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Development of a novel paradigm for the measurement of olfactory discrimination in dogs (*Canis familiaris*): A pilot study

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Abstract Olfactory dysfunction in older human beings has been shown to be associated with an increased risk of cognitive decline, yet age-related changes in olfactory behavior have received little attention in the dog model of human aging. We developed an odor habituation and fine odor discrimination paradigm to test the hypothesis that dogs would show a novelty response toward unfamiliar urine from entire male conspecifics. We tested 26 odor detection dogs (14 females, 12 males) from the New South Wales police dog unit, ranging in age from 1 year 2 months to 11 years 10 months. First, dogs were familiarized with a master odor over 2 presentations. Second, we measured difference in investigation time of a master odor as compared with 5 odor mixtures using the following ratios of novel-to-master odor: 100:0, 80:20, 60:40, 40:60, and 20:80. Dogs habituated to the master odor after the first presentation ($t(25) = 6.048, P < 0.001$). After 2 dogs that failed to habituate were excluded, there was a nonsignificant trend ($t(21) = -1.968, P = 0.062$) for aged dogs (>8 years, $N = 6$) to show reduced habituation as compared with middle-aged dogs (5-8 years, $N = 9$) and with all dogs aged <8 years ($N = 18, t(21) = -1.883, P = 0.072$). Approximately half of the dogs tested ($N = 11$) failed to show a novelty response toward the 100:0, novel:master odor. The remaining dogs ($N = 15$) showed a significant novelty response toward this odor (mean difference = 1.89 seconds, confidence interval = 0.86-2.84). Investigation of the remaining odor mixtures was not significantly different from investigation of the master odor in all dogs. Further development of this paradigm is needed using naive pet dogs before it can be used as a reliable measure of fine odor discrimination. The current, weak trend for an age effect in habituation warrants further investigation in a larger cohort to determine if this effect becomes significant or if it is simply a manifestation of small sample size and low statistical power. It is recommended that future studies use dogs that have not been trained against or actively discouraged from investigating urine because previous learning may have had a significant effect on the outcomes of this study.

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Introduction

Many aspects of age-related cognitive decline in the dog have been shown to resemble human cognitive change (Berchtold and Cotman, 2009). In addition to the subtle

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decline seen with normal aging (Adams et al., 2000), dogs with canine cognitive dysfunction show pathological (Head et al., 2000; Tapp et al., 2004), pharmacological (Araujo et al., 2005; Pugliese et al., 2005), and behavioral (Landsberg et al., 2003) changes that are similar to those seen in human Alzheimer's dementia.

Given these similarities, it is surprising that age-related changes in olfactory function have not been studied in the dog. Human research has shown that olfactory identification tests can be more sensitive to cognitive decline than some global cognitive tests (Graves et al., 1999). Impaired olfactory function (in the 25th percentile) has also been shown to be associated with a 50% increase in the risk of developing mild cognitive impairment as compared with unimpaired function (in the 75th percentile) (Wilson et al., 2007b). Impaired olfactory function has also been shown to be associated with the rate of decline (Swan and Carmelli, 2002; Wilson et al., 2006). These findings have generated interest in olfactory dysfunction as a possible early diagnostic biomarker (Graves et al., 1999).

Pathological analysis of the canine olfactory epithelium has revealed atrophy in dogs aged >14 years, as well as atrophy of the nerve layer (Hirai et al., 1996). Dogs were also found to have a high prevalence of age-related cerebrovascular amyloidosis, ubiquitin deposits, and astrocytic gliosis within the olfactory bulb (Hirai et al., 1996). Although these findings are similar to those seen in human beings and rodent models, major differences are apparent. β -amyloid plaques are prevalent in human olfactory brain regions (Wilson et al., 2007a) and the mouse olfactory bulb (Wesson et al., 2010), whereas in dogs, no β -amyloid deposits have been found in the olfactory bulb, despite high plaque loads elsewhere in the cerebral cortex (Hirai et al., 1996; Hou et al., 1997). One study (Overall and Arnold, 2007) found evidence of β -amyloid in the olfactory epithelium of an aged dog. This study is of particular interest because it presents a novel way of assessing β -amyloid *ante mortem*, and may allow comparisons of pathology and behavior in living dogs. To date, it has not been established whether these pathological differences also translate into differences in behavioral evidence of olfactory dysfunction with age in the dog.

