Odor-Specific Effects on Reentrainment Following Phase Advances in the

Diurnal Rodent, *Octodon degus*

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Abstract

Reentrainment following phase shifts of the Light:Dark (LD) cycle is accelerated in *Octodon degus* in the presence of olfactory social cues (i.e. odors) produced by conspecifics. However, not all odors from conspecifics were effective for facilitating reentrainment after a phase-advance. In the current experiments, we examined whether non-animal odors, odors from another species, or conspecific odors, including those manipulated by steroid hormones, can cause the same increased reentrainment of wheel running activity as odors from an intact, adult female degu. A variety of odors, each selected to probe a particular aspect of the reentrainment acceleration phenomenon, were presented to a group of phase-shifting female degus. The shifting females (test animals) responded to odors of intact, female degu donors with decreased reentrainment time, but odors of ovariectomized (OVX), OVX with a single hormone replacement capsule (estradiol or progesterone) or phase-shifting females had no effect. Multiple males were effective odor donors, whereas a single male was ineffective in earlier studies. Rats and cloves were not effective in accelerating reentrainment. Furthermore, odors from rats delayed reentrainment. We conclude that the odors that effectively accelerate degu reentrainment after a phase advance of the LD cycle is species-specific. We also report that repeated phase shifts, followed by complete recovery of phase relationships, do not alter the rate of recovery from a phase shift over time. These data suggest that in degus, a social species, odors may reinforce and strengthen the salience of the photic zeitgeber, and/or facilitate synchronization of rhythms between animals.

Key Words: circadian, degu, phase-shift, entrainment, non-photic
Introduction

Circadian rhythms such as sleep/wake cycles, temperature fluctuations and hormonal changes are synchronized by time cues (i.e. zeitgeber) that occur at a 24-hour period through the process of entrainment. Zeitgebers cause the internal circadian mechanism of an organism to advance or delay the underlying, endogenous period to match that of the zeitgeber (10). Adherence to the rhythmicity of environmental time cues allows organisms to prepare for daily events such as foraging, mating and nest-building at times in the day appropriate for that organism (10). Although the most ubiquitous zeitgeber is photoperiod, a number of non-photic stimuli can entrain free-running circadian rhythms or facilitate reentrainment following phase shifts of the light cycle. Effective non-photic stimuli include restricted food availability (23, 31), scheduled or forced exposure to running wheels (11, 12, 29, 34, 40-42), stress/arousal (2, 18, 40), social cues (3, 9, 15-17) and conditioned stimuli (1). Because of threats or special opportunities in the environment, it is adaptive for an organism to have redundant mechanisms by which to synchronize their internal rhythms with the outside environment. (7) Hypothetically, non-photic signals may over ride or reinforce the typical effect of the light:dark cycle on the timing of activity.

*Octodon degus* are diurnal, social hystricomorph rodents that communally raise their young. Olfactory communication among degus appears to be an important aspect of their natural behavior and can be observed during many activities. For example, degus participate in group sand-bathing, sniffing and licking the heads and bodies of other degus, urine spraying and scent marking (8). Degus have specialized scent glands that can be used to produce olfactory stimuli that are rubbed on their surroundings, including other degus (25). Females appear to be more sensitive to olfactory communication signals, as evidenced by their ability to discriminate
between male and female urine and to increase scent marking in the presence of male urine as compared to exposure to female urine (13, 24). The influence of olfactory cues on circadian rhythms in degus, as well as in other social species, may serve to strengthen the salience of the photic zeitgeber, and/or to facilitate synchronization of rhythms between animals. Odor-facilitated entrainment between individuals in a social group could enhance reproduction, foraging, and possibly reduce predation risk (7).

Olfactory social cues can also reinforce the effect of light cues on circadian rhythms. Although free-living animals do not experience large phase shifts in their environment, such manipulations of the LD cycle enable us to elucidate to which zeitgebers an organism is attending, as well as the sensitivity of their circadian mechanism to these cues. In degus, odors can accelerate the rate of reentrainment of wheel running activity following a 6 hour phase advance in the LD cycle (14-17). After a 6-hour phase advance, females take longer to reentrain to the new LD cycle than do males when light is the only zeitgeber. However, females accelerate their reentrainment rate over that of males when they are exposed to odors of another female (“donor”) that is already entrained to the new LD cycle (15). Male degus were not effective odor donors to either males or females following a phase-advance of the LD cycle, but were effective as donors for females after a 6-hour phase-delay (16). In these studies only female shifters responded to the olfactory cues; males did not reduce the length of time required to reentrain after a 6-hour phase-advance or delay of the LD cycle in the presence of a male or female odor donor. Furthermore, only entrained animals were effective as donors; test animals that were phase-shifting in the presence of a single donor that was also shifting did not accelerate their rates of reentrainment.
Subsequent studies explored the hormonal aspects of the sex difference in the use of social olfactory stimuli in reentrainment and reported that testicular hormones suppressed responsiveness to odor cues in males (21) and ovarian hormones were necessary for females responding to odor cues with accelerated reentrainment (20). Ovariectomized (OVX) females did not increase their rates of reentrainment in the presence of a social cue donor, nor were they effective as donors.

