Horses’ interest in non-social odours varies with age and pregnancy in the Habituation/Dishabituation test paradigm

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Research Article

Keywords: Horse, Olfaction, Odour, horse-human interaction, Smell, Sensory ability

Posted Date: May 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-957361/v2

Abstract

In spite of the highly developed olfactory apparatus of horses, implying a high adaptive value, research on equine olfaction is sparse. Our limited knowledge on equine olfaction poses a risk that horse behaviour does not match human expectations, as horses e.g. might react fearful when exposed to certain odours which humans do not consider as frightening. The benefit of acquiring more knowledge of equine olfaction is therefore twofold; 1) it can aid the understanding of horse behaviour and hence reduce the risk of dangerous situations occurring, and 2) there may be unexplored potential of using odours in several practical situations where humans interact with horses, i.e. to improve management routines and the situation for the horses. This study investigated the olfactory sensitivity of 35 Icelandic horses who were presented with four odours: peppermint, orange, lavender and cedar wood in a Habituation/Dishabituation paradigm. The response variables were sniffing duration per presentation and behavioural reaction (licking, biting, snorting, and backing), and we moreover analysed the data for any potential effects of age, sex and gestational stage. Results showed horses were able to detect and distinguish between all four odours and showed increased interest for peppermint (Wilcoxon signed rank test: orange vs peppermint: $V = 68$, $P < 0.001$, lavender vs peppermint: $V = 20$, $P < 0.001$, cedar wood vs peppermint: $V = 47$, $P < 0.001$). More horses expressed licking behaviour when presented to peppermint compared to cedar wood and lavender (Fishers Exact test: peppermint vs lavender & peppermint vs cedar wood: $OR = 4.40$, $P = 0.0068$). Young horses (age 0-5 years) sniffed cedar wood for longer than old horses (Anova: $F = 10.03$, p-value = 0.004), and pregnant mares sniffed lavender less than non-pregnant mares (Wilcoxon signed rank test: pregnant vs not pregnant: $W = 93$, $P = 0.02$), whereas sex had no effect (Wilcoxon signed rank test: $p = 0.4$). The results showed that the test paradigm was meaningful for testing olfactory sensitivity in horses, and olfactory interest of horses varied with age and gestational status but not sex.

Introduction

Odours are a constant component of an animal´s environment, and play a key role in the expression of behaviour, both as a stimulus and as a modulator of the behaviour elicited by another stimulus. In spite of this, the role of olfaction is often ignored in animal behaviour studies, and when handling and interacting with horses. The underlying reason may be our own limited ability or less developed ability, to detect scents and shades of scents. Humans have 369 olfactory receptor (OR) genes, while horses have 1,066 OR genes[1]. The OR gene’s code for various olfactory receptors that detect different odours or scents in the environment. In addition, the olfactory epithelium (OE) of the horse[2] is much larger compared with the human, hence the equine nose is believed to be much more sensitive to odours than the human nose. As a result, most of the currently existing knowledge about olfaction originate from human research or rodents constituting human models[3]. The lack of knowledge from animals and the human inability to detect odours and various concentrations of odours poses a risk that the role of olfaction is underestimated in animal studies and animal handling.

In domestic horses, olfaction is important in the development and expression of behaviour (odours can both function as stimuli leading to behaviour, and as modulators of the behavioural response following another stimulus), in many contexts of horses' lives. Food flavour, foraging, and social behaviour[4,5], mating and reproduction[5–7] but also in the evaluation of predation risk[3] to name but a few. It is therefore surprising how sparse research on the olfactory abilities of horses is[8]. As a result, this study builds on the literature available on equids but when knowledge on equids is lacking, we relied on research from rodents or other mammals for forming our hypotheses.

The role of olfaction in equitation

Horses are sensitive to and affected by odours in their surroundings. These abilities are mentioned in equitation science books [9,10], but are also often ignored in practise. How horses respond to odours is important 1) as it plays a key role in their everyday life[11], and hence their welfare, and 2) as horse's reactions, and the ability to predict these reactions, are crucial for humans to ensure safety when handling and training horses[8]. An odour may be neutral to the horse, but it might also elicit either avoidance behaviour or have an attractive effect depending on the horse's perception of the odour. Studies have shown that avoidance and vigilance behaviour was shown by horses when a predator odour (wolf urine) or an unknown odour (eucalyptus oil) was present, but in addition, this was not associated with higher heart rates[12]. The latter might indicate that although horses expressed behavioural signs of fear, the body's physiological response was not responding to a perceived danger.

It is not uncommon practise for various odorants of non-social origin (i.e. odours not derived from horses such as odours contained within excrements) to be used for calming horses in stressful situations (e.g. during trailering[13]) and as aromatherapy for horses (e.g. [14]). Moreover, a few years ago (2020[15]), a new trend in horse training appeared. The so-called nose work exercise, which has been developed as a leisurely activity with horses. Nose- or scent-work for horses consists of placing an odour in/at a designated place in the horse's environment either indoor or outdoor, and subsequently allowing the horse to sniff out the odour, which then elicits a reward[15]. Despite the increasing popularity of nose work in horse training, however, there is no scientific background for its applicability or for selecting suitable odours, hence these exercises are mainly based on knowledge from dogs[15–17]. Some trainers thus use knowledge of olfaction in their daily handling and training of horses, without any scientific information of its efficacy and potential effect of individual variation. In addition, essential oils such as lavender, lemongrass or peppermint is also gaining more and more practical attention in horse training[18], as these oils are often used either as nose work scents or as presumably, innately calming odours. While studies have confirmed the antimicrobial and antibacterial effects[19] as well as their efficacy in insecticides[20], knowledge on horses ability to detect and differentiate between such odours as well as their preferences of sniffing these odours remain unexplored. More knowledge is thus needed in order to establish if horses can detect such odours and which individual factors affect their interest in the odours.

The influence of age, sex and gestational stage on olfactory interests

In human research, it is well established that olfactory abilities (like other senses) deteriorate with age [21,22]. Some diseases can also contribute to dampen the sensitivity, or even change preferences[23]. Studies of how sensory sensitivity might change with age in non-human mammals are rare, but from studies on working dogs, similar tendencies have been found[24]. Following these results, it may be fair to argue that age could affect olfactory interest in the horse. On the other hand, one study has assessed if age affected olfactory abilities of horses[4], and found no effect when mares and foals were exposed to social odours. It is however unknown if age affects horses' interest in complex (multiple molecules in contrast to simple odours i.e. single molecule odours), non-social odours.

Olfaction research in humans shows that females seem to outperform males in their olfactory abilities[25]. This effect was most pronounced in children, where girls were more aware and reactive towards odorants[26]. Findings from other mammalian, non-human species are sparse, but from results in mice studies point to females reacting more rapidly to odour-induced signalling[27]. From chimpanzees, males and females also differ in their behaviour when exposed to odours, with females sniffing more than males during feeding, and vice versa during social interactions[28]. Collectively, the findings from these studies can be used to argue for a potential effect of sex on the olfactory interest of horses.