Traditionally, odor identification and discrimination in the dog has been assessed with operant conditioning paradigms. These have been used to identify dog's olfactory detection thresholds (Walker et al., 2006), odor identification (Williams and Johnston, 2002), and ability to discriminate between similar odors (Brisbin and Austad, 1991; Schoon and De Bruin, 1994). Although a useful technique, there are some limitations to the use of operant techniques in canine aging research. First, all operant conditioning requires for the dog to learn an appropriate behavioral response, but learning in older dogs may also be impaired (Adams et al., 2000), thus leading to confounding results. Second, the rewarded behavioral response (often a sit, drop, or pawing at the object) may be

physically difficult or uncomfortable for older dogs to perform. Staring may be a viable alternative behavior for use in old dogs, but age-related visual impairment would need to be ruled out before dogs are trained to stare. Third, the extended training required to establish a conditioned response may exclude involvement of community-based animals. Given that the aging population of community-based dogs is a valuable source of research subjects (Salvin et al., 2010), testing procedures need to be acceptable to the owners. In this case, electroencephalographic olfactometry has been suggested as an alternative (Hirano et al., 2000), but the requirement for sedation is again likely to be unappealing to owners. The presence of artifacts (nongognitive responses) in electroencephalographic recordings can also make analysis of the findings difficult (Keren et al., 2010).

Based on these limitations, our aim was to develop an odor discrimination paradigm that could be completed in a day and did not require for the dog to undergo any training. Some recently reported rodent paradigms avoid training by using rodents' natural tendency to investigate novel overfamiliar stimuli (Gaskin et al., 2010; O'Dell et al., 2011). Wesson et al. (2010) presented mice with an odor across 4 consecutive trials, allowing for assessment of odor investigation and memory (habituation). To assess odor discrimination, the mice were then presented with a novel odor and differences in investigation time between habituated and novel odors were recorded. Enwere et al. (2004) demonstrated that fine, rather than discrete, odor discrimination was age-sensitive in mice by presenting them with a choice test of different scented drinking waters, one of which had an aversive bitter taste. By mixing the 2 scented waters in varying proportions, the researchers were able to determine the olfactory threshold at which an odor preference was no longer present.

We trialed aspects of both methods to test whether a threshold could be estimated at which dogs could no longer discriminate a novel odor from a familiar odor. Entire male dog urine was used as an ethologically relevant scent of a high interest to dogs (Doty and Dunbar, 1974; Lisberg and Snowdon, 2009). Our paradigm was based on the assumption that dogs would spend more time investigating an unfamiliar urine scent sample than a familiar urine scent sample.

Materials and methods

Subjects

This research was conducted with approval from the University of Sydney's Animal Ethics Committee (Approval # N00/3-2007/3/4571). Animals were drug- or explosive-detection dogs (*Canis familiaris*) from the New South Wales (NSW) police dog unit. These dogs were

either operational, in training, or retired and were selected because they were trained to investigate an odor on command. All dogs included in the study were Labrador retrievers ($N = 26$). Female ($N = 14$) and male ($N = 12$) desexed dogs were used. Dogs ranged in age from 1 year 2 months to 11 years 10 months (average age: 6 years 1 month). When assigned to 3 age groups, the average age of dogs in the young ($N = 9$), middle-aged ($N = 10$), and aged ($N = 7$) groups was 2 years 9 months, 6 years 4 months, and 9 years 11 months, respectively.

Odor sample preparation

Urine from sexually intact male dogs ($N = 18$) aged >12 months and of various breeds was collected over a 2-month period. Urine was collected in a stainless steel ladle and immediately transferred into a sterile 50-mL specimen jar(s) and the lid tightly sealed. Between dogs, the collection ladle was rinsed with water and then cleaned with a 70% alcohol solution followed by a second rinse with water. All samples were frozen at -40°C within 3 hours of collection.

After sufficient volume had been collected, all samples were defrosted at room temperature for 12 hours. To moderate any variation in individual urine odor, urine samples in batches of 3 were mixed in equal proportions (3.3 mL each) to form 1 odor sample (9.9 mL) and placed in a new sterile specimen jar. This process was repeated until there was insufficient volume from the 3 urine samples to make a further 9.9-mL odor sample replication. From each 9.9-mL aliquot, 6 different odor samples (master sample and samples B-F, see Table 1 for the composition of these samples) were generated with 3 to 34 replications of each sample. After mixing, samples were refrozen.