The role of gonadal hormones on production of odors effective in enhancing recovery from a phase shift was also examined. Castrated males were no better than intact males in producing an effective cue for phase-advancing females (21), suggesting that adult testosterone is not suppressing production of the necessary odor. An ovariectomized (OVX) female odor donor also failed to enhance recovery rate of intact females after a phase-advance (21), suggesting the ovarian hormones are necessary for production of the odor cue by donor females.

The current set of experiments further examines the characteristics of olfactory cues that accelerate reentrainment. We had several goals: (1) to determine if a non-animal odor (that had a positive valence for degus) could be effective as an entrainment signal (Experiments 1, 2); (2) to test whether odors that enhance re-entrainment must be from conspecifics (Experiments 3, 4); (3) to test whether ineffective, conspecific odors could be strengthened (Experiments 5, 7); (4) to examine which ovarian hormones produce effective odors in female degus (Experiment 6); and (5) to verify that repeated phase-shifts do not alter the rate of recovery so long as recovery is complete after each shift (Control shifts from across several experiments).

Non-animal olfactory stimuli have been reported to be effective in circadian conditioning experiments (1), so we tested whether such stimuli could also be effective in a reentrainment paradigm. In the first experiment degus were tested with several non-animal odors to determine
which would produce attention and habituation. We used the odor cue from this study that received the most investigation from degus (cloves) as the odor stimulus in the second experiment. In this experiment, we also tested whether odors from a different species (rats) could alter reentrainment of intact, female degus following a 6-hour phase-advance of the LD cycle. We hypothesized that if accelerated reentrainment is a result of increased attention to an odor, then a stimulus that degus investigate readily could provide an effective accelerating stimulus (i.e. cloves). Similarly, if effective odors result from ovarian hormones, then odors from intact, female rats might act to enhance reentrainment of phase-advancing female degus.

In the third experiment, we tested the hypothesis that familiarity with the donor’s odor would accelerate reentrainment better than an unfamiliar donor’s odor (as was used in prior experiments). This experiment compared rates of reentrainment when shifting females were exposed to their own entrained sister or when exposed to odors of an unfamiliar female. The effectiveness of odors from multiple unfamiliar female donors (increased odor intensity) under conditions with or without the presence of the live animal was also tested.

In the fourth experiment, we tested the necessity for fresh odor production by donor animals by using only the dirty cages that previously held donors. Earlier work has focused on using animals that are constantly present as odor donors. In the original experiments, animals could also see and hear each other, but visual and auditory cues were determined to be unnecessary for the acceleration of reentrainment (14, 17). Odors from live animals were routed from a donor chamber to a chamber housing experimental animals to test the effects of freshly produced odors on entrainment in degus (16). In the present experiment, we completely remove the live animal and used only its odor-impregnated bedding as the source.
The fifth and sixth experiments focused on conspecific odors that had previously been ineffective, but we increased the intensity of the donor cues by using multiple animals or systematically altered hormonal state. Because increased odor intensity from intact, female degus caused previously odor-unresponsive, intact males to increase their rate of reentrainment (21), we hypothesized that other previously ineffective conspecific odors might also be effective when the concentration was increased. Odors from multiple males, shifting females, OVX females, or OVX females with implanted estradiol, progesterone or both hormones were used during reentrainment to determine which could provide an effective cue for intact, phase-shifting females (Experiments 5, 6). We hypothesized that only odors from intact, female donors or OVX, hormone-replaced females would be effective for enhancing the rate of reentrainment (species and sex specific odor).

In a related experiment (Experiment 7), we tested the hypothesis that odors from multiple, intact, female donors would accelerate reentrainment in phase-shifting OVX females. We previously reported that OVX shifters do not increase their rates of reentrainment in the presence of a single female olfactory cue donor (21). We hypothesized that only intact females would be able to respond to odors, even when the concentration was increased with multiple intact donors.

