Effects of Anosmia on Odor Preference Learning

Senior Thesis

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Abstract

The sense of olfaction can be thought of as consisting of two distinct sub-modalities: orthonasal (perception of odorants that enter the nose through the nostrils) and retronasal (perception of odorants that are present in the mouth and that reach the nasal cavity from the back of the throat). Researchers in the Katz lab have proposed that retronasal olfaction should share properties with taste; recent testing has confirmed that, like taste preference learning, retronasal learning is: 1) more rapid than orthonasal learning; and 2) dependent on taste cortex. This research has used odorants dissolved in water to isolate retronasal stimuli, which theoretically, should differ from pure water only with regard to odor, but which conceivably could contain taste and mouth feel components. In order to ensure that the observed preferences were learned only to odor, it is therefore necessary to render rats anosmic, and then to repeat these tests with the anosmic rats. Here, I began this set of studies, by first establishing orthonasal (i.e., purely olfactory) preferences to an odor, then rendering rats anosmic *via* deciliation, and then attempting to confirm that the procedure rendered the rats anosmic—my prediction is that the orthonasal preference would be lost. My data did reveal a decline in preference to chance (50%) levels in deciliated animals; unfortunately, this same decrease was also observed in control (anesthesia-only) animals. This suggests that there may have been one of several possible failures in the behavior paradigm for control rats, a failure that made me run out of time before I was able to conduct a test similar to that of previous studies. In the discussion, I explore next steps, and the implications for this research in regard to the current literature on olfaction.

Effects of Anosmia on Odor Preference Learning

Olfaction occurs when molecules of a substance interact with receptors in the nose. In mammals, molecules can reach these receptors *via* two pathways--orthonasal and retronasal (Furudono, Cruz, & Lowe, 2013; Heilmann & Hummel, 2004; Linforth, Martin, Carey, Davidson, & Taylor, 2002; Masaoka, Satoh, Akai, & Homma, 2010; Ni et al., 2015; Rozin, 1982; Scott, Acevedo, Sherrill, & Phan, 2007; Small, Gerber, Mak, & Hummel, 2005): orthonasal olfaction occurs when odorant molecules external to the body are inhaled through the nostrils; retronasal olfaction occurs when odorant molecules are sampled from inside the oral cavity, flowing up into the nasal cavity from the throat (See Figure 1). While orthonasal olfaction is what we typically think of as our sense of smell, retronasal olfaction is often conflated with "taste" in everyday discourse because it has a strong impact on the perception of flavor. This interaction of taste with retronasal olfaction makes a certain sort of sense, given the fact that "oral sourcing" connect both directly to food consumption (Furudono, Cruz, & Lowe, 2013; Heilmann & Hummel, 2004; Linforth, Martin, Carey, Davidson, & Taylor, 2002; Masaoka, Satoh, Akai, & Homma, 2010; Ni et al., 2015; Rozin, 1982; Scott, Acevedo, Sherrill, & Phan, 2007; Small, Gerber, Mak, & Hummel, 2005)—a direct connection that is not shared by orthonasal olfaction, which is used to process stimuli that are external to the body.

Given the above considerations, it is reasonable to theorize that orthonasal and retronasal olfaction could make use of functionally distinct central nervous system circuits (Small, Gerber, Mak, & Hummel, 2005; Veldhuizen, 2010), and thereby evidence different functional properties. As of yet, however, there has been little research done into implications of the dual-modal nature of olfaction, and the research that has been done focuses not on central differences but the periphery: the previous literature has investigated the possibility that odorants with different

physical properties may be filtered in distinct ways by the nasal epithelium, creating spatial and temporal maps of odor binding in the nose itself, depending on the direction of airflow (Scott, Acevedo, Sherrill, & Phan, 2007). There have been a few studies, however, suggesting that orthonasal and retronasal stimuli are processed differently at the level of the olfactory bulb and cortex (Furudono, Cruz, & Lowe, 2013; Gautam, Short, & Verhagen, 2014; Small, Gerber, Mak, & Hummel, 2005)—findings that are consistent with the premise that separate (or at least non-identical) olfactory systems are activated depending on the way odorants enter the nose (Shepherd, 2006).