One week before the start of testing, 1 replication of each of the 5 mixed odor samples (B-F) was defrosted at room temperature for 12 hours. Each sample was separated using a 1,000- μL pipette into multiple subsamples containing sufficient volume (Table 1) for approximately a day of testing. A further set of subsamples was generated as required when the previous subsamples had been used. After separation, samples were refrozen.

On each day of testing, 1 master sample and 1 subsample each of samples B-F were defrosted in a warm water (55°C) bath for 10 minutes. The required volume of master sample (Table 1) was pipetted into samples C-F to produce the final mixtures. All of the samples were then placed in an insulated box and transported to the testing site located an hour away. After testing, any remaining sample was discarded, and fresh samples were mixed for each testing day.

Testing apparatus

A 120-cm-long \times 90-cm-wide \times 150-cm-high cage was set up and covered in a vinyl canvas (Figure 1). On both long sides, 2 plastic covered wooden blocks were positioned 1 m apart, 60 cm from the base of the cage.

A 2-mm-wide, 10-mm-deep groove was cut in the top of each block to allow for the insertion of an odor-bearing card. The odor card was bleached beer matt board (Grammage = 390 gsm, Thickness = 900 μm) cut into a $6 \times 3 \text{ cm}^2$ shape, as shown in Figure 2, and perforated with 9 holes measuring 3 mm in diameter. The holes were designed to allow noises associated with the dog's sniffing to pass through the card and be recorded by the microphones behind. The odor sample (0.15 mL) was syringed onto a new odor card at the start of each trial. A separate 2-mL syringe was used for each odor to avoid cross-contamination.

Above each block, a 25-mm hole was cut in the vinyl canvas and a microphone (SHURE SM57 dynamic microphone, frequency response = 40-15,000 Hz, Shure Inc, Niles, IL) was placed inside the cage, directly behind the hole so that the diaphragm of the microphone was positioned 5 mm behind the odor card. A video camera was positioned directly above the cage to record the dog's behavior.

Between trials, the floor around the cage, the walls of the cage, and the plastic-covered wooden blocks were washed with a 10% deodorant disinfectant solution (DeleteTM, Maraylya, NSW, 2% w/w quaternary ammonium deodorant).

Testing protocol

For each dog, 3 sets of 2 trials (i.e., a total of 6 trials) were conducted 1 hour apart to maintain the dog's

Table 1 Composition of odor samples used in this study

Odor sample	Number of subsamples from one 9.9-mL odor sample in a set	Volume of sample (μL)	Volume of master sample added (μL)	Total volume (μL)	Percentage of sample in final mixture (%)
Master	1	9,900	N/A	9,900	N/A
B	7	1,250	0	1,250	100
C	9	1,000	250	1,250	80
D	13	750	500	1,250	60
E	19	500	750	1,250	40
F	39	250	1,000	1,250	20

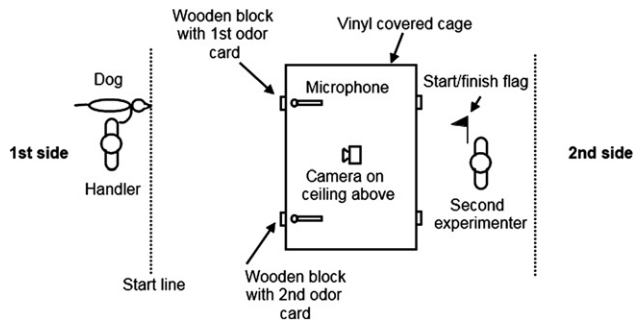


Figure 1 Diagrammatic representation of testing apparatus from above. The position of the handler-dog combination, second experimenter and microphones are reversed when the odors are presented on the 2nd side. The camera is positioned on the ceiling above the center of the cage.

motivation for investigating the odors (Figure 3). The first trial of a set consisted of the dog being presented 2 odors on one side of the cage, and in the second trial the dog was presented 2 odors on the opposite side of the cage. This process was repeated for each set and was established to reduce the chance of the dog disengaging with the location of the odors. In each trial, dogs were positioned at the start line, 1.7 m away from the cage, before being presented each odor. The dogs were presented the left odor first for 20 seconds followed by a 1-minute break at the start point after which they were presented the right odor for 20 seconds.