Another factor, which might affect olfactory interest, is the gestational stage of a horse. It is well known that pregnant women change in their olfactory preferences during pregnancy (reviewed in Cameron 2014[29]), but contrarily not much is known about non-human mammalian species on this topic. Research on dairy cows suggest that pregnant dairy cow change with respect to their olfactory responsiveness towards (or perception of) social odours (here: amniotic fluid) as calving approaches[30–32]. These changes are believed to be caused by hormonal changes in the body of the cow as parturition approaches, resulting in the onset of maternal behaviour[33]. It is thus reasonable to propose that pregnant mares might be under the same hormonal influence and as a result differ from non-pregnant mares in olfactory interests.

The olfactory Habituation/Dishabituation test is a simple, and sophisticated method for the assessment of olfactory capacities in animals[34]. The test paradigm relies on the theory; repeated presentation of the same odour resulting in decreased sniffing duration (habituation), whereas subsequent presentation of a new odour reinstates sniffing duration (dishabituation)[34]. In the 1980s the olfactory Habituation/Dishabituation test was first assessed in gerbils by[35] and later the test has also been tried out in other species (dairy cows:[36], pigs:[37,38], mice:[39]). Yet it has only been tried out once in equids[4]. In the study by Hothersall et al.[4] the odours tested were of social origin (urine, faeces and body odour from rubbing blankets on the fur). Despite the more obvious biological relevance of social odours, other types of odours might also be relevant to horses. These include several complex odours from e.g. herbs and grasses (relevant in a food choice context), potentially certain soil types (as these might contain minerals horses need), and smoke and predators (as horses need to learn which odours to avoid).

This study aimed to test horses' olfactory abilities in the context of non-social odours. The study aimed to make further adaptations of the Habituation/Dishabituation paradigm[34] to ensure applicability and relevance to horses (adapted from Hothersall et al.[4] and Rørvang et al.[36]), and investigate if age, sex and gestational stage affected the olfactory interest of horses. Since this study was the first to test horses' abilities to detect non-social odours and potential effects of age, sex and gestational stage, hypotheses could not be made prior to the study. Instead, the study was a hypothesis generating investigation for future research on horse olfaction and non-social odours.

Materials And Method

Ethical considerations

The owner of the horses was informed and agreed to all experimental procedures, data collection and publication before the experiment started. All procedures were conducted in accordance with national legislation on animal experimentation by the Danish Ministry of Justice, Act. no. nr. 253 (8 March 2013) and § 12 in Act. no. 1459 (17 December 2013), and met the ARRIVE guidelines[40] and the ethical guidelines proposed by the Ethical Committee of the ISAE (International Society of Applied Ethology)[41].

As the experiment was conducted during the COVID-19 pandemic, measures were taken to comply with the current precautions during the period March – April 2021. The experiment was conducted in Denmark, and hence complied with the Danish COVID-19 regulations[42]. The experiment was conducted in a separate building, distanced from other daily activities related to training, and management of horses who were not part of the experiment.

Animals, housing and management

Thirty-five privately owned Icelandic horses aged 6 months - 25 years old (mean ± sd = 10.4 ± 8.1) participated in the tests. Three geldings, seven stallions and 25 mares were included, and the uneven sex distribution was only caused by availability of horses at the farm. The horses were kept in individual pens with sawdust bedding during night time and on days of testing. Horses were pastured during daytime when not participating in the tests. The horses were either native to the stud or had been kept at the stud for minimum 6 months prior to the study. All horses were handled and trained by the same trainer at the time of the study. Feed (concentrates) and fresh hay (approx. 1.5% of total body weight = 5 kg hay per day) were provided twice daily (at 0700 and 1800 h). The horses were tested in a familiar individual pen to avoid stress due to being moved, and horses were never socially isolated (minimum 4 horses (maximum 6 horses) were present in the barn during testing). The specific barn section (hereafter called "stable"), in which the testing took place, was separate from the other farm buildings and contained eight individual pens (four on each side of the rectangle stable, separated by a stable aisle). The pen sides were made of solid wood (bottom half) and metal bars (top half) and allowed the horses physical contact with neighbouring horses, and visual contact with neighbouring and adjacent horses (i.e. horses could see all other pens). The front side of the pen had a lower wooden part allowing the horse access to the stable aisle.

Habituation procedure

Prior to testing, a habituation procedure was carried out to familiarise the horses to the equipment used, and the presence of the human experimenter. One specific odour bucket (see section "Odours buckets" below, Figure 1A) was used as a habituation bucket, which never had any odour added. The habituation procedure consisted of placing the habituation bucket in the stable aisle, in front of the horse's home pen, 25cm away from the metal bars (Figure 1B) allowing the horse to investigate the bucket. The horse was free to touch, sniff, lick and bite the bucket for 3 minutes. In order to meet the habituation criterion, the horse should approach the bucket, at least once, within the 3 min period and investigate it for more than 10 s but no more than 2 min. In addition, the horses should not display any behaviour indicative of fear (such as immobilization or freezing behaviour, flight responses, backwards movements or vigilant behaviour (i.e. head raised above shoulder height while ears pointing forwards) neither during investigation of the bucket nor when the human experimenter approached the pen (i.e. during placement/removal of the habituation bucket). The horses had up to three 3-minute trials distributed over the course of the day (maximum 3 trials per day) in order to meet the habituation criterion (all but one horse met the criterion during these three trials). When the horse met the criterion it was ready for testing and was tested either in the afternoon of the same day (if the criterion was met during the morning trials) or on the next day.

Odour buckets

The odour buckets (Figure 1A) were five white plastic boxes (height x length x width: 16 cm x 34 cm x 23.5 cm, 13 L; model 9950, Jem & Fix, Hørsholm, Denmark), which were covered with a wire mesh (galvanized wire (0.9 mm Ø) mesh (width: 6 mm) model 6321, Rancho®, Odense, Denmark)). The wire mesh lid made the odour buckets permeable to air, and hence odours, but prevented the horses from eating, licking and touching the odour samples. The odour buckets each had a ballast weight in the shape of a concrete block (model SF-Klostersten® Camfered, Type 2, pure and clean concrete, height x width x thickness: 14 cm x 21 cm x 5.5 cm, weight: 3.53 kg; IBF, Ikast, Denmark) in order to prevent the horses from tilting and/or tipping the odour buckets. All odour buckets, wire mesh and ballast stones were purchased 2 weeks prior to experimental start and were placed in the stable (Figure 1A) one week before the experiment commenced to ensure that the equipment in itself smelled familiar and similar to the horses' home environment.