It is logical to hypothesize that the processing of odorant properties of substances on the tongue—retronasal olfaction—might be linked with the sense of taste, as the odor stimulation comes simultaneous with taste stimulation, from a tasty/smelly substance already being ingested. Taste is a sense that has strong implications for survival because of its oral sourcing, implications that imbue taste with unique properties that, it could be reasoned, might be shared by retronasal olfaction. Most notably, taste learning is inherently fast due to the fact that such learning is vital for survival: for example, if an animal mistakes a poisonous substance as being something it can eat more than once, it can die. Since taste learning is also important and rapid, it is reasonable to hypothesize that retronasal learning would be equally quick. In comparison, orthonasal olfaction is far less "urgent" a sense, and thus learning need not be so rapid.

Food choice, and food preference learning in particular, can be easily studied in virtually all animals. Previous studies have used these learning paradigms to investigate the difference between retronasal and orthonasal learning, and have found evidence consistent with (but not definitively testing) distinct learning mechanisms for each modality (Chapuis, Messaoudi, Ferreira, & Ravel, 2007). In the Katz lab, we are delving more deeply into this topic. Our

prediction is that faster preference learning should happen when a retronasal stimulus is paired with taste than when an orthonasal stimulus is paired with taste. To test this prediction, we have studied animals' preferences to two odors before and after pairing one odor with a palatable sweet taste, controlling for the mode of odor presentation (retronasal or orthonasal). The data that we have collected, that have been presented at multiple conferences (see Blankenship, Grigorova, Katz, & Maier, 2017), make it clear that retronasal and orthonasal olfaction are indeed functionally distinct, confirming that retronasal learning (at least in this paradigm) is, in fact, faster than orthonasal learning.

If, as the logic above suggests might be the case, this observed difference does reflect a relationship between retronasal olfaction and taste—a relationship with taste that is not shared by orthonasal olfaction—then it stands to reason that perturbation of the taste system will impact retronasal learning more than it does orthonasal learning. Our research validates this prediction as well: optogenetic inhibition of gustatory cortex disrupts preference following training with a retronasal stimulus, but not following training with orthonasal stimuli (Blankenship, Grigorova, Katz, & Maier, 2017). Activity in the gustatory cortex also influences olfactory cortex odor responses, in a manner that is specifically required for retronasal preference learning (Maier, Blankenship, Li, & Katz, 2015).

There remain possible confounds, however, that complicate interpretation of these results. Retronasal olfaction emerges from multi-modal stimuli, and it could conceivably be suggested that our results demonstrate the association, not between retronasal olfaction and taste, but between sweet taste and non-olfactory components of the retronasal stimuli, such as mouthfeel or trigeminal activation (Slotnick & Darling, 1997). That is, it is possible that animals can detect subtle changes in how a solution feels in the mouth, or activates taste receptors on the

tongue, induced by the addition of odorous molecules. While this confound is rendered unlikely by our careful selection of odor stimulus concentrations (Slotnick & Darling, 1997), it is important nonetheless to rule out this critical possibility.

For my honors thesis, I set out to directly test whether these confounding non-olfactory factors affected our results, designing a modified version of the original study using only olfactory stimuli with anosmic rats--animals that have been rendered unable to smell. My rationale was as follows: if our results reflect a coupling of retronasal olfaction and taste, then anosmic rats should not show a change in preference for sweet paired odor; if, however, rats show a preference to the retronasal stimuli (as we deliver them) even after being rendered anosmic, then non-olfactory factors present in the retronasal stimulations must be contributing to preference formation in prior experiments.

Before this experiment can be done, however, the first step in my project requires establishing an effective procedure to eliminate the animal's sense of smell. Previous literature has suggested that animals can be rendered anosmic by temporarily destroying the cilia of olfactory receptor neurons in the nose, a manipulation that can be achieved by washing a detergent or corrosive agent through the nasal passages (MacRae, Kenkel, & Kentner, 2015; Slotnick, Glover, & Bodyak, 2000). There is in fact a large variety of specific methods that can be used, of course, and it can never be safely assumed that such procedures, which impact a visually inaccessible tissue, are successful.