During each odor presentation, dogs were given the “seek” command accompanied by the handler gesturing at the odor card with an open palm. The seek command was

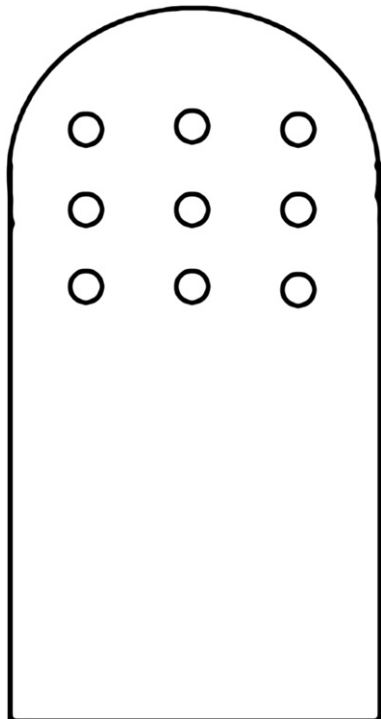


Figure 2 Odor card 6 cm × 3 cm perforated with 9, 3 mm diameter holes.

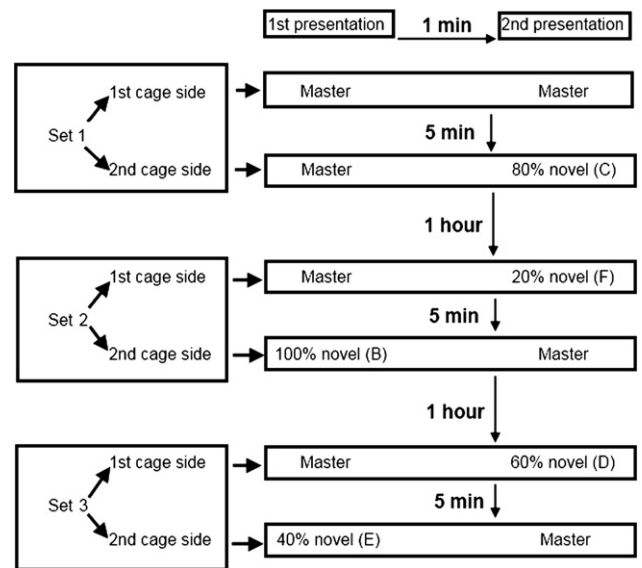


Figure 3 Diagrammatic representation of testing protocol using one of the randomized presentation sequences as an example. Sample odor ratios (novel: Master) are as follows: B = 100:0, C = 80:20, D = 60:40, E = 40:60, F = 20:80.

given at the start of the presentation and then once every 5 seconds, with a total of 4 commands given. Dogs were held on a loose lead unless they tried to move further than 70 cm away from the handler. The handler was always positioned to the left of the dog and to the side of the odor card. The start and end of each presentation within a trial was marked by a second experimenter holding up a flag. This experimenter was out of sight of the handler and dog at all times during the trial.

In the first trial of the first set, dogs were presented with the master odor in both the left position and the right position to allow the dog to become familiar with the odor. For the remaining 5 trials, dogs were given an odor mixture (B-F) in one of the positions and a master odor in the other. The location of the odor mixture in the left or right position was randomized between trials but kept consistent between dogs. The order of odor mixture (B-F) presentation across trials was also randomized, using 1 of 4 computer-generated sequences. Each dog was designated 1 of the 4 randomization sequences at the start of testing. The handler of the dog was blind to the position and sample mixture on the odor cards and the randomization sequence used.

Analysis

The time spent investigating the odor (nose within 5 cm of the odor card) was recorded from the video footage by the primary investigator. A second person scored the time spent investigating the odors for all videos to determine the accuracy of this measure. Pilot study analysis of the audio recordings suggested that a proportion of the sniffing behavior in some dogs could not be detected from the

microphones and so audio data were not analyzed as part of this study. Further work is currently being conducted by our group to determine the level of agreement between audio and video recordings of olfactory behavior. PSAW v18 (SPSS Inc, IBM, New York, NY) was used for all statistical analyses with the significance threshold kept at 0.05.