The final phase of this study tested the hypothesis that the recovery time following a phase shift does not change even after repeated shifts, so long as the individual completely recovers after each phase shift. Animals were exposed to three control phase advances at the beginning, middle and end of the repeated experiments, during which only clean air was drawn through the donor housing unit into the test animal housing unit. The amount of time between these control shifts was over one year, so any effects of multiple phase shifts on recovery time
would likely be evident. We did not expect to find a systematic effect of multiple phase-shifts on reentrainment rates over time.

Methods

Animals and Housing

Degus were obtained from an outbred colony maintained at the University of Michigan. Prior to use in the experiments, all animals were maintained at $20^\circ \pm 1^\circ$ C on a LD 12:12 light schedule, with lights on at 0600h and off at 1800h. Light intensity was 250 lux at the cage bottom. Adult animals were 2 to 3 years old, with an average life expectancy of 6 to 7 years. Cages were cleaned weekly at random times during the light phase of the LD cycle. Rodent chow (Purina 5001) and water were available ad libitum. All procedures involving animals were approved by the Animal Care and Use Committee (IACUC) at the University of Michigan.

In Experiments 2-6, a group of 8 intact, adult females were randomly chosen from the colony for use in experiments as test animals undergoing phase shifts (shifters) for testing responses to test odors. Because of individual variability in reentrainment under control conditions (26), animals served as their own controls by experiencing shifts without test odors. Experiment 7 used 10 OVX female shifters to test the role of gonadal steroids on the effectiveness of donor odors on reentrainment rate. In all experiments, except Experiment 3, shifters were housed individually in 48cm x 26.7cm x 20.3cm Nalgene cages with running wheels (9cm wide, 34.5cm diameter) in an environmental chamber that holds 8 cages. The odor donor animals or odor sources were located in an adjacent enclosure and the two were connected by a 6” wide duct (6ft. long) with fans at both ends to propel air and odors from the donor enclosure to the shifters’ enclosure.
In Experiment 3, animals were housed in pairs similar to earlier experiments for the first 2 shifts (15, 16, 22), with the phase-shifting animal and the entrained odor donor separated by a wire mesh divider in a Nalgene cage with dimensions of 48cm x 38cm x 19.5cm. Only shifting females were provided with running wheels when co-housed with another female.

All phase shifts were 6-hour phase-advances of the LD cycle. Data were collected as number of running wheel rotations per 10-minute periods using Minimitter equipment and VitalView software (Minimitter, Inc., Sun River, OR). Data collection began 3 days after introduction of the wheels, allowing the animals to become familiar with the apparatus. Animals were re-shifted only after complete recovery and 2 weeks at a stable phase angle (typically 4 weeks, see data analysis for details).

Animals were shifted three times with only clean air passed through empty, clean donor housing chambers for comparison control shifts throughout the studies. In other control shifts, the clean air entered the shifters’ chamber without first passing through the empty donor chamber, or the animals were not housed in a multi-animal chamber (Experiment 3). The same animals were used as shifters throughout experiments 2-6 to allow intra-animal comparisons which provide a more accurate view of responses to the varying conditions since there is variability between animals in rates of recovery under the baseline (no odor) condition.

Surgical procedures for ovariectomy (OVX) were carried out with Ketamine HCl (30mg/kg of body weight) and Xylazine (2.5mg/kg of body weight) anesthesia. Yohimbine (2.5mg/kg of body weight) and lactated ringers (3ml, i.p.) were used to aid recovery. All degus were allowed one week for recovery before entering the experiment.
Experimental Procedures

Repeated Control Phase Shifts Testing Stability of Reentrainment Rate. All test animals experienced the same experimental conditions simultaneously (i.e., the design was not counter-balanced) so as to assure that the same odors were being experienced by all animals. To control for the possibility that previous phase-shift conditions might influence later phase shifts, animals were repeatedly phase-shifted while experiencing only clean air between experimental phase-shifts. Data from three of the control shifts in which shifters were phase-advanced while exposed to clean air from a filtered air handling system drawn through the empty donor housing chamber were statistically compared and are presented in the results. The first control shift was compared with those that occurred 6 and 14 months later. The other three control shifts (described in Experiments 2-6) are not included in the analysis because the clean air entered the shifters’ chamber without first passing through the empty clean donor chamber. However, rates of reentrainment for the latter shifts did not differ from those reported.