Odour samples

Substances chosen as test odours were odour oils approved for human use: Orange oil (*Citrus sinensis*), peppermint oil (*Mentha piperita*), cedarwood oil (*Cedrus*) and lavender oil (*Lavandula angustifolia*) (Urtegaarden ApS, Allingaabro, Denmark). These substances are all complex odours (i.e. each composed of many different odorants) which were chosen as they are natural, and non-toxic odours, cheap, accessible and easy to standardize (using the build-in drop mechanism in the odour bottle). The specific four odours were chosen from 36 possible odours as we hypothesised that orange, peppermint, cedar wood and lavender would be novel to the horses, since none of these substances were found in their feed, hay or as ingredients in any products used on the farm (e.g. crèmes or soap). Moreover, we hypothesised, based on human perception, that these four odours would be perceived as different to each other. Fresh odour samples were prepared before each testing trial (approximately twice a week). Odour samples were prepared in a separate, closed room at one end of the stable. The entrance to the odour preparation room was outdoors (no direct access from the stable), hence the room was out of sight to the horses and odour contamination was limited. Odour samples were made by placing one filter paper (unbleached, light brown filter paper model 7607, Harald Nyborg, Viborg, Denmark) in the odour bucket, and adding 10 drops (~0.649 mL) of the odour oil to it at room temperature (Figure 1A), using the build-in drop applicator on the odour oil bottle. The filter paper absorbed and dispersed the odour oil and ensured that all odour samples had similar colouring. A separate odour bucket and corresponding wire mesh lid and ballast stone were used for each different odour in order to prevent any cross-contamination of odours. At the end of a test day, all odour buckets had the filter papers removed, and all equipment used (odour bucket, wire mesh lid and ballast weight) were cleaned with water and odourless soap. When cleaned, the materials were left to dry for at least 24 h before the next test was initiated. Odour preparation and cleaning of equipment were done in a room separate from the testing area, which were ventilated 24h a day by two fully open windows at either end of the room. The person handling odour samples wore latex gloves for all preparation procedures.

Developing the Habituation/Dishabituation test

Using the methods reported in Rørvang et al.[36] and Hothersall et al.[4], we developed a Habituation/Dishabituation test, which investigated if horses were able to detect and distinguish between odours of non-social origin. In order to assess horses' interest in the odours, each first presentation of an odour was used to indicate the horse's immediate interest in the odour (as proposed in Rørvang et al.[36]). In olfactory preference testing (e.g. as proposed by Witt et al. [43]) water is used as the odourless neutral reference point but as in [36] pilot testing of horses (n = 3 Icelandic horses, who were not part of the actual study) indicated that sniffing water resulted in horses being unwilling to approach and engage in further sniffing. To avoid this, we excluded the water sample and instead used each first presentation as indicator of horses' immediate interest. Interest in an odour was thus measured as time spent sniffing the odour (as in the rodent version of this test[44]) at the first presentation of the particular odour, including when the horse was in direct contact with the odour bucket[36].

To limit potential odour contamination of the stable, a maximum of six horses were tested on one test day, and the stable door and windows were kept open. Prior to testing, a balanced odour order presentation order was made (Table 1), to ensure all possible odour presentation orders were tested. Each horse was assigned to a distinct odour order randomly when the experimenter arrived at the horse stud. In the test situation, the particular odour bucket was moved from the preparation room to the stable and placed in front of the horse's individual pen, in the same manner as during habituation (Figure 1B). The same experimenter prepared the odour samples, and performed the tests for all horses throughout the experiment. The experimenter was not naïve to the odours used or the odours presentation order (as the person could smell the odours used), but naïve to the horses' age, sex and gestational stage (which was provided by the horse owner after each trial). Each odour was presented three times in a row for a duration of 1 min each with an inter-trial break of 2 min. After removal of the first odour, the horse again had a 2 min break without odour[45] before being presented with the next odour. During all breaks, the experimenter removed the odour bucket from the stable to the preparation room to limit inter-trial contamination. After placing an odour bucket, the experimenter would move 1 m away from the horse, while still being positioned in the stable aisle directly in front of the horse being tested, in a squat position as during habituation (i.e. all horses were habituated to the experimenter). Two stopwatches (model 38.2016, TFA Dostmann GmbH & Co. KG, Wertheim, Germany) were used, one to continuously record the occurrence of sniffing behaviour during an odour presentation, and another to time the duration of each odour presentation trial (1 min) and inter-trials pause (2 min). Sniffing behaviour was thus visually monitored and continuously recorded by direct observation[46]. It was defined as the horse's muzzle being in close proximity of (i.e. less than the length of a horse muzzle (12 cm); Figure 1B) or in direct contact with the odour bucket. Licking and biting when in contact with the odour bucket as well as flehmen, backing and snorting during the odour presentation (Table 2) were recorded separately but alongside the recording of sniffing behaviour by the same observer using one-zero sampling[46]. Habituation to an odour was defined as a significant decrease in sniffing duration per presentation, measured over the three consecutive presentations of the same odour. Dishabituation was defined by reinstatement of sniffing when a new odour sample was presented.

Table 1. Odour presentation order with 1st, 2nd, 3rd and 4th odour O=orange, P=peppermint, C=cedarwood, L=lavender. Each odour was presented 3 times, represented as 1-3 in the table. Each presentation order sample size represented as n in the last column.

1st			2nd			3^{rd}			4st			n
O1	O2	O3	P1	P2	P3	L1	L2	L3	C1	C2	C3	2
						C1	C2	C3	L1	L2	L3	1
			C1	C2	C3	L1	L2	L3	P1	P2	P3	2
						P1	P2	P3	L1	L2	L3	1
			L1	L2	L3	P1	P2	P3	C1	C2	C3	2
						C1	C2	C3	P1	P2	P3	1
P1	P2	P3	O1	O2	O3	C1	C2	C3	L1	L2	L3	2
						L1	L2	L3	C1	C2	C3	1
			C1	C2	C3	O1	O2	O3	L1	L2	L3	2
						L1	L2	L3	O1	O2	O3	2
			L1	L2	L3	O1	O2	O3	C1	C2	C3	1
						C1	C2	C3	O1	O2	O3	1
L1	L2	L3	P1	P2	P3	O1	O2	O3	C1	C2	C3	2
						C1	C2	C3	O1	O2	O3	1
			O1	O2	O3	P1	P2	P3	C1	C2	C3	2
						C1	C2	C3	P1	P2	P3	1
			C1	C2	C3	O1	O2	O3	P1	P2	P3	1
						P1	P2	P3	O1	O2	O3	2
C1	C2	C3	P1	P2	P3	O1	O2	O3	L1	L2	L3	1
						L1	L2	L3	O1	O2	O3	1
			O1	O2	O3	P1	P2	P3	L1	L2	L3	2
						L1	L2	L3	P1	P2	P3	2
			L1	L2	L3	O1	O2	O3	P1	P2	P3	2
						P1	P2	P3	O1	O2	O3	1

Table 2. Ethogram of behaviours recorded during testing.

Behaviour	Description
Licking	Muzzle of the horse is in direct contact with the odour bucket, or less than the length of a muzzle away from the odour bucket, with tongue protruding and touching the odour bucket at least once.
Biting	Muzzle of the horse in direct contact with the odour bucket, with open mouth and teeth touching the bucket at least once.
Flehmen[a]	The horse curls the upper lip backwards and inhales simultaneously in both mouth and nose. Head may be elevated and neck may be extended.
Backing	The horse takes at least two steps backwards.
Snorting[a]	Short powerful exhalation(s) from the nostrils

[a] Adapted from Christensen et al., 2005[12]

Statistical analysis

The data comprised 12 repeated measures for each experimental animal; 3 tests per odour i.e. first, second and third presentation, of 4 odours in total (Table 1). Normality of data (duration of sniffing, occurrence of biting, licking, flehmen, backing and snorting), assessed by visual inspection of histograms and in a Shapiro-Wilks normality test, could not be assumed and data were analysed using non-parametric tests as described in Siegel and Castellan[47]. All analyses were performed using software R version 3.6.0 (2019-04-26, “Planting of a tree”) and all P-values were evaluated using a significance level of 5%.