Habituation

Paired-sample *t*-test was used to assess habituation to the master odor. To determine whether individual differences in overall dog sniffing time affected the results, dogs were separated into 3 groups: short (<3.85 seconds, *N* = 9), medium (3.85-5.5 seconds, *N* = 9), and long (>5.5 seconds, *N* = 8) investigation time. Analysis of variance was used to identify any significant differences between habituation to the master odor across investigation time groups. To assess any possible effects of age on habituation, dogs were also separated into 3 age groups: young (<5 years), middle-aged (5-8 years), and aged (>8 years). Analysis of variance was used to test any significant differences in habituation across age groups.

Discrimination

The level of discrimination between the familiar (master) odor and the odor mixtures containing a proportion of novel odor (B-F) was determined as the difference between the times spent investigating the 2 odor samples within a trial. A positive discrimination was classified as more time spent investigating the novel as compared with familiar (master) odor. Ratios could not be used in this instance because some investigation times were 0 for either the master or novel odors. For differences in investigation time of the odor mixture, 95% confidence intervals were calculated to determine whether the investigation was significantly different from chance. Binary logistic regression was used to identify any age group differences in positive discrimination toward the 100% novel odor (B).

Results

Inter-rater reliability

There was a highly significant Pearson's correlation between the investigation times when measured by 2 independent scorers ($r = 0.886$, $P < 0.001$). Investigation times from the primary investigator were therefore considered a valid and repeatable measure of olfactory behavior.

Investigation time

Young, middle-aged, and aged dogs investigated the first odor presented to them for an average of 3.57, 3.87, and

3.32 seconds, respectively. There was no significant difference in the investigation times between groups ($F(2,23) = 0.2$, $P = 0.82$).

Habituation

Habituation to the master odor was not different between the investigation time groups ($F(2,23) = 1.045$, $P = 0.368$), and therefore combined data are presented in all cases. Habituation to the master odor occurred after 1 presentation (Figure 4), with a significant difference between the first and second presentations ($t(25) = 6.048$, $P < 0.001$).

Dogs (*N* = 2) that did not show habituation to the master odor after 1 presentation were excluded from age group analysis of habituation. Overall, there was no significant differences in habituation between age groups ($F(2,21) = 1.974$, $P = 0.164$). Figure 5 shows a nonsignificant trend for dogs in the >8 years age group to show reduced habituation as compared with dogs in the 5-8 years age group ($t(21) = -1.968$, $P = 0.062$), and as compared with all dogs aged <8 years ($t(21) = -1.883$, $P = 0.072$).

Fine odor discrimination

For all odor mixtures, the discrimination between the novel (B-F) odor mixture and familiar (master) odor presented in the same trial was not significantly different from chance (Figure 6). That is, the dogs did not spend significantly more time investigating the novel odor (B-F) mixture than the familiar (master) odor.

Further examination of the results showed that 11 of the 26 dogs failed to positively discriminate when presented with the 100% novel odor (B). That is, they spent more time investigating the familiar (master) odor as compared with the novel odor. If these dogs are excluded, the remaining dogs (*N* = 15) showed a significant positive discrimination toward the 100% novel odor (Figure 7), but

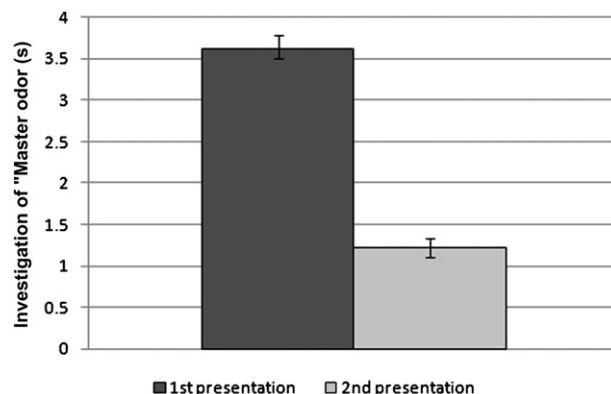


Figure 4 Significant habituation ($P < 0.001$) to Master odor by all dogs (*N* = 26). Standard error bars are shown for the 1st and 2nd presentation.