Experiment 1: Habituation to Non-animal Odors. In a preliminary study, intact females (n=8) were exposed to odors from a variety of sources to find a non-animal odor to which degus would attend for a long time without trying to chew the surface on which the odor was placed. Among the many odor stimuli tested were peppermint, lemon, carrot, potato, apple, onions, vanilla, cloves, and ginger. Animals were individually placed in a cage similar to their home cages and allowed to habituate to a clean petri dish placed in the center of the cage for 15 min. The blank petri dish was replaced with one containing the experimental odor but not the odor source. Five 5-minute sessions with 1 minute inter-trial intervals were videotaped and scored for amount of time spent with head less that 1cm from the dish. Habituation was defined as a significant reduction of time spent near the dish across trials. Each animal was retested 4 days
later with one 5-minute test session. Recovery from habituation was defined as a significant increase in time spent within 1 cm of the dish on the final test day compared with the final test on the habituation day. Cloves were the odor cue that received the most attention from animals thus, it was used in the habituation study below.

For the habituation study, each animal (n=10) was placed in a clean cage similar to its home cage, with no food or water available, given 15 minutes to become familiar with its surroundings and then given a session of five 5-minute trials with 1-minute inter-trial intervals in which a petri dish rubbed with whole cloves was placed in the cage for the animal to explore. Activity was recorded by video, and tapes scored for amount of time spent with head within 1 cm of the petri dish. Habituation was defined as a significant reduction of time spent near the dish over trials. Each animal was then tested 4 days later with a 5-minute session similar to the habituation trials. Recovery from habituation was defined as a significant increase in time spent within 1 cm of the clove-rubbed petri dish on the testing day. Comparisons between trials were made using a paired t-tests, with p<0.05 considered significant.

Experiment 2: Importance of Conspecifics. Non-conspecific and non-animal olfactory stimuli were presented to test whether they would be effective olfactory cues for facilitating reentrainment. Shifters (8 intact females) were phase-advanced while exposed to odors routed from 8 intact, female rats (donors, Rattus norvegicus). The second shift used odors routed from a dish with fresh cloves set in the donor environmental chamber (renewed every 3 days to maintain strong scent), followed by a third shift using only clean air.

Experiment 3: Familiarity with Conspecific. This experiment was designed to test whether familiarity with the donor odor (intact sister) increases the rate of reentrainment compared to odors from an unfamiliar female. Females were housed with their sisters prior to the
onset of these experiments (approximately 12 weeks of separation before the test after 24-36 months of living together). In this experiment only, donors were housed with phase-shifters as in earlier studies (14-16, 21, 22) and described above, because of the use of a sister as an individual donor. The unfamiliar donors were housed with the individual shifters in the same manner for one comparison shift.

Following a phase advance the shifters were housed with their own sister (donor), which had previously been entrained to the new LD cycle (donor). Then the process was repeated using 8 unfamiliar, intact, adult female donors (also entrained to the new LD cycle before use), and lastly they were shifted while exposed to clean air. The shifters were then phase-advanced again while living in an environmental chamber while exposed to odors from 8 unfamiliar, intact females that were housed in a donor chamber. The donors were pre-entrained to the new LD cycle to which the shifters were being advanced.

**Experiment 4: Need for Live Animal.** The shifts in this experiment tested the effectiveness of used animal bedding and its associated odors on accelerating reentrainment to determine whether effective olfactory cues require the presence of the donor. Shifters were phase-advanced while exposed to routed odors from 8 cages containing bedding previously used for 4-7 days by intact, unfamiliar, entrained females. Dirty bedding was replaced weekly. Test animals were subsequently phase shifted with clean air.

**Experiment 5: Previously Ineffective Donor Odors, Increased in Strength.** This experiment examined whether increasing the concentration of odors from donors that were previously found to be ineffective as single donors would facilitate reentrainment. Shifters were phase-advanced with routed odors from 8 intact, adult, male degus randomly chosen from the
colony, 8 reentraining (i.e., phase-shifting), intact females that were shifted at the same time as
the experimental shifters, 8 entrained, OVX females from the colony, and with clean air.

**Experiment 6: Hormone Replacement in OVX Donors.** This experiment was designed to
determine which ovarian hormone (or combination of hormones) is necessary for production of
an odor by female degus that is effective at increasing the rate of reentrainment of shifters. Eight
entrained, OVX females implanted first with estradiol, then progesterone, and lastly a
combination of the two hormones were used as odor donors during a series of phase-advances,
followed by a shift with clean air. Silastic capsules similar to those used in earlier experiments
(20) containing crystalline estradiol benzoate (E; Sigma) or progesterone (P; Sigma) were
prepared as described in Labyak and Lee (26) with an effective length of 15 mm (Dow-Corning;
1.98mm ID, 3.15mm OD). Hormone capsules were put into the odor donors for 48 h prior to
their odors being routed to shifting females.