Habituation/Dishabituation

A Kruskal Wallis test was used to determine if significant habituation (reduction in sniffing duration) occurred between successive presentations of same odours (one comparison per odour per horse; n = 144, Table 1). A post hoc pair-wise Wilcoxon test was secondly used to test if sniffing duration decreased significantly between presentations (1^{st} vs 2^{nd} and 2^{nd} vs 3^{rd} presentations for each odour). When ties were present, data were converted into an exact distribution using the Wilcoxon-Pratt signed-rank test software in the package ‘Coin’ for the R program[48]). The same procedure was also used to analyse whether reinstatement of sniffing (dishabituation) occurred when a new odour was presented by comparing sniffing durations between successive presentations of different odours (three comparisons per horse; n = 108, Table 1).

Analysing interest in the odours

When analysing level of interest in each odour, sniffing duration for the first presentation of each odour was compared separately in a Wilcoxon signed ranks test to determine which odour elicited the most investigation. Ties were dealt with by conversion to an exact distribution using the Wilcoxon-Pratt signed-rank test software. The occurrence of licking, biting, flehmen, backing and snorting behaviour (Table 2) was converted into total occurrences per trial for the first presentations of each odour, respectively.

Effects of age, sex, and gestational stage

To investigate if age affected sniffing duration, data were analysed in linear regression models for each odour separately, with age as the fixed effect. The ANOVA´s F-ratio statistics determined if age significantly affected the sniffing duration of each odour. To investigate whether an effect of sex occurred, a Wilcoxon-Man-Whitney test was used to compare males and females sniffing durations. A Fisher exact test was used when comparing the occurrences of licking, biting, flehmen, backing and snorting per trial for males and females. To investigate if pregnancy affected sniffing duration a Wilcoxon-Man-Whitney test was used to compare pregnant females (n = 8) to non-pregnant females (n = 17), and a Fisher exact test was used to compare the occurrences of licking, biting, flehmen, backing and snorting per trial for pregnant and non-pregnant females.

Behaviour

A Fishers Exact test was used to compare if more horses expressed licking, biting, snorting, or backing behaviour when presented for the first time with either of the four odours (orange 1^{st} vs lavender 1^{st}, orange 1^{st} vs peppermint 1^{st}, orange 1^{st} vs cedarwood 1^{st}, etc. for each odour = 12 comparisons for each behaviour), and when comparing if more females or males expressed the behaviour when presented to an odour for the first time (i.e. number of males vs females expressing; licking, biting, snorting, or backing, respectively, for orange 1^{st}, peppermint 1^{st}, lavender 1^{st} and cedarwood 1^{st} respectively).

Results

One horse showed strong neophobic reactions to the odour bucket and was excluded from the tests for welfare reasons (the one horse, who did not meet the habituation criterion, see section “Habituation procedure” above). Five other horses showed no interest in the test situation (i.e. did not approach the odour bucket) when presented with lavender for the first time (n = 2) or when presented with lavender for the second time (n = 1), or when presented with cedarwood the first time (n = 1), or when presented with cedarwood for the second time (n = 1).

Habituation/Dishabituation

During the cause of the experiment, sniffing duration of the four odours persisted regardless of odour (Figure 2A) indicating that the horses' interest in the test situation/the odours did not decrease over time (total test duration per horse: 20 min).

All horses sniffed the same odour significantly less when presented with it the second and third time (Table 3 and Figure 2B).

Table 3. Results of the Kruskal Wallis tests and the posthoc pair-wise Wilcoxon tests for successive presentations of the same odour (orange, peppermint, lavender and cedar wood, respectively).

<table>
<tr><td>Test</td><td colspan="2">Orange presentations (n=36)</td><td colspan="2">Peppermint presentations (n=36)</td><td colspan="2">Lavender presentations (n=34)</td><td colspan="2">Cedar wood presentations (n=35)</td></tr>
<tr><td>Kruskal Wallis test</td><td colspan="2">X^2 = 46, p-value < 0.001</td><td colspan="2">X^2 = 27, p-value < 0.001</td><td colspan="2">X^2 = 45, p-value < 0.001</td><td colspan="2">X^2 = 36, p-value < 0.001</td></tr>
<tr><td rowspan="2">Pair-wise Wilcoxon test</td><td>1st vs 2nd</td><td>2nd vs 3rd</td><td>1st vs 2nd</td><td>2nd vs 3rd</td><td>1st vs 2nd</td><td>2nd vs 3rd</td><td>1st vs 2nd</td><td>1st vs 2nd</td></tr>
<tr><td>p-value < 0.001</td><td>p-value < 0.001</td><td>p-value = 0.01</td><td>p-value = 0.007</td><td>p-value < 0.001</td><td>p-value < 0.001</td><td>p-value = 0.002</td><td>p-value = 0.001</td></tr>
</table>

When presented with a new odour after the third odour presentation, sniffing duration increased significantly in all cases (Wilcoxon Signed Rank test: third presentation vs the first presentation (Table 4, Figure 2A).

Table 4. Results of the Wilcoxon Signed Rank test for each pair of 3rd and 1st odour presentations, with sample size.

Third vs first odour presentations	n	Wilcoxon Signed Rank Test
Orange 3rd vs peppermint 1st	9	V = 0, p-value < 0.001
Orange 3rd vs lavender 1st	8	V = 11, p-value < 0.001
Orange 3rd vs cedar wood 1st	9	V = 1, p-value <0.001
Peppermint 3rd vs orange 1st	9	V = 359, p-value = 0.003
Peppermint 3rd vs lavender 1st	9	V = 154, p-value = 0.023
Peppermint 3rd vs cedar wood 1st	8	V = 194, p-value = 0.126
Lavender 3rd vs orange 1st	9	V = 68, p-value < 0.001
Lavender 3rd vs peppermint 1st	9	V = 0, p-value < 0.001
Lavender 3rd vs cedar wood 1st	9	V = 1, p-value < 0.001
Cedar wood 3rd vs orange 1st	9	V = 62.5, p-value < 0.001
Cedar wood 3rd vs peppermint 1st	9	V = 4, p-value < 0.001
Cedar wood 3rd vs lavender 1st	8	V = 0, p-value < 0.001

Interest in odours

First odour presentations were used to investigate immediate interest in the odours, and the horses showed a greater interest in peppermint, compared to all other odours represented by a significantly higher sniffing duration (Wilcoxon signed rank test: orange 1st vs peppermint 1st: V = 68, p-value < 0.001, lavender 1st vs peppermint 1st: V = 20, p-value < 0.001, cedar wood 1st vs peppermint 1st: V = 47, p-value < 0.001). Additionally, there was a tendency for a higher sniffing duration for orange as compared to cedar wood (Wilcoxon signed rank test: orange 1st vs cedar wood 1st: V = 378, p-value = 0.083), while all other comparisons of sniffing duration at first odour presentation were insignificant (Figure 3).