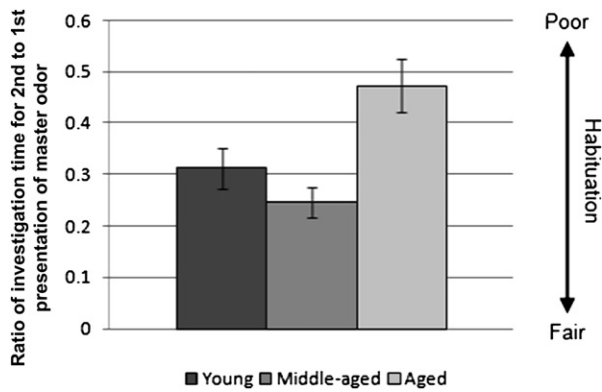


Figure 5 Investigation time for the 2nd presentation of the Master odor as a proportion of the investigation time at the 1st presentation in three age groups: Young, < 5 years $n = 9$; Middle-aged, 5-8 years $n = 9$; and Aged, >8 years $n = 6$. Higher ratios indicate less habituation. Standard error bars are shown for all groups.

discrimination toward the remaining odor mixtures (C-F) was not significantly different from chance.

In dogs ($N = 15$) that positively discriminated toward the 100% novel (B) odor, there was no significant difference between the degree of discrimination toward the 100% novel (B) odor between young (2.06, $N = 6$), middle-aged (1.66, $N = 7$), and aged (1.90, $N = 2$) dogs ($F(2,12) = 0.06$, $P = 0.942$).

Discussion

Olfactory dysfunction in human beings has been reported as a promising early diagnostic indicator of cognitive decline (Arnold et al., 1998, 2001; Swan and Carmelli, 2002; Wilson et al., 2007b). Other than histopathological evidence (Overall

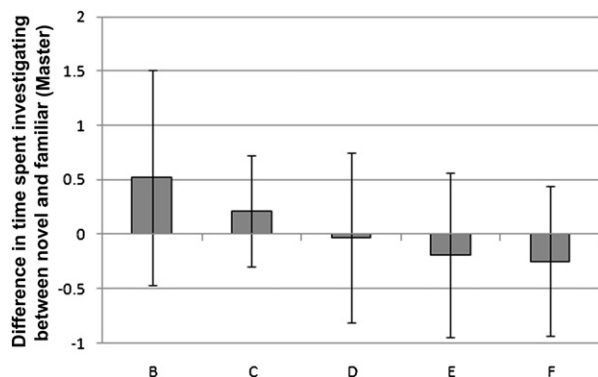


Figure 6 Discrimination between novel (B-F) odor mixtures and the familiar (Master) odor presented in the same trial for all dogs ($N = 26$). A positive value indicates a positive discrimination towards the novel odor mixture. A negative value indicates discrimination towards the familiar (Master) odor. 95% confidence intervals are given for each odor mixture. If the confidence interval crosses zero, the result is not significantly different from chance.

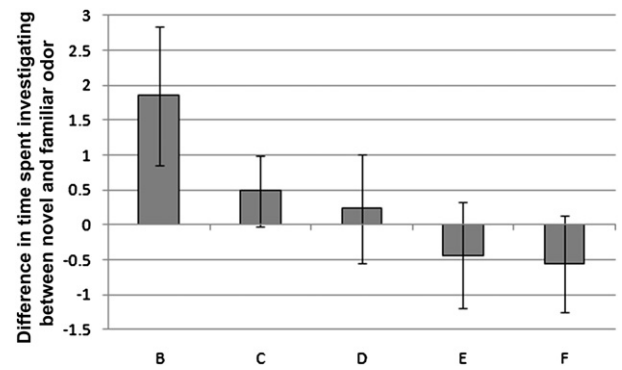


Figure 7 Discrimination between novel (B-F) odor mixtures and the familiar (Master) odor presented in the same trial for dogs ($n = 15$) which showed a positive discrimination towards the 100% novel (B) odor. 95% confidence intervals are given for each odor mixture. If the confidence interval crosses 0, the result is not significantly different from chance.

and Arnold, 2007), changes in olfactory function in aged dogs have not been investigated. We trialed a novel paradigm based on canine urinary scents, designed to be suitable for use in community-based animals. Habituation to a previously presented urinary scent was clearly demonstrated. A weak trend for age-related decline in habituation was also seen, but this was not significant. Several design factors may explain why a more robust olfactory discrimination effect was not observed.