I therefore first set out to confirm that animals were truly rendered anosmic, which can be done by testing the animals' performance on a preference task involving *only* ortho-olfactory stimuli. After an effective deciliation procedure has been established and confirmed by my orthonasal preference test, I will conduct the same behavioral paradigm again with a new group

of subjects but with retronasal odor only—my ultimate prediction being that anosmia will nullify preference expression in retronasal learning conditions. Yet, it is important to reiterate that this experiment essentially provides a critical control for the previous odor learning findings. If retronasal preference expression is not disrupted by anosmia, then it is possible that the prior results can be explained by non-olfactory properties of the retronasal stimuli. If there is no preference, this study would enhance the internal validity of the original study to suggest that retronasal learning occurs faster than orthonasal learning and is uniquely dependent on gustatory cortex, highlighting its connection to taste.

Method

Subjects

33 adult female Long Evans rats from Charles River Laboratories served as subjects. All rats were housed individually in the Katz lab animal vivarium with controlled humidity, temperature and light cycles in accordance with approved IACUC procedures.

Testing Cage

Experiments were performed in a large, opaque cage with wire cage tops which served to hold odor and fluid consumption samples. Each cage was fitted with a filter top to control odor dispersion. During each session, two bottles filled with 20 milliliters of distilled water were placed inside the testing cage for 20 minutes. The top of each bottle was lined with a strip of filter paper, protected by a fitted nose cone to prevent direct contact with the rat. During trials when odor was used, 2.5 microliters of odorant amyl-acetate was placed on the strip of one bottle. This is an example of a purely orthonasal stimulus, since no odorants are available for sampling inside the mouth. The positions of the bottles were switched on each day of the experiment to minimize the formation of side preferences.

Habituation

Prior to the start of the experiment, subjects were habituated to their individual testing cages to encourage comfort with the experimental set-up. During habituation, animals received two bottles containing distilled water fitted with a sipper tube to enable liquid consumption for 20 minutes, with no odor, and consumption was recorded by weight. Animals were placed on a water restriction schedule to encourage fluid consumption the day before habituation began, according to IACUC guidelines.

Preference Training

For the first four nights leading up to the first odor test, subjects received a bottle of 15ml amyl acetate (0.025%) and sucrose solution (10%), along with an odor strip of 2.5ul amyl acetate. This is a modified version of the preference induction protocol used previously in the Katz lab.

Odor Testing

Animals were assessed for preferences between ortho-odorized water bottles (2.5ul of amyl acetate applied to filter paper fitted on the bottle) versus non-odorized bottles. The bottles delivering both stimuli were presented side by side in counterbalanced positions for each test. Animals were permitted to consume water from both bottles for 20 minutes. All bottles were weighed before and after the testing period in order to determine how many millimeters were consumed from each. Relative preference for stimuli will be assessed as a percentage of the total number of milliliters for the odorized sample versus non-odorized for each animal (calculated: ml for odor / (ml for odor + ml for non-odor) * 100). Half of the animals received deciliation (see below) following this first odor test, while the other half were only anesthetized via a ketamine-xylazine cocktail as a control group. Four days after the first test, this same odor test procedure was performed for all animals, calculated in the same way. A two-way mixed effects ANOVA

was performed on the resultant data, analyzing the between subject factor of deciliation versus control and within subject factor of odor test 1 versus odor test 2.