Effect of age, sex, and pregnancy

The analyses showed no effect of sex on sniffing duration for any of the four odours (Wilcoxon signed rank test: p-value = 0.4). There was however a significant effect of age on the sniffing duration of cedar wood, with younger horses sniffing the first presentation of cedar wood for longer than older horses (Anova: F = 10.03, p-value = 0.004). Seven of the 0-5 year old horses sniffed cedar wood for more than 30 seconds at first presentation. In addition, there was a tendency for younger horses to sniff orange longer than older horses (Anova: $F = 2.86$, p-value = 0.1), with horses younger than 5 years (n = 6) sniffing the first presentation of Orange for more than 40 seconds. Age had no effect on sniffing durations for peppermint and lavender ($Anova_{peppermint}$: $F = 0.12$, p-value > 0.1, $Anova_{lavender}$: $F = 1.36$, p-value > 0.1). Of the 25 mares, 8 were pregnant at the time of the study with (mean ± sd) 75 ± 31 days to expected birth when tested. There was a significant effect of pregnancy on the sniffing duration of lavender, with pregnant mares sniffing significantly less (Wilcoxon signed rank test: pregnant vs not pregnant: W = 93, p-value = 0.02). Due to the age effects on sniffing duration of cedar wood and orange, the effects of pregnancy were analysed in linear models including the age effect. These showed no effect of pregnancy on the sniffing duration of orange (Anova: $F = 0.02$, p-value > 0.5), but a tendency for an effect of pregnancy on the sniffing duration of cedar wood, with pregnant mares sniffing less (Anova: $F = 3.11$, p-value = 0.09).

Behaviour

Licking was the most common behaviour, which was expressed by all horses, but not by all horses for all odours (Figure 4). Biting was the second most common although less frequent than licking. Snorting and backing were generally rare and restricted to only 11 horses. More horses showed licking behaviour when presented with peppermint, than lavender or cedar wood (Fishers Exact test: peppermint vs lavender & peppermint vs cedar wood: OR = 4.40, p-value = 0.0068), but there were no other differences between other pair comparisons. Generally, fewer horses expressed biting, but significantly more horses bit the peppermint sample, compared to the lavender sample, whereas no other pair comparisons were significantly different. Due to the low occurrence of snorting and backing, statistical analyses was not possible. Lastly, equally many females and males expressed licking, biting, snorting or backing (Fishers Exact test: p-value > 0.05).

Discussion

In this study, horses were able to detect and distinguish between four complex odours; orange, peppermint, lavender and cedar wood and the Habituation/Dishabituation test paradigm developed for the horses worked well to test olfactory abilities of horses. Over the course of the experiment, the overall sniffing duration did not change significantly regardless of odour (Figure 2A), hence horses' interest in the testing situation (and the odours) persisted during the course of the experiment. Horses showed increased interest (significantly higher sniffing duration) when presented to peppermint. More horses expressed licking behaviour when presented to peppermint compared to cedar wood and lavender, and more horses exhibited biting behaviour when presented to peppermint compared to lavender. Young horses sniffed cedar wood (and orange, tendency) for longer than older horses, and in addition, pregnant mares sniffed lavender less than non-pregnant mares. Behaviours indicative of averseness (i.e. snorting and backing) did not increase over time (regardless of odour), and horses expressing these behaviours during a first presentation did not necessarily express them in subsequent presentations.

In 2010, Hothersall et al.[4] were the first to try adapting the Habituation/Dishabituation test for horses using social odours (i.e. urine, faeces or fleece fabric previously rubbed on the fur of a companion), but the current study is the first to test horses on complex odours with no social reference to the horse. The horses readily habituated to the odours when presented successively, and sniffed new odours significantly longer, hence both habituating and dishabituating to the odours according to the definition of the terms (see section *"Developing the Habituation/Dishabituation test"*). Compared to the test situation made by Hothersall et al.[4], this study made some further adaptations to improve the validity of the results: To avoid any human cueing and other effects from humans physically presenting/holding the samples, the odours boxes in the current experiment were presented to the horses on the stable aisle. The human experimenter only brought and retrieved the samples. The wire mesh on top of the odour buckets further ensured that horses were unable to touch the samples to avoid the mixing of smell and taste. In previous studies of olfaction in animals, it has been common to add a presumably neutral odour control (usually water) (e.g. cattle[36], mice[49], rats[50]). In the current study, however, the water control was excluded to avoid horses losing interest in sniffing something odourless, which has previously been proven a challenge when testing cattle[36]. Since the test paradigm relies on the animal voluntarily investigating the odours, it is essential that appetitive activity is sustained throughout the test. The overall sniffing duration for odour samples presented the 1st, 2nd, 3rd, and 4th time (i.e. regardless of specific odour) did not decrease or increase in the current experiment (Figure 2A.), hence motivation to investigate sustained over the cause of the experiment. Behaviours indicative of aversiveness were infrequent (mean (range) of occurrence per odour presentation: snorting: 1.6 (0-4), backing: 0.8 (0-2), Figure 4), and horses expressing these did not continue to do so, indicating these behaviours might have been a result of novelty rather than averseness. Moreover, only two horses failed to approach the odours (one on lavender and one on cedar wood) after a first presentation, suggesting that the majority of horses were motivated to investigate the odours regardless of time, order of odour presentation and specific odour.

As previously done with cattle[36], our horses were tested in a social setting. This is the greatest change from the original rodent tests, which are done in an enclosed cage with only the focal animal present, e.g. Tarland and Brosda[50]. This setting was chosen to limit the negative effects of social isolation. Horses were tested from a familiar individual pen, and had companions nearby, although not in the neighbouring pens to limit odour contamination. The disadvantage to this setting is a potential risk of social transmission of fear or induced curiosity caused by reduced fear[51]. The effect of the presence of conspecifics may however be more profound when the situation is more frightening, making social transmission of fear less likely in this particular situation as all animals were habituated to the test situation beforehand. Occurrence of behaviour related to fear (i.e. vigilance, snorting, backing and flight) were uncommon during habituation and later during the test. During the test, fear related behaviour might have been an indication of activation of the trigeminal nerve[52]. The olfactory and trigeminal systems have a close relationship, and some odours can trigger the trigeminal nerve[53]. In the current study it is not possible to elucidate if the fear related behaviour observed was a result of a stimulation of the olfactory nerve, the trigeminal nerve, a learned response to the olfactory stimuli or a combination of these. Since the odours were novel to the horses, and as the horses expressing fear related behaviour in a first presentation did not continue to do so in subsequent presentations of the same odour, it is likely that this behaviour was caused by novelty. Horses often react with fear-related behaviour to novelty[54,55], and presentation of an unknown odour for the first time, is likely to elicit such behaviour.