**Experiment 7: OVX Shifters Reentraining with Multiple Female Donors.** Ten OVX
shifters were phase-advanced with routed odors from 8 intact, unrelated, unfamiliar, entrained
female donors. This experiment was designed to test whether OVX females, which were
unresponsive to the rate-enhancing effects of a single intact female’s odor (21), would be able to
respond to a more intense odor stimulus from multiple donors.

**Data Analyses**

The number of days required to reentrain to a new LD cycle following a phase advance,
activity levels, and amplitude of the activity rhythm were obtained from every animal in each of
the experimental conditions (with or without donor odors), allowing each animal to serve as its
own control. Phase angle of activity onset was determined after an animal displayed at least 2
weeks of entrained rhythms prior to and after a phase shift and was calculated by examining 24-
hour activity frequency histograms for the time of activity onset. Activity onset was measured relative to the onset of the light cue and was defined as at least 40 minutes of consecutive activity with a minimum of 40 wheel revolutions per 10-minute block of activity following a lack of activity of approximately 4 hours (15, 16). The time of activity onset over a period of 4 to 7 days was averaged and compared to the LD cycle to obtain phase angle of activity onset before phase shifts.

Reentrainment was operationally defined as the day on which the animal first displayed a morning phase angle of entrainment similar to the phase angle prior to the phase shift for at least 3 consecutive days (see Figure 1). Mean daily activity levels were calculated by averaging activity levels across 10-minute bins over a 24-hour period. Maximum daily activity level was defined as the greatest number of wheel rotations per 10-minute bin in a 24-hour period. Activity rhythm amplitude was obtained by subtracting the activity mean from the maximum activity level.

A repeated measures ANOVA was used for analysis of repeated shifts within an experiment (Expt. 2, 3, 5 and 6). Two-tailed paired t-tests were used to compare animals in each shifting test condition to the control, clean air condition of that experiment, and to positive donor effects where appropriate, with $p<0.05$ considered significant. Data are presented as means ± SEM.

**Results**

*Effect of Multiple Phase Advances on Reentrainment Rates*
There were no significant differences in the number of days to reentrain among any of the control shifts with clean air routed between the donor and shifter environmental chambers, despite 14 shifts between the first and last control shift (F=1.10; p > 0.050; Figure 2). These three shifts did not differ from other control shifts with donors housed alone or exposed to clean air that was not passed through donor chambers.

Experiment 1 – Habituation to Non-animal Odors

The preliminary odor attention study found that clove odors were not aversive and were investigated longer than any of the other odors tested. Animals significantly reduced the amount of time near the clove odors over the 5-trial habituation session (F=6.98, p < 0.01; Trial 1 mean = 39.8 ± 8.4 sec, Trial 5 mean = 10.9 ± 6.1 sec) and significantly increased the amount of time spent near the odors on the testing day, 4 days later (paired t = 4.08, p < 0.003; Retest mean = 30.7 ± 4.0 sec). The habituation response demonstrated that degus were able to discern the odor of cloves, readily approach it, recognize it upon later exposure, and return to it days later.

Experiment 2 – Importance of Conspecifics

Reentrainment with odors from cloves took the same amount of time as with clean air. However, reentrainment while exposed to routed odors from 8 intact, female rats took significantly longer than the control condition (paired t = -3.85; p < 0.01; Figure 3). Thus, non-degu animal odors can alter the rate of reentrainment, although not positively in this case.
Experiment 3– Familiarity with Conspecific

Compared to recovery with clean air exposure, rates of reentrainment were accelerated in all three donor conditions. Housing with an intact sister, an intact unfamiliar female, or alone with routed odors from a group of unfamiliar, intact females resulted in shorter reentrainment periods than when housed alone with clean air (F=6.45, p<0.01; Figure 4). There were no differences in the length of time to reentrain among the three donor conditions. Thus, familiarity with conspecific odors does not appear to further enhance reentrainment rate, and odors routed through air ducts are as effective as co-housing with an entrained female conspecific.

Insert Figure 4 about here

Experiment 4 – Need for Live Animal

Reentrainment with odor exposure from cages containing dirty bedding used by intact females and routed through donor chambers took fewer days than when exposed to clean air (Clean Air = 12.13 ± 0.67days; Dirty Bedding = 8.63 ± 0.38days; paired t = -2.88; p<0.01).