Deciliation

Subjects underwent a nasal deciliation procedure to render them anosmic. For all deciliation procedures, animals were maintained under anesthesia via a ketamine-xylazine cocktail (25% ketamine, 1% xylazine) administered to the intraperitoneal cavity. Animals were kept on a heating pad to regulate body temperature, and anesthetic plane was established by toe pinch reaction, respiration, and monitoring of whisking. Once the animal had reached sufficient anesthetic plane, they were placed in a supine position to enable nasal irrigation with a sterile deciliation agent, 5% solution of Zinc Sulfate (deciliation procedure citations here). A thin-tipped pipette was inserted 1.5mm into the external right nare, and 50ul of deciliation agent was slowly infused. Following infusion, animals were left in the supine position for 5 minutes to allow solution to irrigate the nasal turbinates, during which animals were closely monitored for respiratory distress. Animals were then placed into an elevated prone position for 5 minutes, allowing residual agent to diffuse out of the nose. This process was assisted by absorption of solution from the nare opening with sterile cotton tipped applicators. Following this 5 minute period, the animal was placed in the supine position again to perform the nasal irrigation procedure on the left side nare. Animals were treated with Meloxicam for management of any potential discomfort that may have resulted from the procedure.

Results

The first step of my project was to establish an effective deciliation protocol with the method described above. In order to do this, it was necessary to first establish a learned preference to the odor (amyl acetate) as opposed to air; it could then be asked whether this

orthonasal odor preference could be disrupted by deciliation. Repeated pairings of the odor with sucrose caused rats to successfully demonstrate preference for the odor (see Figure 2). The average trained preference for amyl acetate over odorless air (M=76.34%, SD=16.1) was significantly higher than chance (50%), $t(32) = 9.423$, $p = .000$.

With this odor preference established, I then performed the deciliation procedure on the trained rats. Following one day of recovery, I performed a re-test, hoping to establish whether the rats had been rendered anosmic. Unfortunately, this proved impossible, as in odor test 2 (post-deciliation), I saw a drastic drop in total fluid consumption (see Figure 3): animals consumed significantly less and almost zero fluid following deciliation, $t(10)=4.22$, $p < .01$, in fact demonstrating a floor effect in fluid consumption. Because of this significant reduction in consumption, it was not possible to accurately compare preferences for odors (that are presented at the site of consumption) pre- and post-deciliation, and therefore it was not possible to confidently discern if the procedure actually disrupted olfactory detection.

Therefore, my next step was to establish the amount of post-procedure recovery time it would take for rats to begin to readily consume fluid again in the testing chamber. To do this, I measured consumption in weight of a single bottle for 20 minutes one day before performing the deciliation, and again for the X days after. The results of this test, which demonstrated that it takes approximately 4 days for the animal's rate of consumption to increase back to its starting value, is shown in Figure 4 (note this is preliminary data from one animal).

Armed with this knowledge, I then modified my original experimental design to include a four-day post-deciliation recovery period between the first and second odor tests. This experiment was also performed on a control group, which were anesthetized one day following

the baseline learned preference test, but that did not receive the deciliation procedure described in the Methods.

The results of these tests can be found in Figures 5 and 6, which were analyzed *via* a 2-way ANOVA. This ANOVA revealed a main effect of odor test day, $F(1, 11) = 16.37$, $p = .002$, but no main effect of condition, $F(1, 11) = 1.395$, $p = .262$, or interaction, $F(1, 11) = 0$, $p = .996$. Visual inspection of the data indicates that both control and deciliation group preferences were in decline. Without knowing why olfactory preferences seemed to vanish in my control rats, I cannot confidently conclude that the loss of preference in deciliated rats reflect anosmia, as opposed to (perhaps) some specific effect of the newly imposed recovery period (see below).

Discussion

My initial prediction was that rats rendered anosmic by the deciliation procedure would demonstrate a loss of preference to ortho-olfactory stimuli. Of course, this prediction incorporated the assumption that control group preferences would not decrease in the same manner—that non-deciliated rats would maintain learned preferences across the pre- and post-test interval. My results, alas, demonstrate that this was not the case—control and deciliated rats became similarly unable or disinterested in demonstrating the odor preference. Future work, therefore, must investigate why the learned preference was so fragile in control rats, and how the procedure can be changed to strengthen that learned preference.