Of the four odours presented, peppermint evoked the most investigation (longest sniffing duration) for all horses. Peppermint has also previously been found to increase activity in other species e.g. captive mice[56], dogs[57], and zoo-kept lions[58]. In the lion study, peppermint also stimulated more species-specific behaviour (back rolling). All types of mints, including peppermint, are among the oldest herbs used for medical purposes[59] and is botanically related to catnip[60]. Catnip is known to both encourage play behaviour in cats, but can also increase sleep and hence reduce activity[60]. Peppermint is often used as flavour in horse treats and feedstuffs (e.g. [61]) as well as in many types of fluent electrolyte mixtures or insect repellents (e.g.[62]). Horses may thus have had an already established association with peppermint. This theory was further supported by the high number of horses expressing licking and biting behaviour when sniffing the peppermint samples (20 out of 35 horses at the first presentation of peppermint). Horses may thus have perceived peppermint odour as edible, and hence expressed more behaviour linked to eating. Following communications with the horses' owner/trainer, it was nonetheless noted that none of the horses had been fed any treats or feed with peppermint, eucalyptus or other mint flavours for at least the period they were in this particular stable (minimum 6 months). The latter moreover means that none of the 0-5 years old horses tested (n = 14) had ever been exposed to peppermint (or other mints), since they grew up at this stable, and were never handled by other trainers. This adds further support to a theory suggesting peppermint to evoke an innate interest in horses. Peppermint may thus, in addition to activating the olfactory nerve, have activated the facial and glossopharyngeal nerve (taste innervation of the tongue), resulting in the licking and biting behaviour, without the horse having a prior experience with the taste. This study may thus be the first to indicate that horses, without prior experience with the taste of a substance, may be able to link smell with taste (as e.g. humans[63]) which has never been demonstrated before[8].

This study is the first to illustrate an effect of age on olfactory interest in horses. The age effect on sniffing of cedar wood odour could be a result of a deterioration of the older horses' (mean age of older horses: 16) olfactory abilities making cedar wood odour hard to detect, and hence resulting in less sniffing. Research on cedar wood is not abundant, but there is some evidence for a relaxing effect of inhaling the odour in rats[64]. This effect could be speculated to affect horses' inhalation behaviour, resulting in less sniffing in some horses. In contrast to our finding, Hothersall et al.[4] found no effect of age on investigation of social odours (mares and foals were tested). The underlying reason for the different results could be the social nature of the odours used by Hothersall et al.[4] and the complex, non-social odours used in this study. Due to social odours likely playing a role in sexual behaviour, age differences might be more pronounced and biologically relevant in this context compared with non-social odours[65]. The response found in the young horses are potentially more 'pure' as individuals are affected by their environment throughout their lives, and hence older horses may learn to associate certain situations and emotions with an odour[66]. As a result, horses may come to 'like' or 'dislike' odours, which they did not innately have any association to. More studies are needed to confirm if olfactory abilities of the horse decline with age, and to outline if and how this affects the handling of horses.

This study is the first to report an effect of gestational stage on olfactory interest in mares, which is somewhat in accordance with other findings on dairy cattle[30,32,36]. However, dairy cow olfactory responsiveness (or preference) have only been shown to change with regard to social odours (amniotic fluids), and hence cows may, like horses, differ with regards to complex, non-social odours. Outside parturition, many ungulates are repulsed by odours linked to the placenta and/or the amniotic fluids (e.g. Sheep:[67], gerbils:[68], golden hamster:[69]). As parturition approaches, however, the female becomes increasingly responsive towards cues from the young, and some of these cues are of olfactory nature[33]. We speculate that although some odours may have little or nothing to do with the young, the hormonal change in the female might affect her response to both social odours and non-social odours (i.e. like a side-effect). The effect of gestational stage was, in this case, only linked to lavender, with pregnant mares sniffing lavender less than non-pregnant mares. Lavender is, like cedarwood, an odorant associated with anxiolytic effects (Schuwald et al., 2013[70]). For instance, shelter dogs exposed to lavender have been found to reduce activity and vocalisations and in turn spend more time resting[57], and travel-induced excitement could be lowered in dogs during transit when exposed to lavender[71]. Travel sickness in pigs was also alleviated when pigs had access to straw sprayed with lavender[72], and dressage horses exposed to lavender aromatherapy have lower heart rate variability leader the authors to conclude that lavender have an immediate calming effect on horses[73]. It was therefore surprising that pregnant mares indicated some level of avoidance of lavender, unless it could have potential harmful effects on the foetus. Future studies should therefore focus on testing olfactory interest of mares both pre- and post-partum in order to fully understand how olfactory interest changes with gestational state. This is especially important in relation to aromatherapy as some odours may be more or less suitable for various groups of horses. A large amount of work also remains with testing the already available odorous remedies (i.e. aromatherapies, pheromones[15]) on the market to establish if these have a real purpose of use.

In conclusion, this study adds important information to the basic knowledge and understanding of equine olfaction. The results can aid the understanding of horses' behavioural reactions to different odours, and in the future, it may be possible to relate these to the physiology and health of horses. Odours may constitute a source of environmental enrichment for horses either directly as pleasant scents, or secondary as new scents to already existing enrichment materials.

Data availability

Data are freely available upon request to the corresponding author MVR (mariav.rorvang@slu.se).

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Declarations

Acknowledgements

The authors would like to thank Gösta and Anna-Birgit Henrikssons foundation and the Swedish University of Agricultural Sciences for funding. A special thanks to Bo U. Hansen and the personnel at Katulabo for their assistance during the experiments and for kindly allowing us access to the horses. Lastly, M.V.R. would like to thank Birte Nielsen, UFAW, England for the many discussions about which odours to use when testing olfaction in equids and animals in general. International cooperation on the study was supported by the Ministry of Agriculture of the Czech Republic (MZERO0718).

Author contributions

M.V.R. applied for, and was later awarded the funding for the study. M.V.R. designed the Habituation/Dishabituation test and performed the experimental work with virtual participation from J.Y. and K.N. M.V.R. was in charge of data processing and statistical analyses, and K.N. and J.Y. participated in the discussion of statistical procedures and subsequent results. K.N. and M.V.R. collaborated in writing the first draft of the article, and all authors contributed in discussion, proof reading, and fine-tuning the final draft for publication. All authors contributed to the article and approved the submitted version.

Additional information

All authors declare no competing interests.

Funding: Funding for the experiments was awarded by Gösta and Anna-Birgit Henrikssons foundation and funding for open access were provided by the Swedish University of Agricultural Sciences.

Materials and correspondence: Any enquiry for materials or questions regarding the study should be addressed to M.V.R.

Supplementary information

Supplementary video S1. Illustration of the licking reaction to peppermint odour samples. The odour sample is only on the filter paper, hence there is no odour oil on the wire mesh lid which the horse licks. This behaviour was not exclusive to peppermint samples but occurred significantly more often for horses presented for peppermint than for any of the other odours (orange, cedar wood and lavender).