Experiment 5 – Previously Ineffective Donor Odors, Increased in Strength

Shifters reentraining with routed odors from 8 intact males required fewer days to recover their previous phase angle of entrainment than when reentraining with clean air (paired t = 9.26; p<0.01; Figure 5). Routed odors from 8 intact, phase-shifting female donors or 8 OVX donors did not increase rates of reentrainment over those with clean air.

Insert Figure 5 about here

Experiment 6 – Hormone Replacement in OVX Donors
Odors from OVX females implanted with both estradiol and progesterone capsules reduced the length of time shifters needed to reentrain (F=5.96; p<0.01; see Figure 6), while odors from donors implanted with only estrogen or progesterone were ineffective in increasing reentrainment rate.

Experiment 7 - OVX Shifters Reentraining with Multiple Female Donors
OVX female shifters did not accelerate reentrainment rates in the presence of multiple (n=8) intact, female donors (Clean Air = 13.6 ± 1.2; with Intact Donors = 13.2 ± 0.8; p>0.05).

Activity Analyses Across Conditions
Maximum daily activity levels and amplitude during reentrainment did not differ between the various odor treatment conditions except when shifters were entraining while exposed to odors from multiple males. Mean activity levels of shifting females were lower while exposed to odors from multiple males in Experiment 5 (Clean Air = 303.25 ± 10.92; Multiple Males = 202.50 ± 26.23; paired t =-3.31; p<0.01).

Discussion
This set of experiments further refines our knowledge about the olfactory cues that enhance reentrainment following a 6-hour phase advance of the LD cycle. We report that steroid hormones are necessary for production of an effective olfactory stimulus. Intact males (when increased in number), intact females and females with progesterone and estrogen replacement, but not OVX females, produce odors able to enhance reentrainment rate. It is unclear whether androgens are directly effective in male odor production, or if testosterone is first converted to
estrogen to produce the effective odor (22). We also found that circadian timing of the production of the odor cue by donors is not critical, since continuous exposure to odors from used bedding of intact females effectively accelerated reentrainment. However, entrainment of donors is critically important, since odors from phase-shifting donors (even a group of 8) were ineffective for accelerating reentrainment. These data replicate the earlier findings of Goel and Lee (15) with a single shifting donor. Furthermore, odors that accelerate reentrainment are likely to be species-specific for degus, as shifters did not accelerate their rate of reentrainment in the presence of odors from female rats or cloves. Indeed, rat odors slowed reentrainment.

Analysis of multiple phase-shifts with clean air pulled through clean empty chambers demonstrates that multiple phase-advances do not alter reentrainment rate over time. This was an important control experiment not only for this study, but also for previous and future experiments that rely on comparing a set of animals across more than one shift. Previous studies suggested that there were no effects of multiple phase advances so long as shifters were allowed to completely reentrain before the next shift (using counter-balanced designs; (16, 17, 33). We have now systematically examined the impact of many phase advances over 14 months and can be assured that results are not influenced by the number of previous shifts. Apparently, degus do not habituate or become sensitized to the repeated shifts so long as recovery is complete each time.

Experiment 2 further defines the species specificity of the odors effective in enhancing recovery from a phase shift. Reentrainment with odors from intact female rats did not hasten reentrainment. Therefore, the presence of odors produced by an animal with intact and functioning ovaries, but not necessarily of the same species, is not the only requirement for producing an effective cue. Furthermore, the increased length of time to reentrain with rat donors
is probably not due solely to unfamiliarity of the odors, since degus exposed to odors from
cloves for the first time did not slow their rates of reentrainment. Since shifters took significantly
longer to reentrain in the presence of female rats, there is the possibility that odors from an
unfamiliar species produced an aversive stimulus. It is possible that aversive odor stimuli act as
stressors increasing the amount of time necessary to recover from a phase shift, as previously
describe with 1 h of restraint stress at the onset of a phase shift (33).

Results from Experiment 3 demonstrate that reentrainment rate is not accelerated for
shifters housed with their sister as a donor rather than an intact, unfamiliar female odor donor.
We also found that exposure to odors of multiple, intact females drawn through a duct to the
phase-shifters’ enclosure produced the same recovery time as exposure to one live animal. There
may be no advantage to knowing the donor (sister) or to an increase in odor intensity by using a
group of donors because female shifters are experiencing a ceiling effect with a 6 h phase
advance with odors from a single, unfamiliar, female donor. (In contrast, males were able to use
strengthened odor intensity from multiple females to accelerate their reentrainment (22).
However, the odors from a familiar conspecific or a group of conspecifics might show a greater
benefit under conditions where recovery from a phase shift is more difficult (27). To test this,
female shifters could be given a larger phase advance (e.g., 9 h shift) or be shifted under a lower
light intensity (reducing the effectiveness of the photic zeitgeber) that would require more time
for recovery, allowing a greater opportunity to display differences in recovery time as a function
of the odor intensity or familiarity.