Using this paradigm, we found that dogs can habituate to a novel odor after 1 presentation. Studies in rodents show similar rapid habituation to novel odors. For example, Wesson et al. (2010) found a significant reduction in investigation time (habituation) to novel odors in wild-type mice after 1 presentation. Sundberg et al. (1982) showed complete habituation (no response) to conspecific urinary scent in rodents after an average of 4-5 presentations. Habituation is considered a non-hippocampus-dependent form of implicit or nondeclarative memory (Wesson et al., 2010).

The results of this pilot study also suggest that aged dogs may show reduced levels of habituation as compared with young and middle-aged dogs. This is consistent with findings in rodents (Wesson et al., 2010) in which aged transgenic dementia (Tg 2576) mice showed increased latency to habituate to a novel odor as compared with age-matched wild-type controls. In our study, low sample size was a key issue. The observed power was 20.3%, suggesting that a sample size of 38 animals in each age group is required to enable detection of age differences with 80% power. The effect of training may also have contributed to the weak age differences. Younger dogs may be currently discouraged from urine sniffing behavior more than older retired dogs, resulting in their losing interest in the odors more rapidly.

The significant habituation results and the trend toward an age effect suggest that habituation alone may be a suitable measure for age-related olfactory behavior. The

simplicity of this paradigm is promising because it could be readily applied in a clinical setting. Further investigation is needed to identify whether potential age-related deficits in olfactory habituation in untrained dogs are correlated with behavioral and cognitive dysfunction in aged dogs.

Results from the olfactory discrimination (cross-habituation) part of our study produced mixed results. Almost half of dogs (42.3%) did not behave as predicted by our hypothesis, failing to show a preference for investigating a 100% novel odor. Indeed, these dogs showed a significant preference for the familiar odor. This result was contrary to our initial hypothesis and to our understanding of the olfactory habituation process. It also fails to parallel the findings of published rodent studies (Wesson et al., 2010). A potential explanation for this anomaly is that, as police detection dogs, the current subjects had been trained and rewarded for detecting and responding to a set of familiar odors. It is possible that some dogs were returning to the more familiar odor because they had been rewarded for this response during conditioning to a new odor. A study by Lisberg and Snowdon (2009) found that companion dogs investigated unfamiliar urine significantly longer than a water control, but that there was no significant difference between investigation of urine from familiar dogs (group mates) and urine from unfamiliar dogs. Dogs also had previous experience with conditioning programs that may have enhanced their problem-solving capabilities (i.e., they may have learned to learn). Although no learning was required in this protocol, such previous conditioning may have altered the way the dogs approached the task. Sniffing urine is an actively discouraged behavior in police dogs and this may also have influenced these dogs' responses. However, the use of a less ethologically relevant odor will make testing of naïve companion dogs difficult because of the need for training to maintain interest in the odors. Other unknown factors may also have contributed to the current results and further research is required to identify them. A repeat of our experiment using untrained companion dogs will help to identify whether the lack of a consistent preference for novel odors is an artifact of the training police dogs receive. It will be important to select dogs whose owners have not actively discouraged them from urine sniffing behavior. A veterinary examination to rule out other potential causes of cognitive dysfunction, such as dental disease, would also be of benefit in future studies.

In addition to the problems identified previously, interest in engaging with the odor seemed to be low after the first trial. Again, it is possible that the training received by police dogs, in which sniffing urine is discouraged, reduced the dogs' interest in the odors. It is also possible that the small volume of urine used in each sample was insufficient to fully engage our dogs' attention. The Lisberg and Snowdon (2009) study spread urine over a $12 \times 3 \text{ cm}^2$ area for each sample and did not report any issues in maintaining interest. Although issues were not reported in the Lisberg and Snowdon (2009) study, average investigation

times were still short (approximately 2-5 seconds), suggesting that these dogs' interest in the odors may have waned. Improving dogs' engagement in our paradigm is a critical next step. The use of untrained dogs and increased sample volumes may help in this regard.

Given the issues identified with consistency and motivation, further work will be required before this protocol can be established as an effective measure of olfactory function and dysfunction. However, there is potential for the measure of habituation alone to be sufficient for identifying age-related change. Further development and investigation of this paradigm with a larger sample size and greater statistical power is therefore encouraged. If this provides significant results, a logical next step would be a longitudinal study to determine whether olfactory dysfunction is correlated with, or even pre-dates, cognitive dysfunction.

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