Figures

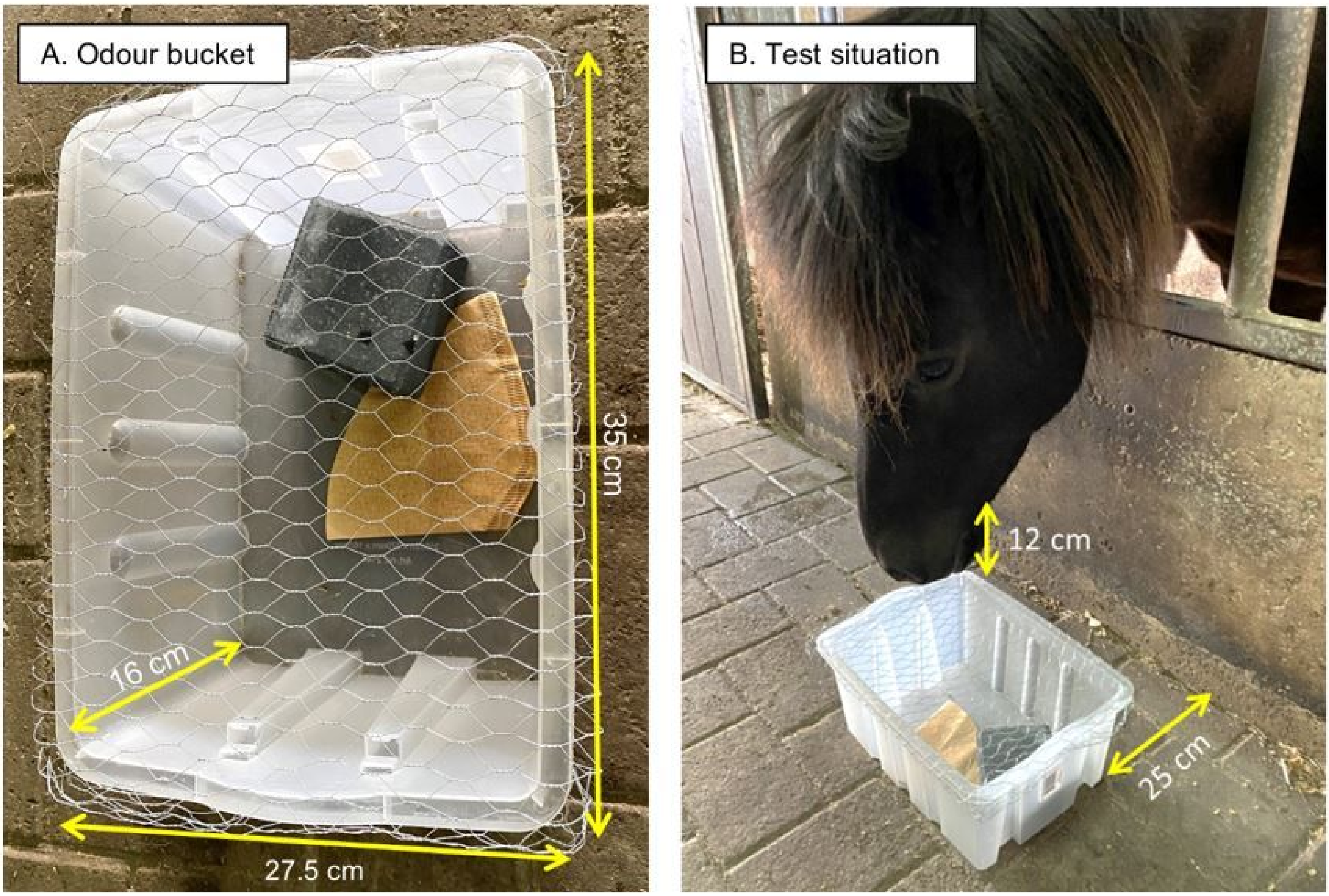

Figure 1

A. Illustration of an odour bucket with wire mesh lid, ballast rock and filter paper (with odour sample). The odour bucket measured 35 x 16 x 27.5 cm. B. Illustration of the test situation. The odour bucket was placed on the aisle floor 25 cm from the grating to the horse's pen. Sniffing duration was measured as from when the horse's muzzle was within the length of one horse muzzle (12 cm) away from the bucket.

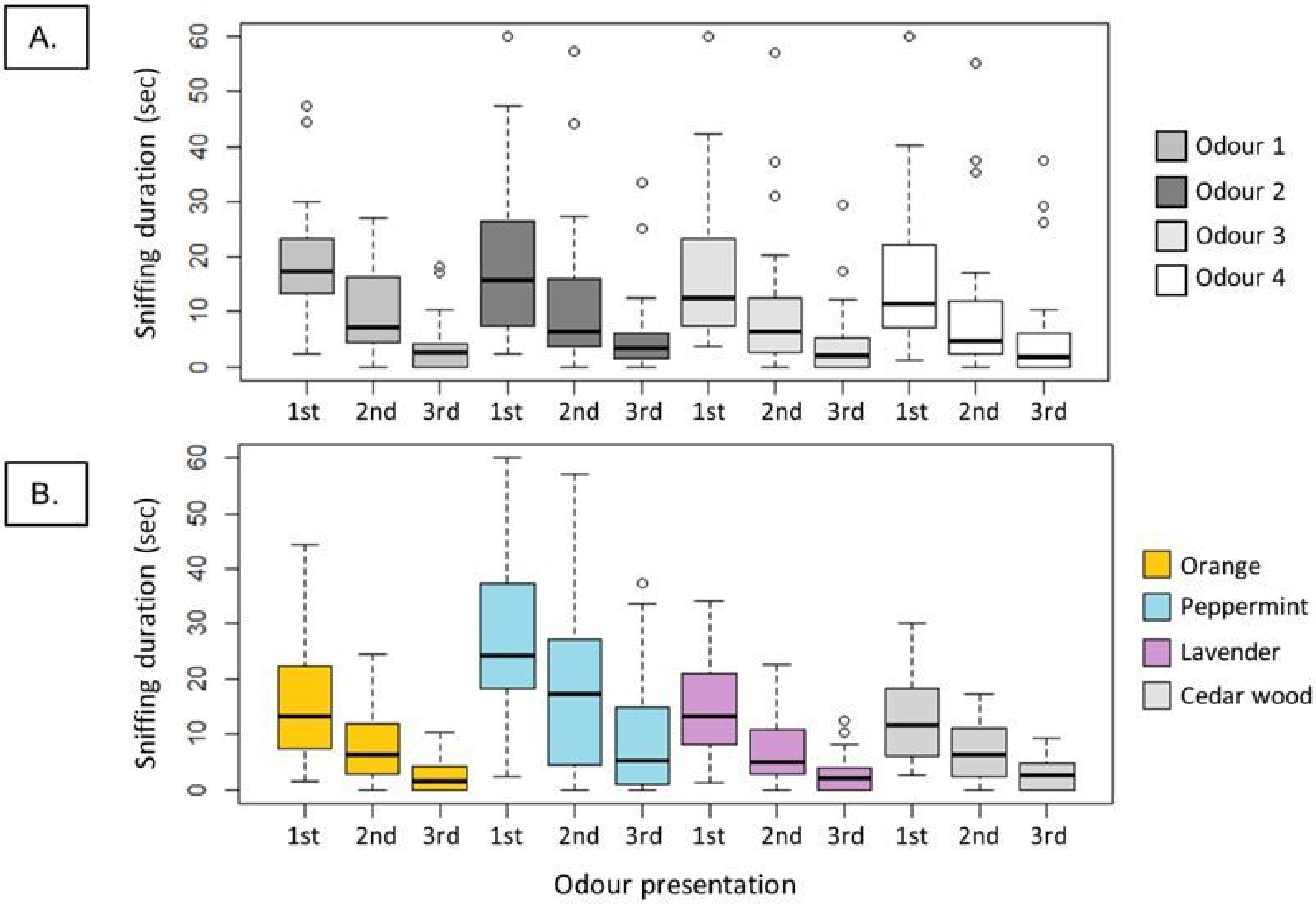

Figure 2

A. Illustration of odour presentations (1st, 2nd, 3rd) over time regardless of specific odour. B. Illustration of odour presentations (1st, 2nd, 3rd) for all odours, orange representing orange, light blue representing peppermint, purple representing lavender and light grey representing cedar wood. For both A. and B. the boxes represent the 25, and 75% quartiles, the thick line inside the box represent the median and the dashed lines illustrate the range.