Experiment 4 examined the need for a live animal to be present to produce a fresh odor.
Cages without animals in them, but containing soiled bedding from entrained, intact females was
sufficient to enhance reentrainment rate in shifters. Therefore, the effective odors do not have to
be produced by donors while the shifter is reentraining. The odors remain effective even if produced up to 7 days earlier. Additionally, the circadian timing of production of the odor is likely not critical for the odor to aid reentrainment, since they were supplied from the soiled bedding produced across several days. However, it is still possible that the reentrainment-enhancing cues are only produced at certain times of day, but remain available for an extended period of time. In the past, donors were pre-entrained to the new LD cycle of the shifting animals. It is now clear that odor-producing entrained donor animals do not need to be entrained to the same LD cycle as the shifters. In this experiment the bedding odors were from animals with lights on at 0600h, while the shifters lights came on at 2400h (LD12:12 for both groups). In Experiment 5, previously ineffective intact male odors were able to accelerate reentrainment in phase-advancing females when more than one donor animal was used to strengthen the stimulus. In earlier studies it was surprising that housing shifters with a single male accelerated reentrainment after 6-hour phase-delays but not advances (15, 16). The current data demonstrate that males produce the effective odor during both phase-advances and delays, but advances may require a stronger stimulus, perhaps because advances already occur faster than delays of the same magnitude (27).

This experiment also illustrated the importance of ovarian hormones for female production of effective odors. As in a previous study using a single OVX donor (19), animals shifting with odors from multiple, OVX animals did not accelerate reentrainment. However, shifting animals were responsive to OVX females with both estradiol and progesterone capsule implants, while the individual hormones were ineffective (Experiment 6). Because males are able to produce a sufficient odor stimulus if more than one donor is used (Experiment 5), we hypothesize that the same odor is made by both males and females, but in smaller quantities by
males. Progesterone, although primarily thought of as an ovarian hormone, is produced in small amounts by the adrenals of both sexes. However, if adrenal progesterone was sufficient to produce the odor, then increased numbers of OVX females should increase odor intensity (as it does with male odors) and should have improved shifter reentrainment, but that was not the case. Since estrogen is often critical to increase sensitivity to progesterone in a variety of tissues, and testosterone produced by males can be converted to estrogen, sensitivity to progesterone could be increased in males compared to OVX females with or without P, whereas with no mechanism for estrogen production in the OVX+P females, their odors would still be ineffective. This hypothesis is supported by the fact that OVX females with both P and E capsules produced odors that improved the reentrainment rate in shifters. More research refining the role of hormones in degu odor production is needed.

As seen in a prior experiment using a single female phase-shifting donor (15), multiple, intact, phase-shifting donors did not produce the odor that facilitates reentrainment. It is possible that increased stress while phase-shifting (33) results in reduced production of the essential olfactory cue, perhaps by reducing steroid hormones or increasing corticosteroids, or both. This should be examined in a future study to determine whether increased stress or cortisol reduces the rate-enhancing odor production of entrained donors.

Experiment 7 examined the ability of OVX phase-shifters to use olfactory social cues from multiple, intact, female donors to accelerate reentrainment. In previous research (21), OVX shifters exposed to a single intact female during reentrainment did not accelerate their rate of reentrainment. In the current study, OVX shifters remained unresponsive to increased olfactory stimuli during reentrainment. Thus, ovarian hormones are necessary for effective use of the reentrainment-enhancing stimulus. Previous research has shown that E+P replacement in OVX
females reinstates their responsiveness to the odors of a singer donor (21), and in Experiment 6, we found that OVX females with E+P hormone replacement also were able to produce the effective odor. Thus, for females, both odor production and the ability to respond to the odor during reentrainment are dependent upon circulating E and P.

The results from these experiments expand our knowledge of the type of odor needed to enhance reentrainment after a phase-advance of the LD cycle. However, a future experiment examining the effect of the timing of presentation of odors within the reentraining period of the shifter would provide important additional information. All the studies involving the use of odors to modify reentrainment have exposed shifters to odors continuously throughout the reentrainment period. However, the period of time during which the odor cue has its greatest effect could be as long as the entire reentraining period or as short as the first few hours following the shift in the light cycle. Responsiveness to odors presented during specific days (e.g. the first 2 days following a phase shift), or even during particular times on specific days (e.g. the first 3 hours following a change in a light schedule) in the reentrainment period could determine whether greater sensitivity to conspecific odors exists at specific times during the recovery period. If degus show differential sensitivity to the timing of the odor presentation, there could be implications for new methods of aiding recovery from phase shift in humans after transmeridian jet travel or during shift work.