It is possible to speculate as to possible culprits. To begin with, it is possible that preference for odor was extinguished in all rats after the four day recovery period necessary for deciliated animals—perhaps I failed to induce robust, long-term preference learning. If this possibility, which is easily evaluated with tests in which control rats that have not been anesthetized are post-tested for the next four days, proves to be the case, it will be necessary in

future experiments to use a less harsh concentration of deciliation agent, and thereby to reduce the amount of time needed to recover consumption in deciliated rats (hopefully without reducing the efficacy of the procedure itself).

Perhaps an easier solution would involve increasing the strength of the learned preference—by pairing the odor with a higher concentration of sucrose (a sweeter taste), or by increasing the number of training trials—thereby reducing the likelihood that the preference disappears across a 4-day waiting period.

It is also worth considering the possibility that the decrease in preference in control animals could reflect effects of the anesthetic. It could be that the ketamine-xylazine cocktail I used to anesthetize all animals had such an adverse effect on the rats that they were unable to demonstrate the preference they had previously learned. This possibility is highly unlikely, given the sheer number of experiments that have involved this anesthesia, but could be investigated in future research by conducting the same experiment multiple times with different anesthetics that might have less harsh of an effect. Finally, it is also possible that repeated presentations of the odor caused animals to lose their preference for it, just as repeated preference for an initially aversive taste causes rats to lose their neophobia. This possibility is also highly unlikely, but it could conceivably be tested (ironically enough) by *lengthening* the pre- and post-test interval.

Regardless of the solution to the mystery of my control rats, there are some more general limitations to my experiment that should be noted. For one, the bottle test design is inherently less controlled than other ways of testing odor learning, such as olfactometer rigs. This is because odor placed on an odor strip has no temporal or concentrational control, but olfactometer rigs are designed specifically for delivery of stimuli controlled by these variables. Thus, it is possible that the preferences learned during my experiment were not as strong as they could have

been, as instead of a consistently controlled burst of odor in a rig, the odor placed on the strip could have faded and/or dispersed around the cage and therefore rendered the odorized bottle non distinguishable from the non odorized bottle. However, the bottle test design was more efficient for the amount of time I had to conduct my experiments, as I could run multiple rats at a time as opposed to just one in a rig.

Relatedly, there may be better ways to test odor preferences than with liquid consumption. MacRae, Kenkel, & Kentner (2015) measured olfactory behaviors by recording the amount of time rats spent sniffing and interacting with an odor strip. This could be a better way to test the success of my deciliation procedure, since the recovery time for engaging in exploratory behaviors might be less than the time needed to recover liquid consumption levels, and the preference test could then be performed before preference extinguishes.

It is also important to consider the remote possibility that olfaction in rats might not in fact be dual-modal. Rodents, unlike humans, are 'obligate nasal breathers', meaning that their upper air passageway has been suggested to not share a common pathway with food in the oropharynx (Gautam & Verhagen, 2012). It remains the case, however, that retronasal stimuli are successfully discriminated from orthonasal stimuli in a behavioral paradigm with rats and mice (Gautam & Verhagen, 2012). Furthermore, rats show reliable responses in the olfactory bulb to retronasal odor stimuli (Gautam & Verhagen, 2012).

I ultimately was not able to conduct the second retronasal experiment that I had originally planned, as it was not clear whether an effective deciliation procedure had been established. Once such a deciliation protocol had been solidified, and the retronasal test is conducted with anosmic animals, it will be possible to test the possibility that rats in that study learned preferences to non-olfactory components of the stimuli. This could have significant implications

for the previous findings by the Katz lab, since we could no longer be sure that the rapid learning observed for retronasal stimuli was actually for the odor itself.