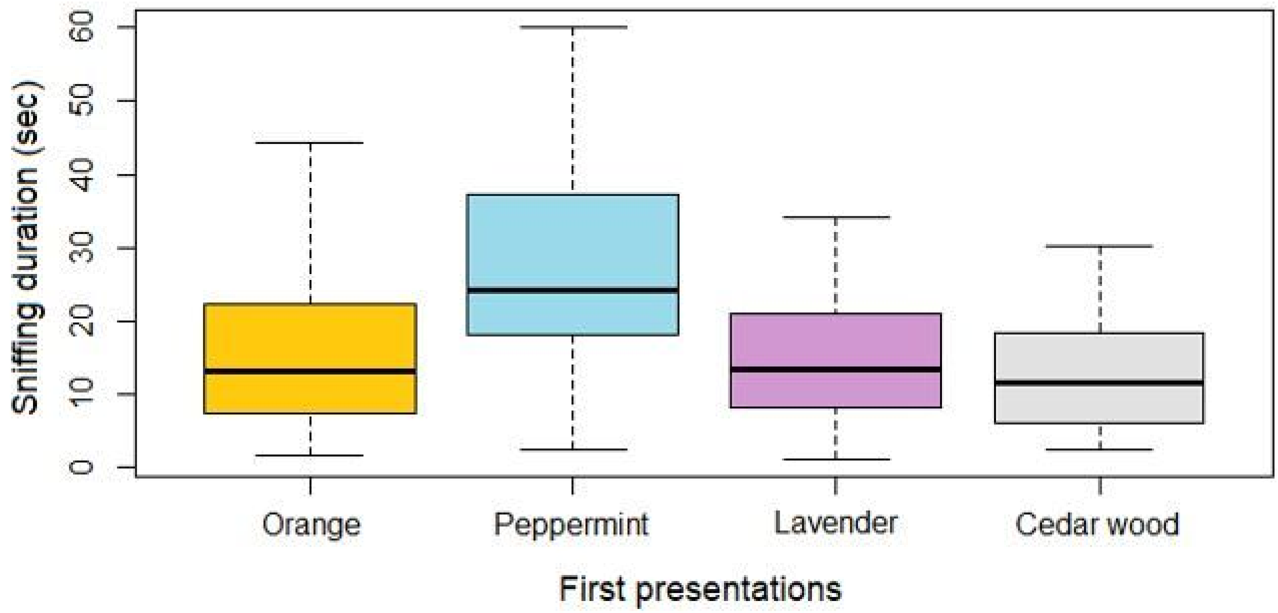

Figure 3

Illustration of the sniffing duration of 1st presentations of each odour. Orange represent orange, light blue represents peppermint, purple represents lavender and light grey represents cedar wood. The boxes represent the 25, and 75% quartiles, the thick line inside the box represent the median and the dashed lines illustrate the range.

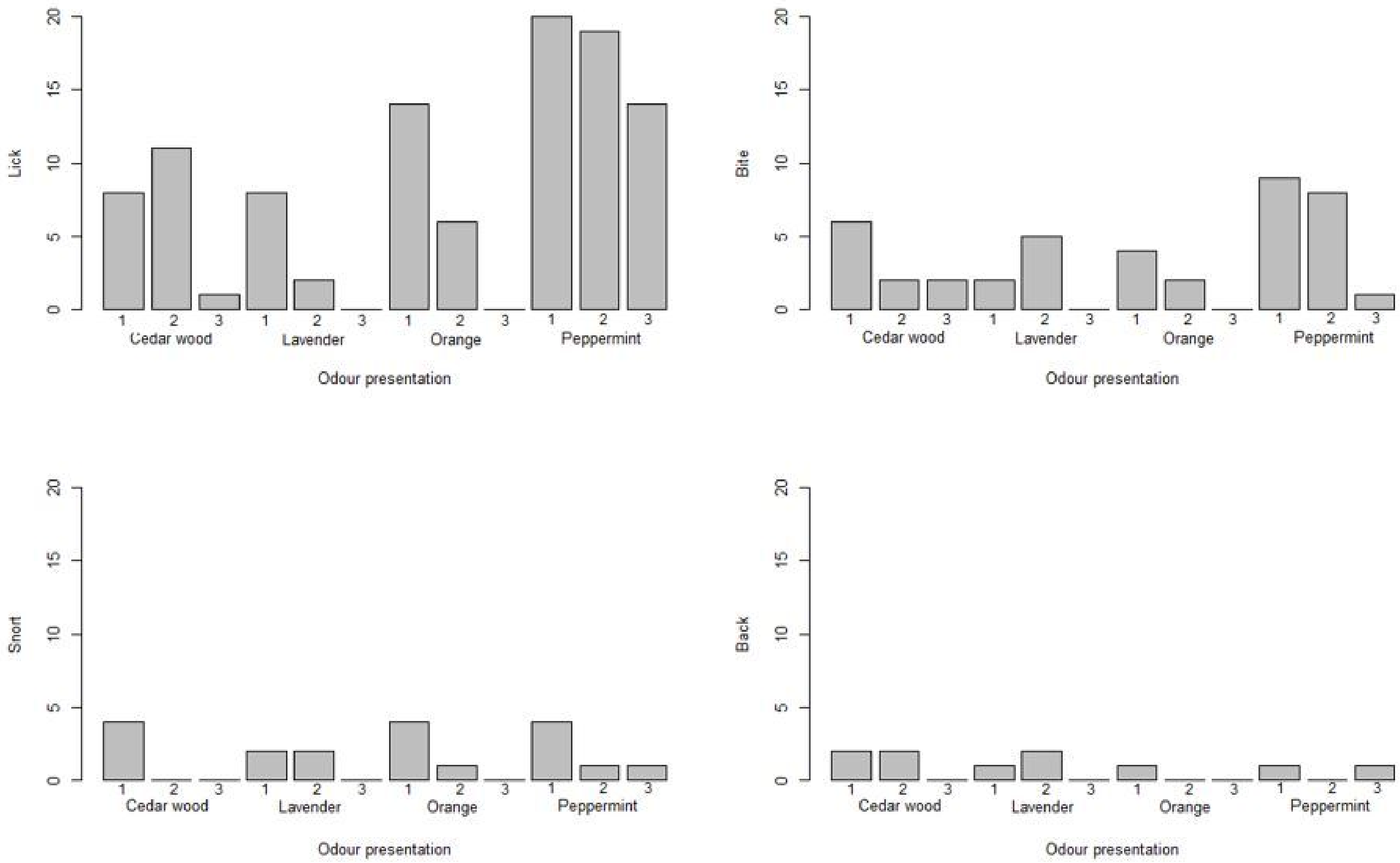

Figure 4

The number of horses expressing licking, biting, snorting and backing when presented with the odours 1st, 2nd, and 3rd, time.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- VideoS1.mov