In summary, steroid hormones in adult degus, both male and female, influence sensitivity to the non-photic odor cues produced by conspecifics. Testosterone decreases sensitivity in both males and females (22), and probably decreases odor production (Experiment 5; 21). Ovarian hormones are critical for both odor sensitivity (Experiment 7; (21) and production (Experiment
6). Lastly, non-conspecific odors may have no effect at all (such as cloves) even if they are attractive, while others (such as rat odors) may have a negative influence on reentrainment rate. Extrapolation of the data to humans suggests some interesting possibilities for treatments designed to reduce the duration of jet lag. A variety of human behavioral and/or physiological systems are sensitive to odors from other humans. For example, both male and female axillary stimuli can influence the timing of menstrual cyclicity (19, 20, 30, 35, 37, 39). Steroid hormones have also been shown to influence human psychological aspects such as mood, attractiveness, memory and sociosexual behaviors (4-6, 20, 28). Thus it seems not only possible, but likely, that humans would be responsive to olfactory stimuli during reentrainment following trans-meridian travel. Odors may act to reduce the stress response that occurs during a phase shift, thereby accelerating recovery (32). Another example is seen in the social marmosets; when they are housed in a strange environment they have lower cortisol response when they are exposed to familiar conspecifics or their vocalizations than when housed alone (36, 38). It is also possible that the use of odors could aid entrainment of people with disorders of the circadian system or who fail to entrain to 24-hour schedules for other reasons. Lastly, the research conducted with odors could possibly be applied to hospitalized people to help stabilize their circadian rhythms, which could lead to better sleep, eating, weight gain and development.
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References


Figure Legends

Figure 1. Representative example of wheel-running activity during a 6 h phase advance while animal is exposed to clean air. Data are shown as a double-plotted (48 hour) actogram. The light:dark cycle prior to the shift is shown at the top of the actogram with the open bar and the upper shaded box representing when lights are on. The lower shaded area is the new period of lights on in the phase-advanced light cycle. Stable entrainment with the original phase angle of entrainment occurs on the fourteenth day after the phase shift.

Figure 2. Mean number of days ± SEM to reentrain after a 6-hour phase-advance of the LD cycle with clean air passed through empty donor chamber (control) at the beginning of the set of experiments (1\textsuperscript{st}), after 6 months (2\textsuperscript{nd}) and 14 months (3\textsuperscript{rd}). The first and 3rd shifts had 14 intervening shifts while exposed to a variety of conditions. All animals were allowed to completely recover following each shift by waiting 3-4 weeks between shifts.

Figure 3. Mean number of days ± SEM for reentrainment after a 6-hour phase-advance of the LD cycle with exposure to clean air (CLEAN AIR), 8 female rats (RATS) or cloves (CLOVES). (* = significantly more days to reentrain, p < 0.01)

Figure 4. Mean number of days ± SEM to reentrain with exposure to clean air (CLEAN AIR), an intact sister housed with the shifter (SISTER), an unfamiliar, intact female housed with the shifter (UNFAMIL 1), and with routed odors from a group of 8 unfamiliar, intact females housed in a separate environmental chamber from the shifters (UNFAMIL 2). Asterisks (*) represent shifts that significantly differ from the control, clean air condition. (p < 0.01).
Figure 5. Mean number of days ± SEM for reentrainment after a 6-hour phase-advance of the LD cycle with clean air (CLEAN AIR), routed odors from 8 intact males (MALES), routed odors from 8 intact females that were also undergoing a phase-shift in a separate environmental chamber from the test animals (SHIFTERS), and with routed olfactory cues from 8 OVX donors (OVX). The data from exposure to multiple males is significantly different from all other data (* = p < 0.01).

Figure 6. Mean number of days ± SEM for reentrainment following a 6-hour phase-advance of the LD cycle with routed clean air (CLEAN AIR), odors from 8 OVX donors (OVX), 8 OVX donors with estrogen capsule implants (E), 8 OVX donors with progesterone capsule implants (P), and 8 OVX donors with both estrogen and progesterone replaced (E+P). Only the E+P donors provided olfactory stimuli that significantly reduced the number of days to reentrain (* = p < 0.01).
Figure 1
Figure 2

Mean number of days to retrain

Time (months) of clean air phase shift

0 6 14
Figure 4
Figure 5
Figure 6