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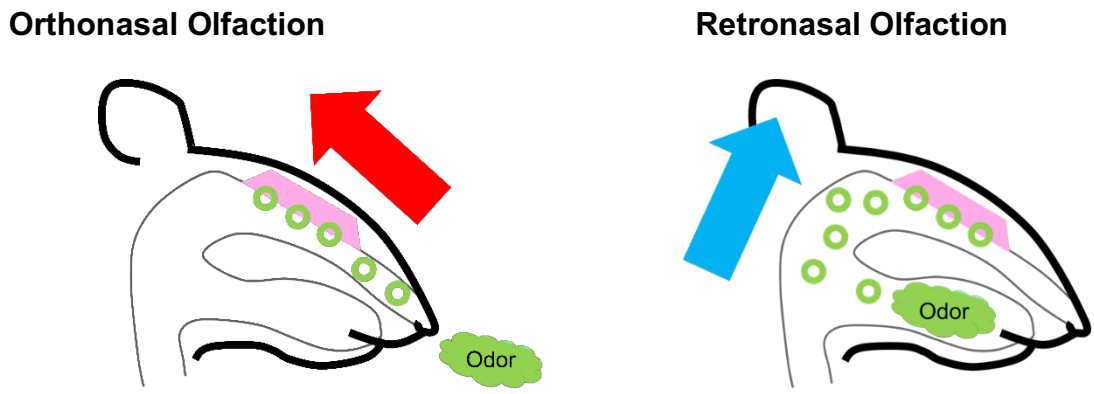

Figure 1. Pathways of Orthonasal and Retronasal Olfaction. For orthonasal olfaction, odor molecules travel through the nose and to the olfactory epithelium. For retronasal olfaction, odor molecules already inside the mouth travel up through the throat and to the olfactory epithelium.

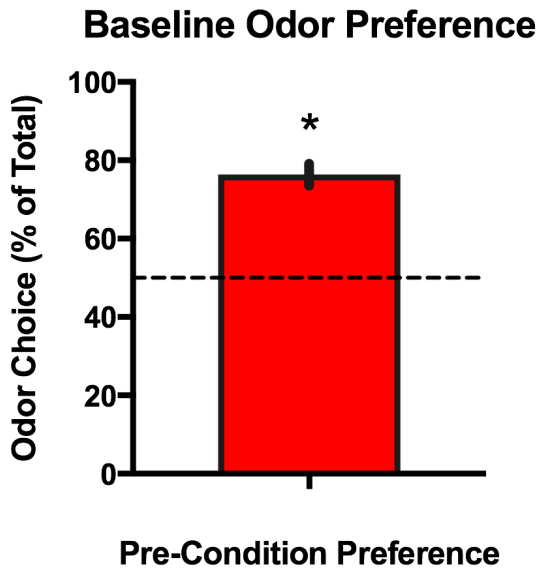

Figure 2. Baseline Odor Preference, n=33. The average preference for odor over air was 76.34%. The dashed line represents the average for chance sampling of odors. Star indicates a significant difference from chance (50%).

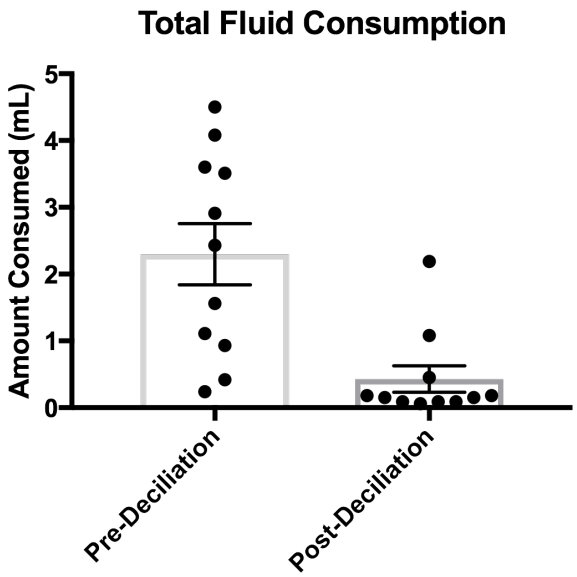

Figure 3. Total Fluid Consumption for Deciliated Subjects. Animals consumed significantly less fluid one day post-deciliation, demonstrating a flooring effect, $t(10) = 4.22$, $p < .01$.

