexually dimorphic in the concentration of major urinary proteins and volatile ligands (pheromones) [68], and females can discriminate the urinary odor of the sexes (e.g., soiled bedding: [69]). Thus, we suspect that urinary scent provides a more reliable signal for sex recognition and potential mating opportunities than USVs do.

4.4. Adaptive functions and evolution of odor-mediated estrus induction

We found that females produced their first litter sooner if they were previously exposed to male odor alone, or in combination with male USVs. Females exposed only to male USVs did not show such an effect, and the type of stimulus did not significantly affect the number of successful matings or the litter size. We caution against drawing strong inferences from these results, as our sample sizes were small. Nevertheless, our results are consistent with the hypothesis that odor-mediated estrus induction enhances female sexual receptivity and rate of reproduction. Future studies are needed to determine whether odor-induced estrous cycling enhance male reproductive success in more natural contexts [60]. Studies are also needed to test whether odor-induced estrus induction provides an example of sexual manipulation or whether this response is beneficial for both sexes. Evolutionary theory of sexual conflict has not received enough attention in studies on mammals and pheromones [70]. Finally, further research is also needed to determine whether odor-mediated estrus induction in house mice and other mammals, such as goats (*Capra hircus*; [71]), sheep *(Ovis aries*; [72]), and hamsters (*Phodopus sungorus*; [73]) evolved convergently or whether it is a shared, ancestral trait among these different mammals.

5. Conclusions

This is the first study to our knowledge to experimentally test whether exposure to male USVs influences female estrus (sexual receptivity) in house mice. While we found no significant evidence that male vocalizations influenced females' estrous cycling, further studies are needed to test this hypothesis and also to investigate the potential interaction of other male sexual signals on estrus induction. This was also first study to our knowledge to test odor-mediated estrus induction (Whitten effect) in wild house mice to our knowledge, thus opening the door for future research on the adaptive functions and evolutionary origins of these mechanisms.

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Ethics Statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Experiments were conducted at the *Konrad Lorenz Institute of Ethology*, Austria, and the protocols and experimental procedures have been approved and were in accordance with ethical standards and guidelines in the care and use of experimental animals of the Ethical and Animal Welfare Commission of the University of Veterinary Medicine, Vienna (Austria) and by the Austrian Federal Ministry of Science and Research (approval no. BMWF-2021–0.588.540).

CRediT authorship contribution statement

Simon Wölfl: Conceptualization, Methodology, Visualization, Formal analysis, Investigation, Writing – original draft. **Sarah M. Zala:** Conceptualization, Methodology, Resources, Visualization, Writing – review & editing, Supervision, Funding acquisition. **Dustin J. Penn:** Conceptualization, Methodology, Resources, Visualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

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