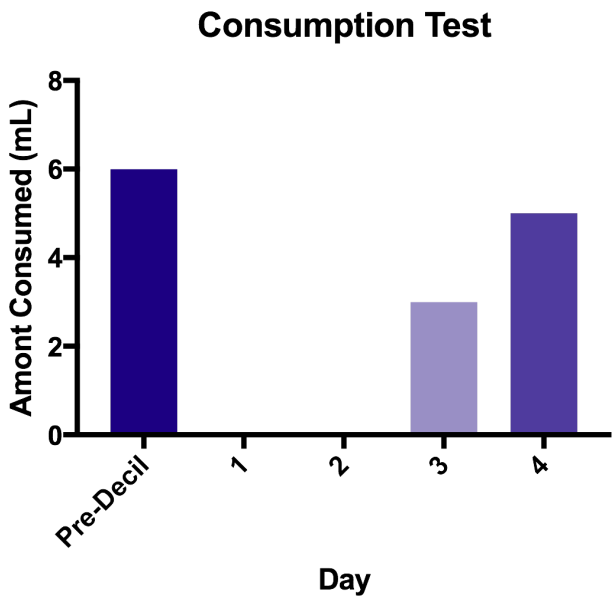

Figure 4. Consumption Test for Post-Deciliation Recovery, n=1. One and two days post-deciliation, consumption drops to zero. However, three days post-deciliation, consumption returns. By the fourth day, consumption reaches levels similar to baseline.

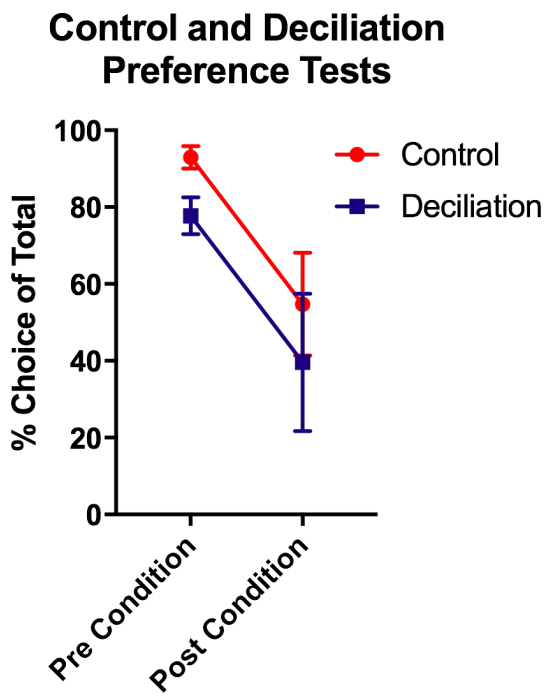

Figure 5. Results of Control and Deciliation Preference Tests. Graph bars in red represent control (kx only) data. Graph bars in blue represent deciliation data.

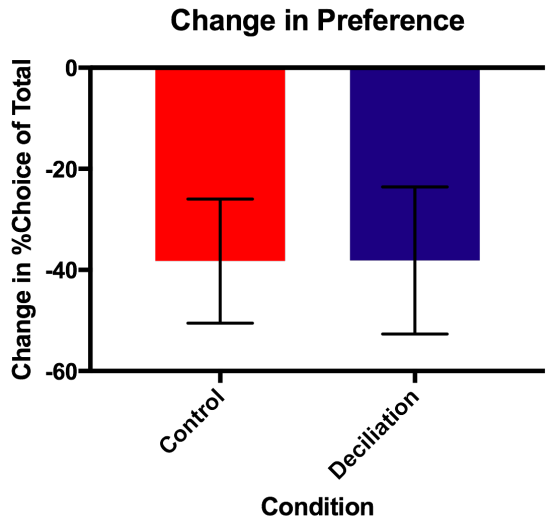

Figure 6. Change in Preference for Control and Deciliation Groups. The difference in preference between the two odor tests was calculated by subtracting the percent preference from odor test 1 from the percent preference from odor test 2. The red bar represents the average change in preference for control rats and the blue bar represents the average change in preference for deciliated rats.